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รายงานวิจัยฉบับสมบูรณ์

การศึกษามิวซินของมะเร็งท่อน้ำดีที่สัมพันธ์กับการติดพยาธิใบไม้ตับ

Characterization of mucin expressed in liver fluke associated cholangiocarcinoma

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กิตติกรรมประกาศ

คณะผู้วิจัยขอขอบพระคุณสำนักงานกองทุนสนับสนุนการวิจัยที่ให้งบประมาณสนับสนุนโครงการวิจัยนี้ (2543-2546) ภาควิชาชีวเคมี และ คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น สถาบันต้นสังกัดของคณะผู้วิจัยที่ให้การสนับสนุนเอื้อเฟื้อสถานที่ เครื่องมือและอุปกรณ์ในการวิจัย รวมทั้งการบริหารจัดการเวลาเพื่อให้คณะผู้วิจัยสามารถดำเนินการวิจัยภายใต้งานหลักที่รับผิดชอบได้

ขอแสดงความขอบคุณอย่างสูงต่อโครงการปริญญาเอกกาญจนาภิเษกที่ให้ทุนสนับสนุนการศึกษาของ นายชายชัย บุญหล้า และขอบคุณ Prof. John Sheehan ที่ให้คำปรึกษา-คำแนะนำเกี่ยวกับการวิเคราะห์มิวซินและให้ความอุปถัมภ์ นายชายชัย บุญหล้า นักศึกษาโครงการปริญญาเอกกาญจนาภิเษก สาขาชีวเคมีทางการแพทย์ในระหว่างการทำวิจัยในสหราชอาณาจักรและสหรัฐอเมริกา

ท้ายสุดขอขอบคุณ ศูนย์วิจัยพยาธิใบไม้ตับและมะเร็งท่อน้ำดี ผู้ป่วยมะเร็งท่อน้ำดี ผู้ช่วยนักวิจัย และคุณมะลิวัลย์ คุณทวงษ์ ผู้ช่วยฝ่ายบัญชีและการเงิน ที่ช่วยให้โครงการวิจัยนี้ดำเนินการและสำเร็จลุล่วงได้ตามวัตถุประสงค์

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วัตถุประสงค์ เพื่อให้ได้องค์ความรู้พื้นฐานที่จำเป็นเกี่ยวกับการแสดงออกและคุณสมบัติทางชีวภาพที่จำเพาะของมิวซินของมะเร็งท่อน้ำดีในผู้ป่วยไทยเพื่อใช้พัฒนาคุณค่าทางการแพทย์

วิธีทดลอง ศึกษาการแสดงออกของมิวซินชนิด MUC1, MUC2, MUC5AC, MUC6 ในเนื้อเยื่อมะเร็งท่อน้ำดีโดยวิธี immunohistochemistry วิเคราะห์มิวซินในซีรัมโดยวิธีอิเล็กโทรโฟลิซิส, western blotting และ immunodetection ศึกษาคุณสมบัติทางชีวภาพและการวิเคราะห์องค์ประกอบน้ำตาลในมิวซินที่แยกสกัดจากซีรัมผู้ป่วยมะเร็งท่อน้ำดี โดยใช้วิธีทางชีวเคมี วิเคราะห์ทางสถิติเพื่อคุณค่าทางการแพทย์โดยหาความสัมพันธ์ของการตรวจพบมิวซินแต่ละชนิดกับคุณลักษณะทางคลินิก

ผลการทดลอง พบว่ามีการแสดงออกเพิ่มขึ้นของมิวซิน MUC1 และมีการสร้างขึ้นใหม่ของมิวซิน MUC5AC ในเนื้อเยื่อมะเร็งท่อน้ำดี การแสดงออกของมิวซิน MUC1 มีความสัมพันธ์กับการลุกลามของเซลล์มะเร็งผ่านทางหลอดเลือด และสามารถใช้เป็นตัวบ่งชี้การพยากรณ์โรคที่ไม่ดีในผู้ป่วยมะเร็งท่อน้ำดีได้ ในขณะที่การแสดงออกของมิวซิน MUC5AC มีความสัมพันธ์อย่างมีนัยสำคัญกับการลุกลามของเซลล์มะเร็งผ่านทางปมประสาท สามารถตรวจพบมิวซินชนิด MUC5AC ได้ในซีรัมผู้ป่วยมะเร็งท่อน้ำดีซึ่งให้ค่าความจำเพาะ และค่าทำนายผลบวกในการวินิจฉัยมะเร็งท่อน้ำดี 96.86% และ 92.56% ตามลำดับ การตรวจพบมิวซิน MUC5AC ในซีรัมมีความสัมพันธ์อย่างมีนัยสำคัญกับการแสดงออกของ MUC5AC ในเนื้อเยื่อมะเร็งและระยะท้ายของมะเร็ง ซึ่งบ่งชี้ว่า MUC5AC ที่ตรวจพบในซีรัมมีต้นกำเนิดมาจากก้อนมะเร็งท่อน้ำดีและเป็นตัวพยากรณ์โรคในผู้ป่วยมะเร็งท่อน้ำดีได้ การศึกษามิวซิน MUC5AC ในซีรัมพบรูปแบบของการเคลื่อนที่ในอิเล็กโทรโฟเรซิส 2 แบบที่ต่างจากแบบปกติ ซึ่งเกิดจากความแตกต่างในขนาด ประจุ ความหนาแน่น และหมู่น้ำตาลที่เป็นองค์ประกอบ

สรุปและวิจารณ์ผลการทดลอง การศึกษานี้พบว่ามะเร็งท่อน้ำดีมีความผิดปกติในการสร้างมิวซิน MUC1 และ MUC5AC ทั้งในเชิงปริมาณและคุณภาพ การตรวจพบมิวซิน MUC5AC ในซีรัมสามารถใช้เป็นตัวตรวจชีวภาพเพื่อวินิจฉัยการเป็นมะเร็งท่อน้ำดีได้ด้วยความจำเพาะและการทำนายผลบวกสูงถึง 96.86% และ 92.56% ตามลำดับ และสามารถใช้เป็นตัวพยากรณ์โรคในผู้ป่วยมะเร็งท่อน้ำดีได้

ข้อเสนอแนะสำหรับงานวิจัยในอนาคต พัฒนาการตรวจมิวซิน MUC5AC ในซีรัม ให้ง่าย สะดวก และสามารถปฏิบัติได้ในห้องปฏิบัติการในสถานพยาบาลเพื่อวินิจฉัยและพยากรณ์โรคมะเร็งท่อน้ำดี

คำหลัก: mucin, MUC1, MUC5AC, cholangiocarcinoma, tumor marker

Abstract

Project Code: BRG/06/2544

Project Title: Characterization of mucin expressed in liver fluke associated cholangiocarcinoma

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Project Period: 3 years

The present study was aimed to investigate the expression profiles of mucins :MUC1, MUC2, MUC5AC and MUC6 in cholangiocarcinoma (CCA) tissues, and its clinical significance, and investigate the biological properties of CCA-associated MUC5AC mucin.

Using immunohistochemical approach, MUC1 and MUC5AC apomucins were found to be overexpressed and neoexpressed in CCA tissues and were considered to be CCA-associated mucins. High expression of MUC1 apomucin was correlated with vascular invasion and was an independent predictor for poor prognosis of the patients, whereas the expression of MUC5AC apomucin was related to neural invasion.

MUC5AC mucin could be detected in serum of CCA patients using agarose electrophoresis and immunoblotting. The assay provided a high specificity and positive predictive values of 96.86% and 92.56% for diagnosis of CCA, respectively. The presence of serum MUC5AC (sMUC5AC) mucin was significantly associated with the expression level of MUC5AC apomucin in CCA tissues and the advanced stage of the tumor suggesting that sMUC5AC mucin was originated from CCA mass. Serum MUC5AC mucin also provided an independent prognostic potential for patient with CCA.

Two aberrant electrophoretic patterns of sMUC5AC mucin from CCA patients were identified. Physico-biochemical characterization demonstrated that they were different in their charge, size, density and sugar moieties.

This study concluded that MUC1 and MUC5AC were CCA-associated mucins, of which MUC5AC mucin could be detected in serum of CCA patients with high specificity. sMUC5AC mucin provided the diagnostic and prognostic values for patients with CCA.

Future direction: Research for an easy, chief, and friendly detection method of serum MUC5AC for diagnosis and prognosis of CCA in routine laboratory should be developed.

Keywords: mucin, MUC1, MUC5AC, cholangiocarcinoma, tumor marker

Output

1. Publications

Major publications

1. Wongkham S, Sheehan JK, Boonla C, Patrakitkomjorn S, Howard M, Kirkham S, Sripa B, Wongkham C, Bhudhisawasdi V. 2003. Serum MUC5AC mucin as a potential marker for cholangiocarcinoma. Cancer Lett 195(1):93-9.
2. Boonla C, Wongkham S, Sheehan JK, Wongkham C, Bhudhisawasdi V, Tepsiri N, Pairojkul C. 2003. Prognostic value of serum MUC5AC mucin in patients with cholangiocarcinoma. Cancer 98(7):1438-43.
3. Boonla C, Sripa B, Thuwajit P, Cha-on U, Bhudhisawasdi V, Miwa M, Sheehan JK, Wongkham S. Mucin expression and prognostic factor of intrahepatic cholangiocarcinoma after hepatic resection. (manuscript submitted to Virchows Archives)
4. Boonla C, Wongkham C, Sheehan JK, Wongkham S. Alteration in glycan of serum MUC5AC mucin in cholangiocarcinoma (manuscript in preparation)

Minor publication

1. Wongkham S, Boonla C, Kongkham S, Wongkham C, Bhudhisawasdi V, Sripa B. 2001. Serum total sialic acid in cholangiocarcinoma patients: an ROC curve analysis. Clin Biochem 34(7):537-41.

2. Knowledge and collaborations

The outcome of this project which has high impact to Thai society and patients with cholangiocarcinoma as a whole is the intervention for detection of serum MUC5AC which can be used as a specific tumor marker and poor prognostic marker for this cancer.

The project also created a good collaboration between Dr. Nalinee Prempracha, Faculty of Medicine, Chiang Mai University and our research team to develop monoclonal antibodies for detection MUC5AC mucin in serum

3. Young researcher built up

One new Ph.D. researcher, Mr. Chanchai Boonla was graduated under this project with the support from RGJ-Ph.D. program

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List of Abbreviations

ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CA19-9	Carbohydrate antigen 19-9
CEA	Carcinoembryonic antigen
CHAPS	3-[(3-Cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate
CsCl	Caecium chloride
DTT	Dithiothreitol
DW	Distilled water
GuHCl	Guanidinium hydrochloride
GuRB	Guanidinium hydrochloride reduction buffer
IAA	Iodoacetamide
°C	Degree Celsius
PBS	Phosphate buffer saline
U/L	Unit per liter
x g	Relative centrifugal force
α	Alpha
β	Beta
γ	Gamma
μ	Micro, micron
μg	Micro-gram
μl	Micro-liter
(v/v)	Volume per volume
(w/v)	Weight per volume

I. INTRODUCTION

1.1 Background and Rationale

Cholangiocarcinoma (CCA), malignancy of bile duct epithelia, is a major cancer in northeast of Thailand and still a challenge public health problem of the region. Animal studies (Thamavit et al., 1978, Thamavit et al., 1987, Thamavit et al., 1993, Thamavit et al., 1994, Flavell, 1981, Flavell and Lucas, 1983, Ohshima et al., 1994; Ohshima and Bartsch, 1994) and epidemiological evidences (Elkins et al., 1990, Haswell-Elkins et al., 1994, Elkins et al., 1996) support the association of liver fluke, *Opisthorchis viverrini*, infection and the development of cancer.

CCA is a slow-growing tumor which frequently is diagnosed when the tumor is big enough to obstruct the biliary tract and produces signs and symptoms. Most of the patients, thus, are diagnosed at the late stage of tumor and their survivals are poor due to the originated as well as disseminated tumors. At present, there is no effective tool or specific biomarkers that can indicate the early stage and status of the CCA. A specific marker for either early detection or monitoring of the tumor may significantly improve patients' prognosis and therapeutic management.

Mucins are heavily O-glycosylated proteins, which are mainly produced by epithelial cells lining ducts and glands. To date, nineteen human mucin genes have been identified and designated. Mucin is classified into two groups, membrane-associated and secreted mucins. The role of mucins is documented to be cytoprotection and lubrication, which is accomplished by its high viscoelasticity, high density and highly hydrodynamic volume. The alternative role, molecular sensor, signaling and immune modulation have also been proposed for membrane-associated mucins (Hollingsworth and Swanson, 2004). Basically, mucin is expressed as cell- or tissue-specific manner, for instances, MUC2 and MUC3 in bowel (Chang et al., 1994), MUC5AC and MUC6 in gastric tissue (Buisine et al., 2000a; Buisine et al., 2000b), MUC5B and MUC7 in saliva (Wickstrom et al., 2000) and MUC1, MUC4, MUC13, MUC15, MUC16 and MUC17 in conjunctival epithelia (Corrales et al., 2003b; Corrales et al., 2003c).

Vast production of mucus is frequently found in various carcinomas. The alterations in quantity and quality of mucins have been demonstrated in cancer tissues, including CCA (Pereira et al., 2001, O'Connell et al., 1998, Lee and Liu, 2001, Kim, 1998; Kim et al., 1996; Kim et al., 1999, Kim et al., 2002a, Jeannon et al., 2001, Jass and Walsh, 2001). Neoexpressed and overexpressed mucins have been proposed to be of clinical values as a maker for supportive diagnosis, prognosis or monitoring therapy (Victorzon et al., 1996, McGuckin et al., 1995b, Lee et al., 2001, Kawamoto et al., 2001, Higashi et al., 1999, Ajioka et al., 1996). In addition, aberrant glycan moiety of mucin has also been documented and suggested for the clinical utility.

In normal biliary epithelia, MUC1 are widely expressed along intrahepatic biliary tree whereas MUC3 and MUC6 are constitutively expressed in large bile ducts and peribiliary glands, respectively (Sasaki et al., 1996, Sasaki et al., 1998b). A weak expression of MUC5AC and MUC2 has been documented in bile duct epithelia (Vandenhaute et al., 1997). In CCA, MUC1, MUC3 and MUC6 are overproduced whereas MUC5AC is neoexpressed. Moreover, cryptic mucin carbohydrate epitopes such as T, Tn and STn antigens are increasingly emerged in transformed cells (Itzkowitz et al., 1991, Yamashita et al., 1993, Terada and Nakanuma, 1996, Sasaki et al., 1999). These alterations have been used as markers for either diagnosis or prognosis in CCA patients. For instance, MUC1 has been demonstrated to be related to poor prognosis while MUC2 has been related to favorable outcome (Higashi et al., 1999).

Up to now, there is no report focused on mucin producing in CCA, especially in OV-associated CCA in Thai patients, which is different in etiology, biology and carcinogenesis regarding to the previous reports of Japanese and western cases. Identification and characterization of mucins expressed in CCA of Thai patients will provide basic knowledge and information that may be applied for clinical diagnosis, prognosis or effective treatment in the future.

1.2 Research Objectives

1. To identify mucins -namely MUC1, MUC2, MUC5AC and MUC6- expressed in carcinoma tissues using immunohistochemical techniques.

2. To verify the possibility of using serum MUC5AC mucin as a CCA-associated marker.
3. To characterize and compare the sugar composition of the partially purified MUC5AC mucins from serum of CCA patients.

1.3 Research Design

Expression of mucins in CCA tissues was determined by immunohistochemistry using antibodies to mucins: MUC1, MUC2, MUC5AC and MUC6. Paraffin embedded tissues from histologically proved CCA were used in this study. The mucins with neo-expressed or over-expressed in CCA tissues were considered as the CCA-associated mucins and further analyzed for clinical significance.

The agarose gel electrophoresis and immunoblotting were used to detect mucin in serum. The potential role of serum mucin as tumor marker of CCA was explored. Sera from patients with Opisthorchiasis, benign biliary diseases, various gastrointestinal cancers and healthy persons were recruited as control.

Alteration in glycosylation of mucins was verified from the electrophoretic patterns of mucin on agarose gel electrophoresis. Each mucin was partially purified by CL-2B gel filtration and characterized for charge, size and density. The sugar moieties were determined by HPAEC-PAD, lectin blotting and desialylation experiment. Finally, carbohydrate moiety of each MUC5AC pattern was proposed.

1.4 Anticipated Outcomes

1. Profiles of mucin expressed in CCA tissues will be obtained from immunohistochemical study, of which CCA-associated mucin(s) will be defined.
2. The CCA-associated mucin may provide a potentially clinical usefulness in the patients suffering with CCA.
3. The CCA-associated mucin detected in sera may be used as supportive diagnosis or prognosis of CCA if its clinical value is determined.
4. The difference of MUC5AC forms could deduce their molecular structures and explain how cancer perturbed mucin biosynthesis.

II. LITERATURE REVIEWS

2.1 Cholangiocarcinoma

2.1.1 Definition and Classification

The term "cholangiocarcinoma" is defined as carcinoma originating anywhere in the biliary tree excluded the gallbladder and ampulla of Vater (Uttaravichien and Buddhisawasdi, 1990, Uttaravichien et al., 1999). CCA can be classified into three broad groups; intrahepatic, perihilar or central and distal extrahepatic tumors (Figure2-1) (Nakeeb et al., 1996, de Groen et al., 1999). Intrahepatic tumors are defined as those confined to the liver that do not involve the extrahepatic biliary tree, do not present with obstructive jaundice, and have no evidence of primary tumor elsewhere. Perihilar or central or Klatskin's tumors are defined as those involving or requiring resection of the hepatic duct bifurcation (Becker et al., 2003). Distal CCAs are those involving the distal extrahepatic or intrapancreatic portion of the bile duct. The incidence of each CCA type in Thai cases has been reviewed: 82.3% of distal extrahepatic, 11.3% of perihilar and 6.7% of intrahepatic (Wiwanitkit, 2003).

All CCA, however, are quite similar in the inherent characteristics e.g. adenoma and mucin-producing tumor (Kokubo et al., 1988, Sheung-To and Gibson, 1970, Sasaki et al., 1998a, Bhudhisawasdi, 1997, Nagakura et al., 1999). The cause, symptom, diagnosis, prognosis and treatment has been recently reviewed (Dubaniewicz, 2003).

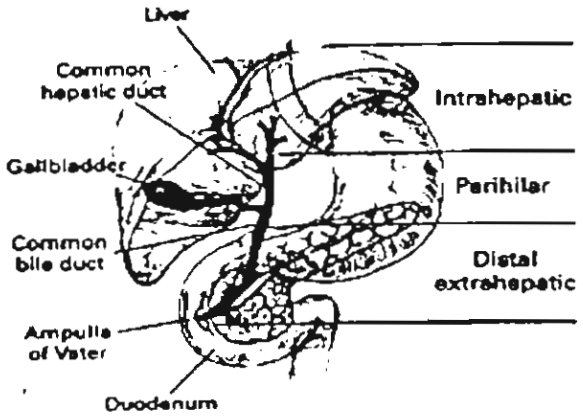


Figure 2-1 Anatomically classification of CCA. CCA is classified into 3 groups: intrahapatic, perihilar or central and distal extrahapatic CCA (de Groen et al., 1999).

2.1.2 Epidemiology and Etiology

CCA is a relatively uncommon cancer with an annual incidence of about 2 cases per 100,000 in Western countries. In contrast to Southeast Asia, CCA is frequently accounted, especially in northeast of Thailand, where the liver fluke, *Opisthorchis viverrini* (OV), is hyperendemic. Approximately, 70% of the population in this region was infected with the liver fluke and the incidence of CCA was believed to be at least 50 times that of Western countries (Uttaravichien et al., 1996, Kullavanijaya et al., 1999). In Khon Kaen province, for instance, 89% of liver cancer was CCA and the age-standardized incidence rate of CCA was estimated at 84.6 and 36.8 per 100,000 male and female, respectively (Vatanasapt et al., 1990, Sithithaworn et al., 1994). Recently, prevalence of OV infection and the factors influencing transmission (such as season and raw freshwater fish dishes) from intermediate host to man were reviewed (Sithithaworn and Haswell-Elkins, 2003).

Risk factors for CCA of Asian cases were obviously different from those of the Western in both epidemiological and experimental studies. In Western countries, primary sclerosing cholangitis (PSC) was the most strongly predisposing factor, which had a relative risk (RR) of 10-30 to develop CCA as compared with the general population (Rosen et al., 1991, de Groen, 2000). In addition, gallstone (Rabeneck, 1994, Holzinger et al., 1999), thorotrast exposure (Holzinger et al., 1999, Rubel and Ishak, 1982, Sahani et al., 2003), hepatolithiasis (Chen et al., 1989, Kubo et al., 1995), autoimmune diseases with primary biliary cirrhosis and chronic ulcerative colitis (Akwari et al., 1975) have been shown to be risk factors of CCA.

In Asia, liver fluke, OV, infection is a major risk in Thai, Laos and Malaysian, while *Chonorchis Sinensis* infection is prominent in Japanese, Korean and Vietnamese (Sithithaworn et al., 1994, de Groen et al., 1999, Kullavanijaya et al., 1999). The infection is acquired by eating raw or undercooked cryprinoid fish contaminated with encysted metacercariae of the parasites. The metacercariae excysts in the duodenum and migrate into intrahepatic bile duct via the ampulla of Vater. The juvenile worms travel along the biliary tree and attach themselves with their suckers to the bile duct epithelium. The adult worms live mainly in the intrahepatic bile ducts and less frequently in gallbladder and pancreatic ducts. These worms are believed to survive about 10 years and the maximum lifespan may be over 25 years (Vatanasapt et al., 1999).

Liver flukes chronically habitat in the biliary tree leading to chronic inflammation and bile duct proliferation and dysplasia, eventually, CCA is developed. Liver fluke *per se*, however, is not sufficient to cause cancer (Thamavit et al., 1978, Chaimuangraj et al., 2003). Potent carcinogens such as nitrosamine compounds, both exogenous and endogenous sources (Satarug et al., 1998; Satarug et al., 1996), are believed to enhance the carcinogenic effect of the flukes (Thamavit et al., 1993).

In the northeast of Thailand, a high frequency of CCA associated with heavy OV infection has been reported (Elkins et al., 1990). Gallbladder disturbances, together with chronic infection and fibrosis of the bile ducts may enhance the susceptibility to CCA among people, especially heavily OV infected males (Elkins et al., 1996). In animal studies, a relationship between heavy OV infection and humoral immune response has been demonstrated. Specific antibodies against somatic and OV egg antigens were suppressed in the heavily and chronically infected hamsters (Sripa and Kaewkes, 2000). Suppression of immunity in the patients with chronic opisthorchiasis may prone them to develop CCA. The actual mechanism, however, remains to be investigated.

2.1.3 Carcinogenesis

Multi-stage cascade of transformation has been proposed for the molecular mechanism of biliary carcinogenesis. Chronic inflammation of biliary cells caused by liver fluke infection accommodates nitric oxide (NO) and other oxygen radicals in infected and inflamed tissues (Ohshima et al., 1994; Ohshima and Bartsch, 1994). The studies on NO demonstrated that the activation of inducible NOS (iNOS) and excess NO production in response to inflammatory cytokines cause oxidative DNA damage and inactivation of DNA repair proteins (Jaiswal et al., 2000, Ohshima et al., 1994). In addition, the inflammatory cytokines can initiate several processes such as modulation of gene expression, alteration of detoxification gene expression and activation of carcinogen metabolism, and finally enhancing the effect of DNA adduct (Pinlaor et al., 2004; Pinlaor et al., 2003).

Several lines of evidence suggests a putative mechanism of CCA development as a four stages cascade, shown in Figure 2-2 (Holzinger et al., 1999, Watanapa and Watanapa, 2002). It is suggested that CCA arises from a precancerous lesion which follows the "hyperplasia-dysplasia-neoplasia" sequence.

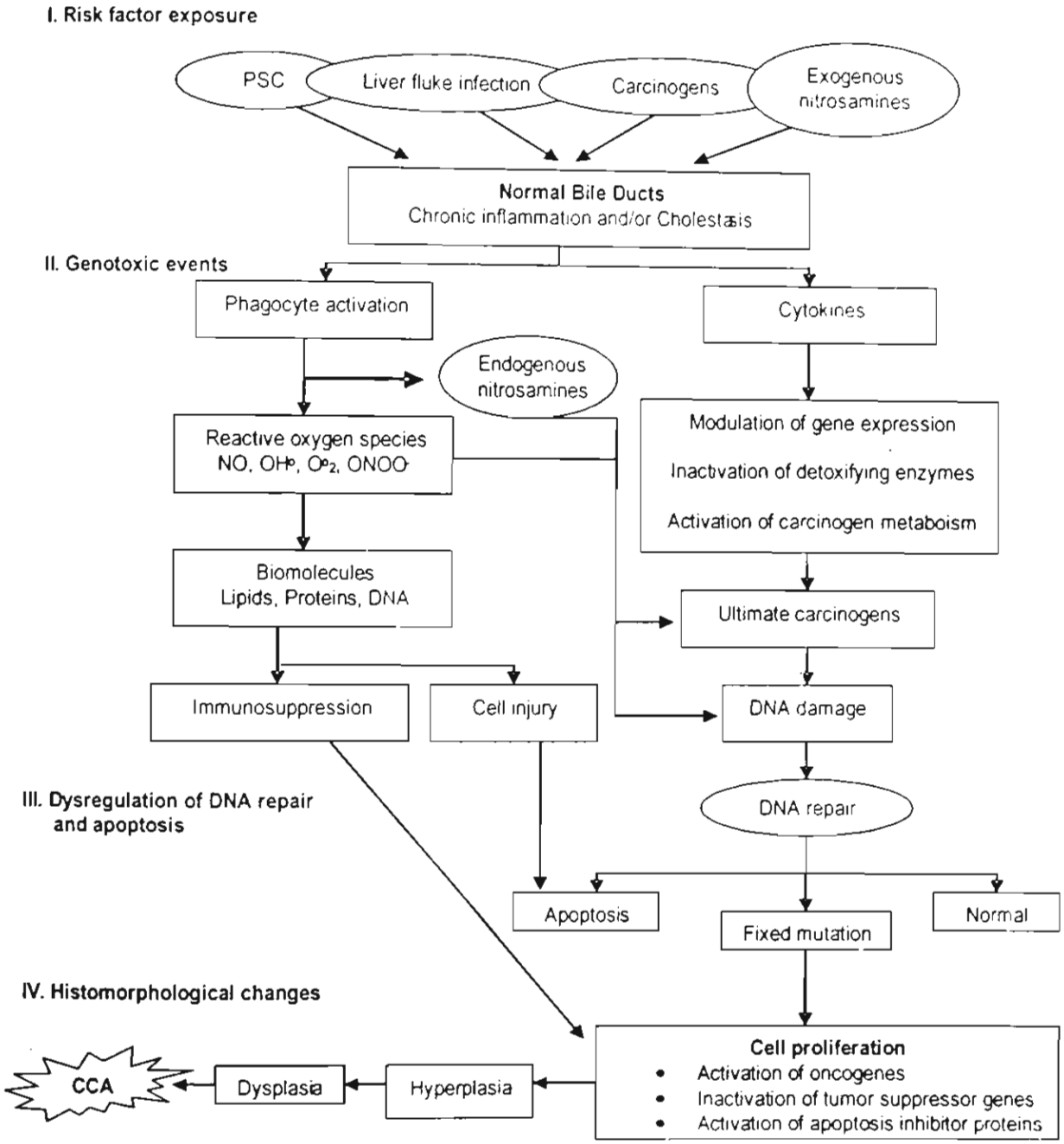


Figure 2-2 The possible mechanism of CCA carcinogenesis. The proposed mechanism is a minor modification from Holzinger et al., 1999 and Watanapa and Watanapa, 2002.

2.1.4 Diagnostic Approaches for CCA

A combination of investigations is used for diagnosis of CCA, e.g. imaging tools, cytological techniques and serological markers. Tissue markers have been used for differential diagnosis and indication of progression. However, most of the investigations are valid when the patients are late advanced stage, resulting in poor prognosis and ineffective management.

2.1.4.1 Imaging approaches

Basically, ultrasonography, computer tomography (CT) and magnetic resonance imaging (MRI) have been used in the clinical diagnosis for tumor mass detection (Tio et al., 1991, Sato et al., 1998, de Groen et al., 1999, Boberg and Schrumpf, 2004). The combination of three-dimensional CT and cine-cholangiography was suggested as the accurate assessment tool of the biliary system in the patients, which is helpful for planning the surgical schedule (Furukawa et al., 2000). All described imaging tools, however, are occasionally negative for the malignancy. A new imaging technology, positron emission tomography (PET) that uses ^{18}F-fluoro-2-deoxy-D-glucose (^{18}F-FDG) is a promising technique in identifying small bile duct cancer (de Groen et al., 1999, Gores, 2000, Torok and Gores, 2001, Kim et al., 2003).

2.1.4.2 Cytological approaches

Detection of tumor cells by fine needle aspiration, bile cytology, endobiliary brush cytology and endoscopic transpapillary biopsy has been reported (Rabinovitz et al., 1990). The overall sensitivity of cytological techniques was 72% with high positive predictive value of 98% (Desa et al., 1991). Recently, a new and less-invasive approach, endosonography-guided fine-needle aspiration (EUS-FNA) has been suggested for the preoperative diagnosis of hilar cholangiocarcinoma, which could pick up the patients with negative brush cytology (Fritscher-Ravens et al., 2004).

2.1.4.3 Immunohistological markers

Immunohistological markers, e.g., α-fetoprotein (AFP), carcinoembryonic antigen (CEA), α-1-antitrypsin, and erythropoiesis-associated antigen have been reported as differential diagnostic markers of CCA from hepatocellular carcinoma (HCC) (Ganjei et al., 1988). Tissue CA19-9 and CA50 antigens are normal constituents of the bile ducts. More than 80% of CCA tissues expressed this antigen where as it was absent in HCC (Haglund et

al., 1991). A new marker, S100A6 (calcyclin) was up-regulated in intrahepatic CCA tissues (Kim et al., 2002b). Detection of telomerase activity combined with p53 overexpression were also suggested for the diagnosis of biliary tract cancers (Itoi et al., 2000).

As summarized in Table 2-1, several markers have been reported to indicate the progression of CCA.

2.1.4.4 Serum markers

Serum markers, especially CA19-9, CEA and CA125 have been suggested for CCA diagnosis (Pungpak et al., 1991). CA19-9 with cut-off point of 100 U/ml was suggested to be a useful diagnosis of CCA without PSC (Patel et al., 2000, Rumalla and Petersen, 2000) whereas the cut-off of 180 U/ml was suggested for CCA with PSC, with specificity of 97.7% (Siqueira et al., 2002). The specific patterns of elevation of CA19-9 and CEA were also observed in liver metastases and CCA (Torzilli et al., 2002). However, several studies indicated the ubiquity of these markers in gastrointestinal tract cancers. The elevations of CA19-9 and CEA were affected by the presence of inflammation (Carpelan-Holmstrom et al., 2002, Hulterantz et al., 1999, Dufek et al., 1994, Pasanen et al., 1993b). In order to improve the accuracy of diagnosis, therefore, using the combination markers of CA19-9, CEA and CA72-4 has been suggested.

A number of serum biomarkers associated with CCA has been continuously reported: CA242 and CA50 (Hulterantz et al., 1999), tissue polypeptide antigen (TPA) (Pasanen et al., 1993a), biliary glycoprotein (BGP or TS135 Ag or CD66a) (Kondo et al., 2001), CYFRA 21-1 (Uenishi et al., 2003), RCAS1, (Watanabe et al., 2003), serum total sialic acid (TSA) (Wongkham et al., 2003; Wongkham et al., 2001, Kongtawelert et al., 2003) and Biliary alkaline phosphatase (BALP) (Bhudhisawasdi et al., 2003).

In addition to serum markers, bile markers were also assessed for CCA diagnosis. Less than 70% of sensitivity of biliary CEA, CA19-9 and CA125 were obtained with specificities of 75.5%, 33.3% and 60%, respectively (Chen et al., 2002). The biliary CA125 was shown to be less affected by inflammation than CEA and CA19-9. The elevated levels of DUPAN-2 and CA195 in bile were reported in all stages of CCA (Dufek et al., 1994).

Table 2-1 Markers related to carcinogenesis and prognosis of CCA

Marker	expression	association	References
G protein gamma 7 (G-γ 7)	Suppression	progressive intrahepatic CCA	Utsunomiya et al., 2002
ERBB-2 receptor protein	Overexpression	early carcinogenic	Endo et al., 2002
cyclooxygenase 2	Overexpression	early carcinogenic	Endo et al., 2002
Fas/Fas ligand (Fas L)	expression	tumor progression	Shimonishi et al., 2000, Ito et al., 2000
oncoprotein MDM2	up-regulated	late stage intrahepatic CCA	Horie et al., 2000
Tp53	overexpression	advanced stage	Horie et al., 2000
cyclin-dependent kinase inhibitor (p27)	Low expression	poor prognosis	Fiorentino et al., 2001
c-Met proto-oncogene product	overexpression	favorable prognosis	Aishima et al., 2002b
c-erbB-2 protein	overexpression	tumor progression	Aishima et al., 2002b
keratin 903	reduction	favor prognosis in mass-forming-type intrahepatic CCA	Aishima et al., 2002a
tenascin, an extracellular matrix (ECM)	expression	poor prognosis in intrahepatic CCA	Aishima et al., 2003
syndecan-1	reduced expression	worse outcome in intrahepatic CCA	Harada et al., 2003

At present, there is no known specific tool or marker for CCA, particularly in an early stage cases (Han et al., 2001, Torok and Gores, 2001, Rumalla and Petersen, 2000). Therefore, a combination tests from different approaches e.g., clinical finding, imaging and tumor markers are required for identification of CCA.

2.2 Mucin

Mucin is a major constituent of mucus, coated the surface of cells lining ducts and glands. The role of protecting the epithelial cells from infection, dehydration and either physical or chemical injury as well as aiding the passage of materials through a tract are known as classical functions of the mucin. Additionally, communicating, signaling and immune modulating roles are also proposed (Gendler and Spicer, 1995, Deplancke and Gaskins, 2001, Chen et al., 2003a, Hollingsworth and Swanson, 2004).

Mucin, a large complex glycoprotein constitutively produced by epithelia, composes of protein backbone structure (apomucin or core protein) and carbohydrate side chains or glycan portion (Figure 2-3). The apomucin contains amino acid tandem repeat or variable number of tandem repeat (VNTR), which abundances for Ser/Thr and also Pro residues. VNTR region is a major site for O-glycosylation. The glycans are heavily linked via O-glycosidic bonds through Ser/Thr residues presented in the repeat domain and the first attached sugar usually is N-acetylgalactosamine (GalNAc). On a basis of mass, approximately 40-80% of mucin molecule consists of O-linked mucin carbohydrates and the length of the mucin carbohydrate chain may vary from one to more than 20 residues. The heterogeneity of mucin glycans has been considered in terms of the length, sequence of glycans as well as the configuration of glycosidic bond (Kim and Gum, 1995).

A repeat sequence in apomucin presents at both nucleotide and amino acid levels which are precisely maintained. The polymorphisms have been shown to be due to either the difference in number of repeated unit (VNTR polymorphism) or the alternative splicing of mucin genes (Ligtenberg et al., 1990, Debailleul et al., 1998, Van Klinken et al., 1997, Choudhury et al., 2000). The repeat unit of each mucin is unique, however they have a similar feature of a high percentage of Ser/Thr residues harboring for the O-glycosylation.

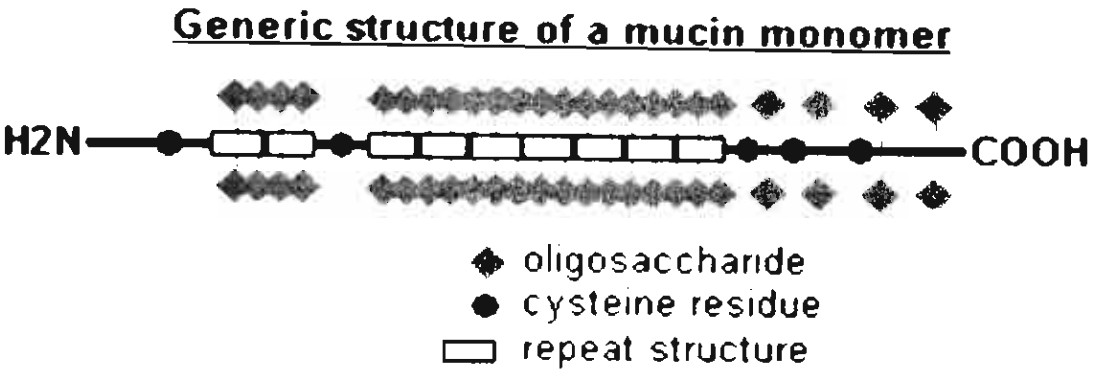


Figure 2-3 The generic structure of mucin. Mucin composes of two main parts, apomucin and oligosaccharide portions. Tandem repeat or VNTR (yellow blocks) is a common feature of apomucin and the VNTR domain abundances with Ser/Thr and Pro residues which are responsible for a heavy O-glycosylation (pink).

2.2.1 Nomenclature and Classification

A number of mucin types has been isolated and identified. The relationships between the deduced polypeptide sequences of the mucins presently assigned to the *MUC* gene family by the Human Genome Organization Gene Nomenclature Committee (HUGO/GNC; http://www.hugo-international.org/hugo/). In humans, 19 mucin genes thus far have been characterized according to nucleotide sequence and designated MUC1, MUC2, MUC3A, MUC3B, MUC4, MUC5AC, MUC5B, MUC6, MUC7, MUC8, MUC9, MUC11, MUC12, MUC13, MUC15, MUC16, MUC17, MUC19 and MUC20 (Bobek et al., 1993, D'Cruz et al., 1996, Lapensee et al., 1997, Williams et al., 1999, Yin and Lloyd, 2001, Yin et al., 2002, Pallesen et al., 2002, Gum et al., 2002, Chen et al., 2003b, Higuchi et al., 2004). In addition, two mouse mucin genes, Muc10 and Muc14 have been isolated and characterized (Denny et al., 1996, Melnick et al., 2001, GenBank accession number NM_016885). These mouse mucins however have not been documented in human.

MUC3 mucin was firstly isolated from intestinal tissue and the second gene of MUC3 was demonstrated and designated as MUC3B therefore the ordinary MUC3 gene was renamed to MUC3A (Pratt et al., 2000). However, they showed more than 94% identity in nucleotide sequence. For MUC5 mucin, three cDNA were firstly cloned from human tracheobronchial

mucosa: MUC5A, MUC5B and MUC5C as recommended by the Human Gene Mapping Nomenclature Committee. Analysis of CpG islands by pulse-field gel electrophoresis verified later that MUC5A and MUC5C are part of the same gene (so called MUC5AC) which is distinct from MUC5B (Guyonnet Duperat et al., 1995).

According to the location of apomucins, they are classified into 2 groups, membrane-associated (MUC1, MUC3A, MUC3B, MUC4, MUC11, MUC12, MUC13, MUC15, MUC16 and MUC17) and secreted mucins (MUC2, MUC5AC, MUC5B, MUC6, MUC7 and MUC19) (Figure 2-4). MUC8, MUC9 and MUC20 have not been clearly classified and explored. The membrane-associated mucins have a hydrophobic membrane-spanning domain and locate across plasma membrane and most of them contain epidermal growth factor (EGF)-like domain. The secreted mucins are also called gel-forming mucins due to their gel-forming property generating a mucus layer coated on cell lining; however MUC7 mucin does not possess this characteristic. The gel-forming type mucin are major constituents of the cell surface mucus acting as biological blanket to protect the cells from harmful attack. They typically form the extremely large oligomers through the linkage of mucin monomers via disulfide bonds.

The chromosomal location and the tandem repeat peptide sequence of each mucin gene are shown in Table 2-2.

2.2.2 Molecular Structure

Mucin composes of apomucin- a protein backbone and mucin glycan. The molecular nature of mucins were complicated by their biophysical properties: relatively large mass (well over 10^6 Daltons), a complex biochemical composition (50-80% O-linked oligosaccharides) and a tendency to form higher-order structure through polymerization (Carlstedt et al., 1985, Carlstedt and Sheehan, 1989, Chace et al., 1989). Mucin typically is a linear flexible molecule (length from 200 nm to beyond 10 μm) due to massive glycans attached and possesses a viscoelastic gel structure (Sellers and Allen, 1989, Sheehan et al., 1991).

2.2.2.1 Apomucin

Apomucin is a core protein backbone of mucin molecule. The VNTR domain, a Ser/Thr-rich domain, is a7 region of where O-glycosylation is taken place. N-glycans are usually found in non-VNTR region and required for mucin sorting and dimerization (Bell et al., 2003, Ho et al., 2003). Several specific mucin domains presented in apomucin have been identified and investigated for their functions. The schematic comparison of specific mucin domains is presented in Figure 2-5.

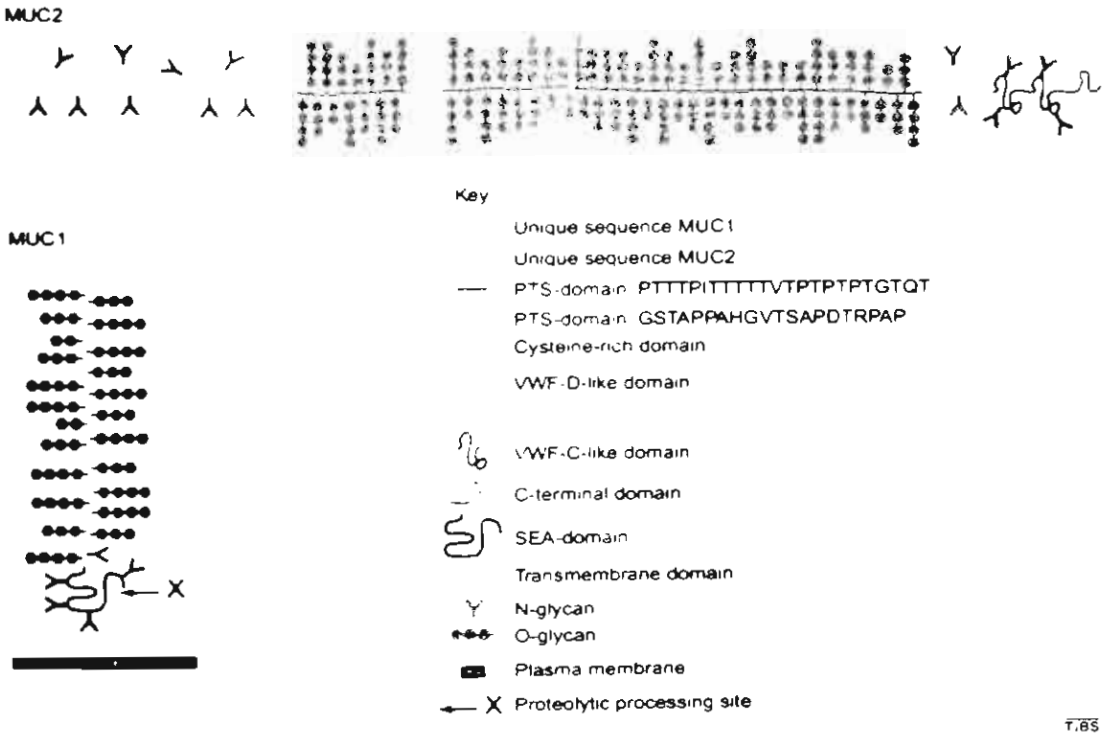


Figure 2-4 Membrane associated and secreted mucins. MUC1 represents a membrane associated mucin with a transmembrane domain. MUC2 represents a secreted mucin with Cysteine-rich domain and VWF-like domain. PTS domain; proline, threonine and/or serine-rich domain, SEA; sea-urchin-spermprotein–enterokinase–agrin and VWF; von-Willebrand-factor, (Dekker et al., 2002).

Table 2-2 Chromosomal location and sequence of tandem repeat of the mucin genes

Mucin Types	Chromosomal location	Tandem repeat peptide sequences (No. of amino acid)
MUC1	1q21	PDTRPAPGSTAPPAHGVTSA (20) (Gendler et al., 1987; Gendler et al., 1991)
MUC2	11p15.5	PTTTPITTTTTVTPTPTPTPTGTQT (23) (Griffiths et al., 1990)
MUC3A and MUC3B*	7q22	HSTPSFTSSITTTETTS (17) (Pratt et al., 2000)
MUC4	3q29	TSSASTGHATPLPVTD (16) (Porchet et al., 1991, Gross et al., 1992)
MUC5AC	11q15.5	TTSTTSAP (8) (Guyonnet Duperat et al., 1995)
MUC5B	11q15.5	SSTPGAHTLTVTTTATTPTATGSTTATP (27) (Porchet et al., 1995)
MUC6	11q15.5	SPFSSTGPMTATSFQTTTTYPTPSHPQTTLPTHVPPFS TSLVTPSTGTVITPTHAQMATSASIHSTPTGTIPPPTTL KATGSTHTAPPMTPTTSGYSQAHSSTSTAAKTSTSLH SHTSSTHHPEVTPTSTTTITPNPTSTGTSTPVAHTTSAT SSRLPTPFTTHSPPTGS (169) (Toribara et al., 1993, Pigny et al., 1996)
MUC7	4q13	TTAAPPTPSATTPAPPSSSAPG (23) (Bobek et al., 1993)
MUC8	12q24.3	TSCPRPLQEGTPGS (14) (Shankar et al., 1997)
MUC9	1p13	15 amino acid residues repeat unit (Arias et al., 1994, Lapensee et al., 1997)
Muc10	ND	A mouse mucin called MucCAM (Melnick et al., 2001)

Table 2-2 Chromosomal location and sequence of tandem repeat of the mucin genes (Cont.)

Mucin Types	Chromosomal location	Tandem repeat peptide sequences (No. of amino acid)
MUC11	7q22	SGLSEESTTSHSSPGSTHTTLSPASTTT (28) (Williams et al., 1999)
MUC12	7q22	SGLSQESTTFHSSPGSTETTSSPASTTT (28) (Williams et al., 1999)
MUC13	3q13.3	151 amino acid residues repeat unit (Williams et al., 2001)
Muc14	ND	A mouse mucin GenBank accession number NM_016885
MUC15	11p14.3	Lack of repetitive segment (Pallesen et al., 2002)
MUC16	19p13.3	165 amino acid residues repeat unit (Yin and Lloyd, 2001)
MUC17	7q22	contains an extended repetitive extracellular glycosylation domain (Gum et al., 2002)
MUC19	12q12	Contains many mucin-like threonine/serine-rich repeats (Chen et al., 2003b)
MUC20	3q29	Contains a mucin tandem repeat of 19 amino acids (Higuchi et al., 2004)

*; The tandem repeat domain has the same amino acid consensus sequence but MUC3B shows more substitutions.
ND; no data

Membrane Associated Mucin

Membrane-bound mucins basically contain a hydrophobic membrane-spanning domain that serves to anchor the mucin to the membrane and mostly contain epidermal growth factor (EGF)-like domain which is believed to mediate interaction between mucin subunits and EGF receptors such as erbB (Carraway et al., 2000, Li et al., 2003). EGF-like domain - erbB interaction is likely to play a role in regulation or signaling that is related to growth, mobility, differentiation, inflammation or other higher-order functions (Jepson et al., 2002). In addition, a common domain found in membrane mucin is sea-urchin-spermprotein–enterokinase–agrin (SEA) domain in which a conserved proteolytic cleavage site is located (Figure 2-6). Membrane mucins exist as a heterodimer with large rod-like mucin domain held together with the cytoplasmic membrane by putative noncovalent interaction which could allow for rapid dissociation of mucin portion from the cell-tethering domain.

The extracellular domain of human MUC1 extends 200-500 nm above the plasma membrane, beyond the predicted glycocalyx of about 10 nm (Bramwell et al., 1986). A calculated molecular mass of MUC1 apomucin was of about 120 kDa. The transmembrane (TM) and cytoplasmic domain of MUC1 are highly conserved (88% identical) suggesting important functional roles (Spicer et al., 1995). The cytoplasmic tail (CT) of MUC1 contains several signal motifs such as tyrosine phosphorylation sites (Zrihan-Licht et al., 1994, Pandey et al., 1995, Schroeder et al., 2001), beta-catenin binding site (Yamamoto et al., 1997), potential docking sites for SH2-containing proteins (Spicer et al., 1995) and binding site for Grb2/SOS signal protein (Pandey et al., 1995) as well as the actin cytoskeleton protein and erbB receptors (Gendler and Spicer, 1995, Schroeder et al., 2001

MUC3A and MUC3B have a similar C-terminal domain and intron/exon structure as well as possess the same tandem repeat, however slight difference has been found in unique exonic and intronic sequences (Pratt et al., 2000). MUC4 is the largest membrane-associated mucin and presents a GlyAspProHis proteolytic site where the large precursor is cleaved into two subunits, MUC4α and MUC4β (Moniaux et al., 1999). MUC4 has a very large variation in number of tandem repeats (7-19 kb) but encodes only one cysteine residue, making MUC4 different from the mucin genes belonging to the 11p15.5 family (Nollet et al., 1998).

Secreted Mucin

Secreted mucins or gel-forming mucins, vary greatly in size from as few as 322 residues to 13,288 residues. Most of them, except MUC7, own the gel-forming property which creates the mucus layer acting as a physical barrier to shield the cells. Carbohydrates in many secreted mucins show structural microheterogeneity and most mucins have negative charge sugars, either sialic acids or O-sulfosaccharides. The secreted mucin has typical domains which do not contain in membranous mucins. The additional major domains found only in secreted type mucins include N-terminal disulfide-rich D-domain (D1, D2, D', D3) and C-terminal disulfide-rich/CK-domain (cysteine-rich/cysteine-knot domain). Several evidences demonstrated that Cysteine-rich/cysteine-knot domain and D domains played a critical role in mucin dimerization and multimerization, respectively (Asker et al., 1995, Toribara et al., 1997, Asker et al., 1998a, Asker et al., 1998b, Perez-Vilar et al., 1998, Bell et al., 1998, Perez-Vilar and Hill, 1998, van Klinken et al., 1998). Figure 2-6 represents the domains of secreted mucins which show sequence identity and possibly similar function among such mucins. The mucins moreover was documented a homology to von Willebrand factor (vWF) suggested the relationship of gene evolution (Desseyn et al., 2000).

Basically, tandem repeat or VNTR domain exists as a continuous domain. In contrast the repeat domain of MUC2, MUC5AC and MUC5B are interrupted with cysteine-rich domains suggesting the close evolutional relationship. The repeat unit in some mucins has identical sequences whereas in others the repeat sequence is consensus. The lack of secondary structures (extended structure) in the repeat regions and their flanking domains suggests that these domains serve as a scaffold for O-linked oligosaccharides. Light scattering and electron microscopy suggest that these glycosylated domains are semi-rigid-extended structure (Sheehan et al., 1986, Roussel et al., 1988, Thornton et al., 1991, Sheehan et al., 1991, Sheehan et al., 1999, Perez-Vilar and Hill, 1999, Sheehan and Thornton, 2000, Round et al., 2002).

All of the half Cys in the D1-, D2-, D3- and C-terminal disulfide domain (CK-domains) are thought to form either inter and intrachain disulfide bonds that are involved in multimerization of mucins (Sheehan and Gum, 1995, Sheehan et al., 1995, Lagow et al., 1999, Round et al., 2002). The C-terminal disulfide-rich/CK-domain, like the D-domains they are

predicted to have globular structures with α-helices and pleated sheets and few or no free thiols. The cysteine rich in the CK-domain is provided to form interchain disulfide bonds between the polypeptide chains of mucins.

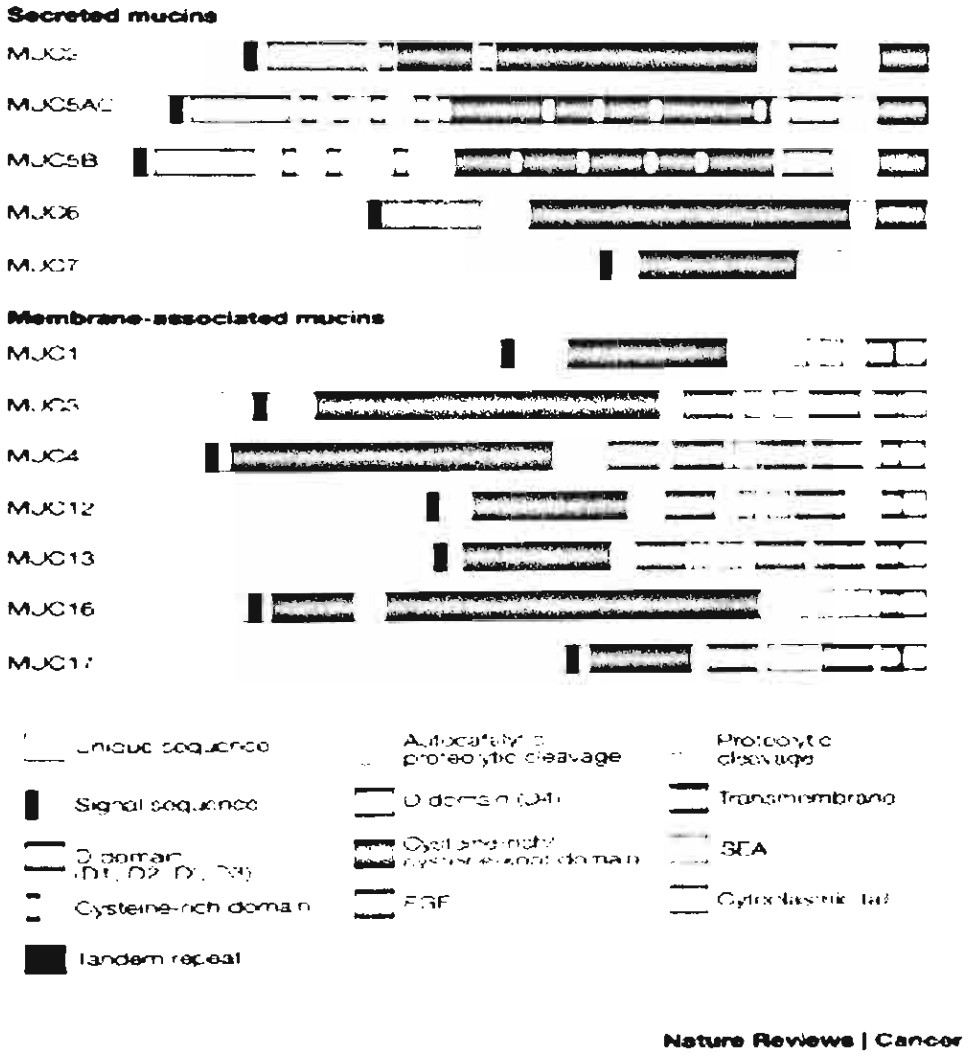


Figure 2-5 The schematic comparison of specific mucin domains. (Hollingsworth and Swanson, 2004)

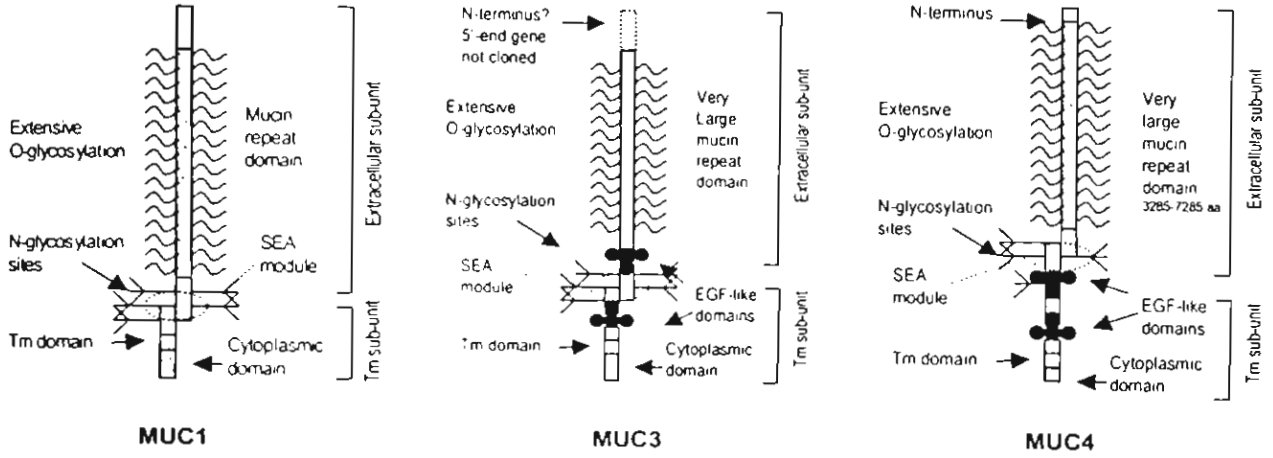


Figure 2.6 The structure of membrane-associated mucins, MUC1, MUC3 and MUC4. Membrane-associated mucins exist as a heterodimer, an extracellular domain (Extracellular subunit) held together with a membrane-bound portion (Tm subunit) with non-covalent interaction. The common domains found in membrane type mucins include tandem repeat domain, transmembrane (Tm) region, cytoplasmic tail and SEA module. SEA domain contains the proteolytic cleavage site which has been suggested a role in shading process of membrane mucin. The EGF-like domain is not found in MUC1 mucin.

In addition, there are extra domains found specifically in some mucins such as- a B-domain with sequence identity to those in von Willebrand factor is found in several mucins, cysteine-rich domains other than the D- and CK-domain are noted in a few mucins and P-domain or trefoil domain, a three-leaved structure like those in the trefoil factor family is found in some frog mucins (Sands and Podolsky, 1996; Thim, 1997; Wong et al., 1999).

Human mucin gene MUC5AC is clustered with MUC2, MUC5B and MUC6 on chromosome 11p15.5. It has a large central tandem repeat array composed of four tandem repeat domains (TR1-TR4). The 5'-region reveals high degree of sequence similarity with MUC2 and MUC5B and codes for 1,336 amino acids organized into a signal peptide, four pro-von Willebrand factor-like D domains (D1, D2, D' and D3) and a short domain which connects to the central repetitive region (Escande et al., 2001).

2.2.2.2 Mucin Carbohydrate

Beyond the core protein, bulky oligosaccharides are attached to apomucin. Most of them are O-glycans that are covalently linked via O-glycosidic interactions. The O-glycosylation is a process that monomeric carbohydrate moieties are chemically linked to the hydroxyl side group of Ser and Thr residues of proteins. O-glycans can be further divided into multiple subgroups depending on the nature of the amino acid residue and sugar group involved in the carbohydrate-protein linkage: i) in mucin-type O-glycoproteins N-acetylgalactosamine (GalNAc) is linked to serine or threonine; ii) for intracellular glycoproteins N-acetylglucosamine (GlcNAc) is linked to serine or threonine and iii) for proteoglycans xylose is linked to serine or threonine. The O-glycosylation process is enzymatically catalyzed by glycosyltransferases, which act sequentially on specific substrates.

Six types of monosaccharide are commonly found in mucin O-glycans of mucins including co-structure sugars, GalNAc, galactose (Gal), GlcNAc and glucose (Glc), and terminal sugars, N-acetylneuraminic acid (NANA, Sialic acid) and fucose (Fuc). Mucin-type O-glycosylation is initiated by addition of GalNAc residue to Ser or Thr residues, followed by sequential addition of sugar residues. The process is taken place in ER. N-glycosylation is suggested to be a critical former step to signal the nascent apomucin entering O-glycosylation process (Axelsson et al., 1998, Perez-Vilar and Hill, 1999) and required for the surface localization (Ho et al., 2003).

Sequential stepwise glycosylation by specific glycosyltransferases leads to formation of at least eight core structures (Figure 2-7). After formation of these cores they are capable of being elongated to oligosaccharides. The process is completed by capping of terminal sugars, fucose and sialic acid. Additional modification by sulfate adding is frequently found in mucin glycans. The sulfation process is an early event taking place at the stage of mucin subunit assembly and is required for mucin polymer formation. It is also suggested that disturbances in mucin sulfation process could be detrimental to the maintenance of mucus integrity (Liau et al., 1991; Liau et al., 1992). In addition, mucin sulfation has been shown to be important in: creating ligands for selectins and contributes to other ligand-receptor interaction and creating a substantial acidic property of the mucins (Lo-Guidice et al., 1994, Liau et al., 1991; Liau et al., 1992, Mendicino and Sangadala, 1999, Dekker et al., 1989).

O-glycosylation with complex oligosaccharides is vital to mucin structure and function. Mucin glycan determinants are involved in specific ligand-receptor interaction (McDermott et al., 2001, Nilius et al., 1994, Fukuda, 2002, Linden et al., 2002, Van de Bovenkamp et al., 2003, Rump et al., 2004) and confer hygroscopic properties (Carlstedt et al., 1985). The variety of sugar types, diversity of glycosidic and anomeric linkages and the extensive branching of oligosaccharides together with the non-template glycan biosynthesis lead to the heterogeneity of mucin carbohydrate. The biosynthesis of glycan depends not only on the types of sugar substrates but also on the glycosyltransferase enzymes available in the cells. Therefore, heterogeneities of glycan of different stages, cell types, individuals and species are revealed (Ho et al., 1993, Beum et al., 1999). These heterogeneities implicate many important biological functions of cells such as receptor (for growth factors, hormones, toxins as well as bacteria and virus lectins), growth regulation, cellular differentiation, cell-cell interaction, cell-substratum interaction and various immunological functions (Kim, 1998, Taylor-Papadimitriou and Finn, 1997).

2.2.3 Biosynthesis and Assembly of Secreted Mucins

It is well know that the molecular weight of secreted mucins is reduced in the presence of reducing agents such as DTT, β-2-mercaptoethanol indicating that the interchain disulfide bonds maintain in multimeric state. The multimerization is accomplished by disulfide linked between monomers through their cystein-rich domains (Dekker and Strous, 1990, Sheehan and Gum, 1995, Van Klinken et al., 1995). Two directional interaction, head to head and tail to tail due to disulfide-linkage via the D-domains (D1, D2, D' and D3) and CK-domains, respectively. Dimerization of MUC2 has been shown to occur in endoplasmic recticulum (ER) via disulfide bonds after nescent apomucins are N-glycosylated (N-glycosylation dependent dimerization) and transferred to golgi apparatus, where multimerization and O-glycosylation are completed (Asker et al., 1998a; Asker et al., 1998b; Asker et al., 1995 Figure 2-8 shows fate of secreted mucin biosynthesis and oligomerization using a study of porcine submaxillary mucin (PSM) as a model- the mucin polymer is accomplished by the dimmer assembly (Perez-Vilar et al., 1998; Perez-Vilar and Hill, 1998; Perez-Vilar and Hill, 1999). The gel-forming mucins could be expressed in the same cell without the formation of heterodimer.

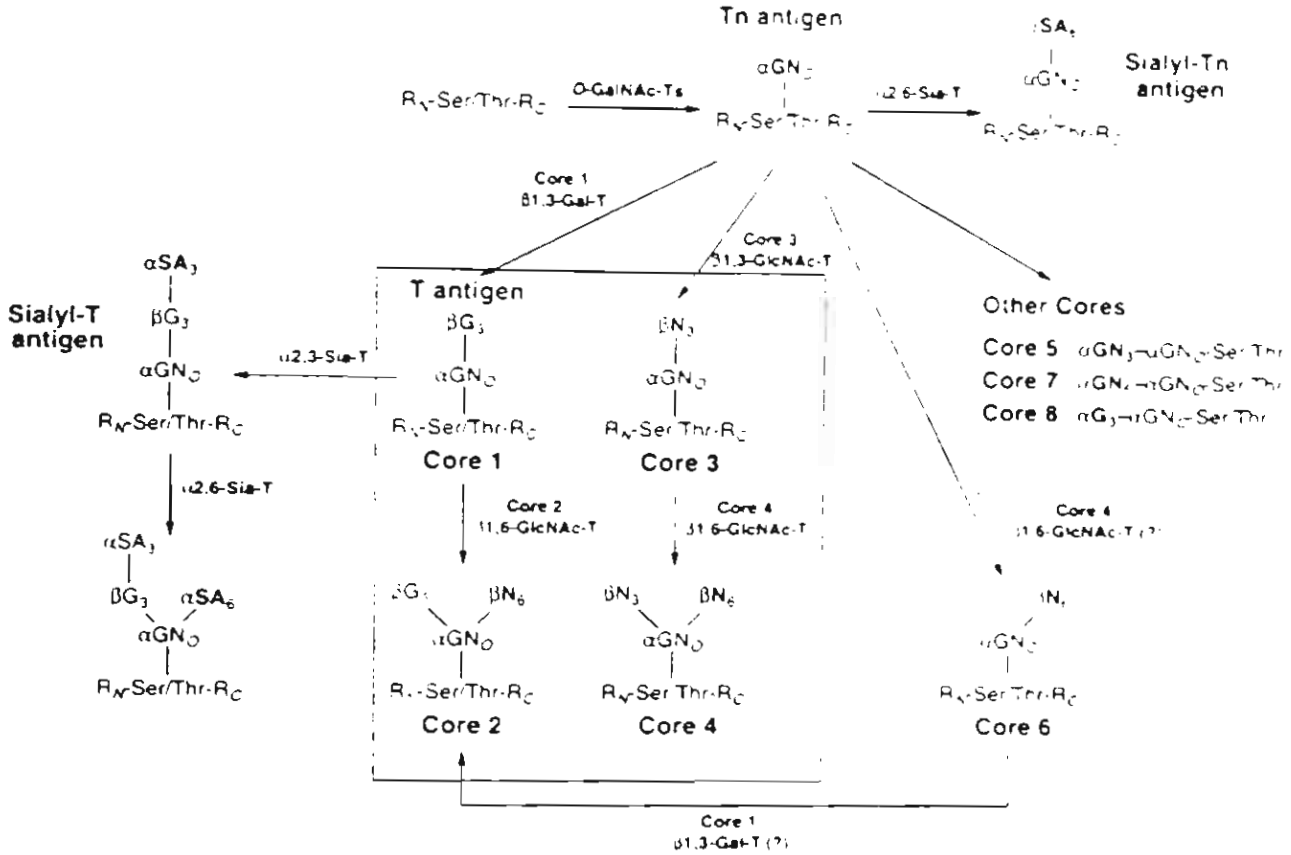


Figure 2-7 Roadmap of biosynthesis of mucin O-linked core structure. G: Galactose, GN: N-acetylgalactosamine, N: N-acetylglucosamine; SA: Sialic acid; RN and RC represent extension of apomucin in N- and C-terminal direction, respectively (Bill et al., 1998).

In summary, based on sequence homology, the ability of MUC2, MUC 5AC, MUC5B and MUC6 to dimerize most likely resides in their C-terminal domains, particularly CK domain and the RER-localized dimerization of gel-forming mucins likely proceeds by similar mechanisms, which is an essential step in the formation of the mucus gels.

2.2.4 Expression and Physiological Functions

Mucin is basically expressed in a tissue- or cell- specific fashion. The differential distribution is likely to reflect the functional difference (De Bolos et al., 1995, Ho et al., 1995, Reid and Harris, 1998).

MUC1 is expressed on the apical cell surface of almost epithelial cells that line ducts and glands (Nakamori et al., 1994, Osako et al., 1993, Sasaki and Nakanuma, 1996a; Sasaki and Nakanuma, 1996b, McGuckin et al., 1995a). MUC2 and MUC3 mucins are detected in different cell types of the intestine in which MUC2 is confined to the villi of goblet cells and crypts of Lieberkuhn, whereas MUC3 is expressed by absorptive cells and also goblet cells (Chang et al., 1994). Transcripts of MUC1, MUC2, MUC4, MUC5AC, MUC7, MUC13, MUC15, MUC16 and MUC17 were presented in normal conjunctival epithelium (Corrales et al., 2003a; Corrales et al., 2003b; Corrales et al., 2003c), whereas MUC1, MUC2, MUC4, MUC5AC, MUC5B, MUC6, and MUC7 mRNAs were reported in healthy human lacrimal sacs and nasolacrimal ducts (Paulsen et al., 2003).

In respiratory tract, MUC5AC and MUC5B are major mucin constitutively produced by airway epithelia (Groneberg et al., 2002; Groneberg et al., 2003). In intrahepatic biliary tree, MUC3 is constitutively expressed in the large bile ducts and peribiliary glands, while MUC2 is rarely expressed. In gallbladder, high amount of MUC3 and MUC5B but almost no MUC2 is observed. MUC1 is a major apomucin found in normal pancreas with heterogenous of MUC3 (Osako et al., 1993, Balague et al., 1994). MUC4 is broadly expressed in the small intestine, colon, esophagus, cervix, and lung (Gendler and Spicer, 1995, Arul et al., 2000).

In gastric tissue, MUC5AC and MUC6 but not MUC2, MUC3, and MUC4 are highly expressed in the stomach. MUC5AC and MUC6, so called "gastric mucin", are localized to distinct cell types. MUC5AC expression is restricted to surface mucus cells, whereas MUC6 expression is seen only in the neck of mucus cells. MUC5B is expressed mainly in the bronchus glands, submaxillary glands, endocervix, and gallbladder (Desseyn et al., 1997, Carrato et al., 1994) while it is weakly expressed or undetectable in pancreas (Nakamori et al., 1994, Balague et al., 1995). MUC7 seems to be very specifically expressed in the salivary glands. This mucin can not be detected in uterus, ovary, stomach as well as intestine (Van Klinken et al., 1995, Reid and Harris, 1998).

Mucins, particularly gel-forming mucins, are expressed to form a gel matrix (acts as a slime blanket) covering the cell sheet which contribute to physiochemical protection of the epithelial cell surface from harmful conditions. The volume and composition of the gel matrix would be determined by the types of mucin produced, the nature of their post-translational

modifications, the degree of intramolecular and intermolecular crosslinking and the types of molecules that were captured by the gel matrix. Beyond the layer of membrane-associated mucins and other cell surface molecules, a secreted mucin layer is consecutively coated and they might be linked together through ligand-receptor interactions.

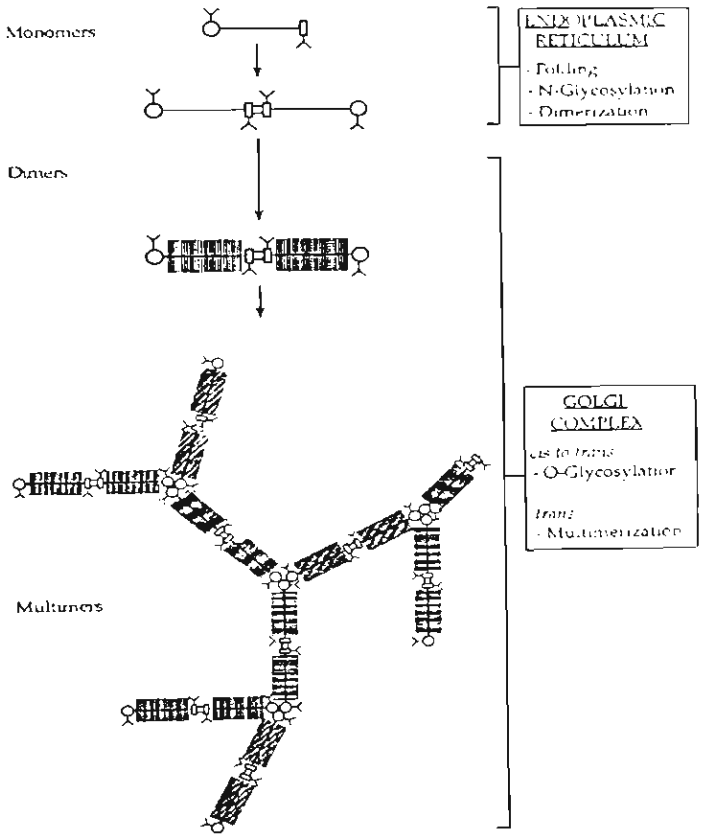


Figure 2-8 Schematic represents mucin dimerization and polymerization of porcine submaxillary mucin. After N-glycosylation nascent apomucins are dimerized as tail to tail direction via the formation of inter-disulfide bonds between cysteine residues in CK domains, which occurred in RER. The mucin dimer is further transported to golgi complex appararus where the O-glycosylation is completed. The multimerization of mucins is accomplished in *trans*-golgi complex by the formation of disulfide bonds between mucin dimers via cysteine residues in D domains (Perez-Vilar and Hill, 1999).

Outer mucin layer provides a physical or steric barrier against microorganisms and hostile materials and it is predicted to configure and maintain the local molecular environment with regard to hydration, ionic composition and concentration as well as the accessibility of macromolecules. It is likely that mucin gel plays a role in molecular discrimination by regulating the afflux and efflux of specific molecules. The molecular discrimination might be undertaken by tandem repeat moieties on apomucins, which, through their stoichiometric power, allows for the establishment of locally high concentration of neutral and charged oligosaccharide structures. Stoichiometric effect might provide a function like ion-exchange or gel-filtration machinery that can facilitate or inhibit the molecular diffusion thereby the molecular sieving role of gel matrix is speculated (Hollingsworth and Swanson, 2004).

Complex mucin gel has been demonstrated to capture and hold biologically active molecules. Molecules that are bound to mucus layer might function as indicator of molecular or physical breach of the mucin matrix and when released, might incite inflammatory, repair or healing processes. Trefoil factors (TFFs) have been reported a close relation and also cooperative interaction with mucins (Kindon et al., 1995, Langer et al., 2001). TFFs comprise part of the mucus gel and protect the cells by contributing to mucus viscosity (Tomasetto et al., 2000, Wiede et al., 2001, Thim et al., 2002). Following their release, TFFs were demonstrated to promote wound healing and mucosal restitution at damaged site (Tran et al., 1999, Taupin et al., 1999).

Roles of binding and sequestering of mucin gel to cytokines, growth factors, differentiation factors and inflammatory mediators have been proposed. It has been shown that IL-1, IL-4, IL-6 and IL-7 have specific lectin activities that allowed them to bind to oligosaccharides presented on mucins (Cebo et al., 2001). Several cytokines detected in mucus gels, IL-1, TNF-α, IL-4, IL-6, IL-9 and IL-13 have been shown to mediate inflammatory responses in epithelial tissues such as lung and gastrointestinal tract, and eventually caused mucus hypersecretion and increased production of specific apomucins (Reader et al., 2003, Dabbagh et al., 1999, Song et al., 2003, Kibe et al., 2003, Enss et al., 2000).

Table 2-3 Differential expression of mucins in epithelial tissues

Tissues	Mucin Types										
	MUC1	MUC2	MUC3	MUC4	MUC5AC	MUC5B	MUC6	MUC7	MUC8	MUC11	MUC12
Salivary gland	+	-	-	++	-	++	-	++	ND	ND	ND
Esophagus	+	-	-	ND	-	+	-	ND	ND	ND	ND
Stomach	+	-	+	+	++	+	++	-	ND	ND	ND
Small intestine	+	++	++	+	±	+	±	-	-	+	-
Colon	+	++	-	-	±	+	±	-	ND	++	++
Pancreas	++	±	+	-	-	++	-	ND	ND	+	+
Gallbladder	+	±	++	ND	+	+	++	ND	ND	ND	ND
Respiratory	+	+	-	++	+	++	±	ND	++	ND	ND
Breast	+	-	-	ND	-	-	±	ND	ND	ND	ND
Uterus	+	-	-	ND	-	+	-	-	±	+	±
Cervix	+	±	-	++	+	++	+	ND	++	ND	ND
Ovary	-	-	-	ND	ND	ND	-	-	±	ND	ND
Prostate	+	±	±	ND	ND	ND	ND	ND	ND	+	±

-: Negative, ±: Trace positive, +: Positive, ++: Strongly positive and ND: No data

The involvement in signal transduction events has been lighted up for membrane-associated mucins. It is thought that membrane mucins may provide signals to epithelial cells in response to alterations in mucin layer or local molecular environment. These signals might response to changes in conformation or ligand status of their extracellular domains. Signals might be transmitted to indicate that a normal status exists at the cell surface, for example, the cell has achieved normal differentiation morphology, or a normal secretory function is intact. Therefore, mucins may serve as an outside-to-inside signal that alters the proliferation,

differentiation or cell-adhesion status of epithelial cells (Yamamoto et al., 1997, Hollingsworth and Swanson, 2004).

2.2.5 Mucin and Disease

It has been found that the mucins obtained from different tissues bared the different oligosaccharide structure, and was altered in pathological conditions. The alterations of mucin biosynthesis affecting the protein core and/or the carbohydrate content have been observed in various pathological situations, for examples, chronic inflammation diseases (Rose, 1992, Tytgat et al., 1996, Longman et al., 2000) and cancers (Carrato et al., 1994, Kim and Gum, 1995, Kim, 1998). Modification of sugar moieties has been demonstrated to involve in several biological processes such as adhesive property, immune modulation and metastatic potential of cancer cells (Porowska et al., 2004, Brockhausen, 2003, Ciborowski and Finn, 2002, McDermott et al., 2001, Hanisch, 2001). From this picture, studies of mucin have been extended to several diseases for several purposes such as: to understand the biological behavior of the disease, to find the mucin marker for either diagnosis or prognosis and to determine the roles of mucin in cancers and other diseases.

The alterations of mucin reveal both quantitative and qualitative changes, respectively. Changing of apomucin expression between normal and disease condition can be divided into three categories (Kim, 1998).

1. An overexpression or up-regulation of apomucins, which caused by an increase of the gene regulation (Gaemers et al., 2001).

2. A decreased expression or depletion of apomucins, which caused by decrease transcription of the mucin genes e.g., MUC2 and MUC3 in colon cancer (Chang et al., 1994), MUC5AC and MUC6 in gastric carcinoma (Reis et al., 1997).

3. A neoexpression of apomucins, which caused by inappropriate or ectopic expression of mucin genes due to dysregulation (Buisine et al., 1996, Retz et al., 1998).

Incomplete and aberrant glycosylation are frequently created during pathogenesis. Mucin carbohydrate antigens, Tn and T (core 1) antigens are incomplete glycosylation, whereas sialyl form, STn and ST antigens are considered as aberrant glycosylation (Figure 2-7). Additionally, the abnormal capping processes (sialylation, fucosylation and sulfation) of mucins have been documented in pathological situation (Parker et al., 1995, Delmotte et al.,

2001, Shori et al., 2001, Davril et al., 1999, Mendicino and Sangadala, 1999). Sialylated-MUC1 mucin was frequently found in invasive carcinomas (Yonezawa and Sato, 1997) and metastatic colon cancer cells suggesting that sialylated glycan structures on mucin may play a role in adhesive interactions which involved both basement membrane and endothelial-associated ligands (Bresalier et al., 1996).

2.2.5.1 Mucin in Cancers

Neoplastic transformation of epithelial cells is commonly associated with alteration in the synthesis (overexpression, decrease expression or neoexpression) and abnormal glycosylation of mucins. The abnormal glycosylation directly affects the mucin structure as well as composition of mucin carbohydrates.

In general, the tandem repeats of mucins are heavily glycosylated with many glycan chains per molecules. During neoplastic transformation, the tandem repeats are more sparsely glycosylated and glycan chain may be much shorter and/or modified in the outer region. These changes may be due to alteration of carbohydrate metabolism, or to changes in glycosyltransferases expression, or in O-acetylation of sialic acids, or to alteration of mucin processing in cancer cells. Thus, the modified structures, exposed inner sugar core structure, or protein backbone moiety may involve in various biological properties of cancer (Kim, 1998). The expressions of mucins in cancer are summarized in Table 2-4.

2.2.5.2 Mucin in CCA

In biliary tract, mucins are produced by both bile duct epithelial cells and peribiliary gland cell linings. In the fetal liver, new bile ducts in the portal tracts, at the hilar level or peripheral level, frequently expressed MUC1 apomucin at their luminal surface (Sasaki et al., 1995). By contrast, in the postnatal liver, the biliary epithelial cells of intrahepatic large bile ducts constantly expressed MUC3 apomucin, whereas those of small bile ducts did not. The data suggested that the biliary epithelial cells switch MUC1 apomucin expression before birth to that of MUC3 after birth. MUC1 is focally expressed in small and large bile ducts, whereas MUC6 apomucin is principally expressed in large bile ducts. MUC2 and MUC5/6 apomucins were absent in the intrahepatic biliary elements of the fetal as well as postnatal livers. Apomucin expression in intrahepatic biliary tree during transformation is shown in Table 2-5.

Apomucin expression in CCA is related to type and histological grading of the tumor (Table 2-6). Peripheral type CCA and combined hepatocellular–cholangiocarcinoma (HC-CC) had similar apomucin pattern with low expressions of MUC2 and MUC3. This implied that cancer may arise from small bile ducts or other elements. Interestingly, MUC1 apomucin can be found in almost all of the cancer cases and this indicates the important role of MUC1 in carcinogenesis of CCA (Sasaki and Nakanuma, 1994, Sasaki et al., 1996).

Table 2-4 Apomucin expression in organ-site cancers

Tissues	Normal		Cancer		
	Apomucin	Mucin carbohydrate	Apomucin	Apomucin neoexpression	Mucin carbohydrate
Stomach	MUC1 MUC5AC MUC6	ND	MUC1 ↓ MUC5AC ↓ MUC6 ↓	MUC2 MUC3 MUC4	ND
Pancreas	MUC1, MUC3 MUC5B	Tn Ag T Ag	MUC1 ↓ MUC3 ↑ MUC5B ↑	MUC2 MUC4 MUC5AC	Tn Ag ↑ T Ag ↑ STn Ag ↑
Colon	MUC1 MUC2 MUC3 MUC4 MUC5B MUC5AC	ND	MUC1 ↑ MUC2 ↓ MUC3 ↓ MUC5B ↑ MUC5AC ↑	MUC5AC (in RVA)	S-Le^a ↑ S-Le^x ↑ STn Ag ↑
Bladder	MUC1 MUC2	ND	MUC1 ↑ MUC2 ↑	MUC7	ND

↓ : Decrease of expression

↑ : Overexpression

ND: No data, Ag: Antigen, RVA: Rectosigmoid Villous Adenoma

Table 2-5 Apomucin expressed in biliary tree under normal and pathological situations

Biliary tree	Normal	Chronic inflammation		Carcinoma	
		Overexpression	Neoexpression	Overexpression	Neoexpression
Large bile ducts	MUC1 MUC3 MUC5AC rare MUC6	MUC1 MUC3 MUC6	MUC2 MUC5AC	MUC1 MUC3 MUC6	MUC2 MUC5AC
Small bile ducts	MUC1 focally	MUC1	MUC2 MUC5AC	MUC1	MUC2 MUC5AC
Peribiliary glands	MUC1 MUC2 rare MUC3 MUC5AC rare MUC6 mostly	MUC1 MUC3 MUC6	MUC2 MUC5AC	MUC1 MUC3 MUC6	MUC2 MUC5AC

Table 2-6 Apomucin expression in CCA and hepatocellular cholangiocarcinoma

Cases	n	Percentage (%) of positive cases			
		MUC1	MUC2	MUC3	MUC5/6
CCA					
- Hilar type	19	100	21	68	79
- Peripheral type	10	100	10	10	50
- Well-diff.	19	95	21	53	89
- Poorly-diff.	12	100	8	42	42
Combined HC-CC	14	93	7	7	21

diff.: Differentiated type

HC-CC: Hepatocellular cholangiocarcinoma

Expression of apomucin in CCA patients with and without cirrhosis has been investigated (Sasaki et al., 1998a). As shown in Table 2-7, MUC3 apomucin was not detected in CCA patients with cirrhosis, suggesting that tumor cells are not originated from the large bile duct epitheliums. The neoexpression of MUC5AC apomucin is clearly shown in CCA without cirrhosis. Furthermore, patients with cirrhosis-presented CCA and with combined HC-CC have similar apomucin profiles indicating that they have the same histogenesis.

Expression of mucin is also related to survival of CCA patients. Patients with high expression of MUC1 or sialylated MUC1 had a shorter survival period than those with negative, in contrast, MUC2 positive patients had a longer survival time than those with MUC2 negative (Higashi et al., 1999). Thus, MUC1 and MUC2 apomucins respectively are reflected to poor and favorable prognosis, however, the biological roles responsible for this phenomenon is remained to be investigated.

Alteration in glycosylation of mucins has been described in various cancers. Non-cancerous large bile ducts are completely absent of STn antigens whereas Tn and T antigens can be found at low percentage (Table 2-8) (Yamashita et al., 1993). Number of positive cases of Tn and T antigens were gradually increased during malignant transformation. These carbohydrate antigens therefore seem to be good markers of intrahepatic bile duct tumor, however, the T antigen is likely less useful than Tn antigen because of it is more frequently found in normal bile ducts. Increase expression of STn antigen in intrahepatic-CCA is obvious (Table 2-7). STn antigen was more frequently expressed in CCA without cirrhosis compared with the other two groups (Sasaki et al., 1999). This data reflects the inherent biological features of these tumors, such as ability of invasion and metastasis.

2.2.6 Mucin expression and Clinical Relationship

Based on the fact that mucin was aberrantly produced in pathological conditions, changes of mucin expression and its molecular structure would provide a clinical linkage. Therefore, various studies have been focused on the clinical significance or a potential of mucin molecule in particular diseases.

Table 2-7 Apomucin expressed in CCA associated with/without cirrhosis

Cases	n	Percentage (%) of positive cases			
		MUC3	MUC5AC	MUC6	MUC7
CCA with cirrhosis	4	0	25	100	75
CCA without cirrhosis	24	67	63	92	63
• Hilar type	17	88	70	94	59
• Peripheral type	7	14	43	86	71
Combined HC-CC	16	6	6	81	88

Table 2-8 Expression of mucin carbohydrate antigens in CCA and non-CCA cases

Tissues	Percentage (%) of positive cases		
	Tn Ag.	STn Ag.	T Ag.
Noncancerous large bile duct[a]	10	0	30
Atypical bile duct epithelium of hepatolithiasis[a]	50	13	24
Atypical bile duct epithelium around intrahepatic bile duct carcinoma[a]	41	12	53
Intrahepatic bile duct carcinoma[a]	92	88	92
CCA without cirrhosis [b]	95	89	51
CCA with cirrhosis [b]	75	25	0
Combined HC-CC [b]	12	0	6

a = Yamashita et al., 1993

b = Sasaki et al., 1999

MUC1 is a well known marker for breast cancer (Murray et al., 1995, Bon et al., 1999, Cheung et al., 2000, Norum et al., 2001, Croce et al., 2003). On the other side, MUC1 is paid attention on to be a target antigen for immunotherapy in several cancers such as breast, pancreas and ovary (Apostolopoulos and McKenzie, 1994, Apostolopoulos et al., 1996, Apostolopoulos et al., 1999, Taylor-Papadimitriou et al., 1999, Hu and Xing, 2003). A vaccine therapy using MUC1 as immunogenic antigen has been undertaken in several lines of clinical trials (Mitchell, 2002, Pecher et al., 2002, Kontani et al., 2003). Humoral and cellular immune responses against MUC1 have been detected in cancer patients and the circulating anti-MUC1-IgG antibody levels has been found to relate to survival of pancreatic cancer patients (Hamanaka et al., 2003). The expression of MUC1 apomucin in renal cell carcinoma is associated with tumor progression (Leroy et al., 2002). In gastric carcinoma, MUC1 mucin expression is significantly associated with poorer outcome (Lee et al., 2001, Matsukita et al., 2003, Akyurek et al., 2002). However, more recent study found the inverted result- patients with gastric carcinoma who maintained high immunoreactivity for anti-MUC1 antibody had a better prognosis (Wang and Fang, 2003).

MUC2 does not appear to be a useful marker for recognizing pancreatic ductal carcinoma whereas MUC1, which is overexpressed in pancreatic ductal carcinoma can be used as an ancillary marker for diagnosing pancreatic ductal carcinoma in cytologic preparations (Chhieng et al., 2003). The expression of MUC2 inversely related with the tumor progression factors and poor outcome in extrahepatic bile duct carcinomas has been reported (Tamada et al., 2002). The overexpression of MUC2 has been suggested to be a reliable molecular marker for pseudomyxoma peritonei (O'Connell et al., 2002) while the reduction of MUC2 expression may relate to malignant transformation of colorectal neoplasia (Li et al., 2001).

MUC3 apomucin has been suggested having a poor prognostic significance in gastric carcinoma (Wang et al., 2000, Wang and Fang, 2003). Aberrant expression of membrane mucins MUC3 and MUC4 in pancreatic intraepithelial neoplasia and adenocarcinoma indicated the progression for pancreatic adenocarcinoma (Park et al., 2003) whereas a decrease in expression of MUC3 and MUC4 was associated with increasing stage of ovarian cancer (Giuntoli et al., 1998). Recently, MUC4 has been assessed to be of a new independent

factor for poor prognosis and of a useful marker to predict the outcome of the patients with intrahepatic CCA-mass forming type (Shibahara et al., 2004).

Expressions of MUC2 and MUC6 were used as prognostic factors in small adenocarcinoma of the lung with lymph node metastasis (Nishiumi et al., 2003). A significant reduction of MUC5AC immunoreactivity was shown to be a marker of worse survival in gastric cancer (Baldus et al., 2002), however a combined evaluation of MUC1 and MUC5AC mucin stainings could be more clinically helpful to predict outcome in these patients (Wang et al., 2003). In colorectal carcinoma, the patients with MUC5AC-negative tumors had poor clinicopathological parameters and showed worse survival than patients with MUC5AC-positive tumors (Kocer et al., 2002). The overexpression of tracheobronchial mucins, MUC5AC and MUC5B were demonstrated to increase the likelihood of post-operative lung-cancer recurrence or metastases (Yu et al., 1996). The expression of MUC6 apomucin in gastric cancer has been suggested a prognostic usefulness by the combination use with HGM, MUC2 and CD10 expression (Tajima et al., 2001).

The differential expression of MUC7 with the onset of malignant transformation of the bladder urothelium was documented (Retz et al., 1998) and reduction of MUC8 mucin has been observed in non-small cell carcinomas, regardless of their histologic subtype (Lopez-Ferrer et al., 2001). MUC11 and MUC12 were found to down-regulate in colorectal cancer tissue (Williams et al., 1999). MUC16 or CA125, a tumor marker for ovarian cancer, has been suggested a role to contribute metastasis of ovarian cancer to the peritoneum by initiating cell attachment to the mesothelial epithelium via binding to mesothelin (Rump et al., 2004).

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Patients and Biological Materials

Patients underwent surgical resection of hepato-pancreato-gatrointestinal cancer at the Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, between 1998 and 1999, were asked to volunteer in the research study. The consent was formally informed and was approved by the patient. Details of the study were approved by the Ethics of Human Research Committee, Khon Kaen University. The gold standard method for CCA diagnosis was histological inspection and the staging of CCA tumor was classified according to TNM system. The diagnosis of benign diseases was based on clinical manifestation and surgical finding. Active opisthorchiasis was asymptomatic persons who were found *O. viverrini* eggs in feces and possessing a negative result of abdominal ultrasound. All clinical characteristics and features used in statistical analysis were recorded by surgeons, Department of Surgery, Faculty of Medicine, Khon Kaen University. Survival of individual CCA patient was determined after surgery. The accrual time was started on January 1, 1998 and ended on May 15, 2001.

Surgical specimens including cancerous and non-cancerous hepatic portions were inspected and cut by histopathologists and then fixed in 10% neutral buffered formalin (n = 58). Fixed tissues were embedded in paraffin block and sectioned into 4 μm thickness. The tissues sections were incubations at 60°C for 30 min and 37°C overnight to dry and fixed tissue onto slide. Sections were kept at ambient temperature until use.

Fresh tumor tissues used for RT-PCR were cut into small pieces and immerged in RNAlater reagent and stood at ambient temperature for 15 hours in order to allow the reagent to completely suffuse into tissue. Samples were then kept at -20°C until use.

A number of CCA patients was informed and asked to volunteer for tracheal lavage collection. After operation, the breath tube was taken off and bronchial mucus inside and surrounded was collected. Sputum samples from airway of healthy volunteers were also collected for MUC5AC mucin detection. Blood samples were obtained from 179 CCA patients, 60 hepato-pancreato-gastrointestinal cancers, 61 benign hepatobiliary diseases, 60 active opisthorchiasis and 74 healthy persons. Withdrawn blood was stood at ambient

temperature until it was completely clotted (5 hours) then centrifuged (3,000 rpm, 10 min). Serum was collected and kept at -20°C until use.

3.1.2 Chemicals

General chemicals were reagent or analytical grades. Only specific chemicals and reagents were listed in Table 3-1

Table 3-1 Chemicals used in the study

Chemicals	Product of
5-bromo-4chloro-3indolyl phosphate	Sigma Chemical Company
6-Deoxy glucose	Sigma Chemical Company
Agarose powder for protein separation	GIBCO BRL
Agarose powder for nucleic acid separation	USB, Cleveland
BCIP buffer solution concentrate (10x)	Zymed
Biomax film	Kodak
Bovine Serum Albumin	Sigma Chemical Company
CHAPS	Sigma Chemical Company,
CsCl	Boehringer Mannheim,
DEPC	USB
Developer	Fuji Hunt, Singapore
Dialysis membranes (molecular weight cut off 12-14,000)	Spectra/Por, Serva, Heidelberg
Diaminobenzidine	Sigma Chemical Company
Dithiothreitol	Sigma Chemical Company
DNA polymerase	Amersham Phamacia Biotech
dNTP	Amersham Phamacia Biotech
ECL kit	New England Nuclear Life Sciences
ECL reagent	Amersham Biosciences
Ethidium bromide	Sigma Chemical Company
Fixer	Fuji Hunt
Goat anti-rabbit IgG conjugated with AP	Sigma Chemical Company
Goat anti-rabbit IgG conjugated with HRP	Sigma Chemical Company

Table 3-1 Chemicals used in the study (Cont.)

Chemicals	Product of
Guanidine hydrochloride	Sigma Chemical Company,
HRP-conjugated streptavidin	Zymed
Hybond ECL Nitrocellulose membrane	Amersham Pharmacia Biotech
Hyperfilm ECL	Amersham Bioscience,
Iodoacetamide	Sigma Chemical Company,
Lectin kit I and II	Vector Laboratories, Inc.
M-MuLV reverse transcriptase	Amersham Phamacia Biotech
MOPS (Morpholinopropane sulfonic acid)	Serva, Boehringer Ingelheim Bioproducts
MUC1-core mouse monoclonal antibody	Novocastra
MUC2 mouse monoclonal antibody	Novocastra
MUC5AC mouse monoclonal antibody	Novocastra
MUC5AC VNTR mouse monoclonal antibody	Chemicon
MUC6 mouse monoclonal antibody	Novocastra
NBT solution concentrate (10x)	Zymed
Neuraminidase	Seikagaku Corporation
Nitroblue tetrazolium	Sigma Chemical Company
Nitrocellulose membranes (pore size 0.2 μ and 0.45 μ)	Schleicher and Schuell
Periodic acid 50% solution	BDH
Polyoxyethylene sorbitan monolaurate (Tween 20)	Sigma Chemical Company
PVDF membrane discs (pore size 0.22 μ, 1.3 cm diameter)	Millipore
Rabbit anti-mouse IgG biotinylated	DAKO Corporation
Rabbit anti-mouse IgG conjugated with HRP	Zymed
Rabbit MUC5AC polyclonal antibody*	Raised from synthetic peptide; RNQDQQGPFKMC

Table 3-1 Chemicals used in the study (Cont.)

Chemicals	Product of
Rabbit MUC5B polyclonal antibody*	Raised from synthetic peptide; ELGQVVECSLDFGLVCR
Restriction enzymes (Alu I, Cla I and EcoR I)	Phamacia LKB Biotechnology
RNeasy kit	Qaigen
Schiff's reagent	Sigma Chemical Company
SDS	BDH
Sepharose CL-2B	Sigma Chemical Company
Swine anti-rabbit IgG biotinylated	DAKO Corporation
Tn Ag mouse monoclonal antibody	Sigma Chemical Company
Trifluoroacetic acid	Perkin Elmer

* Gift from Dr. John K Sheehan

3.2 Methods

3.2.1 Immunohistochemical Technique

Paraffin sections were deparifinized and rehydrated by submerging in xylene and ethanol with stepwise decreasing concentration. Boiling in 0.1 M citrate buffer, pH 6.0 for 3 min and heating in a microwave two times for 10 min each were subsequently performed to unmask the antigens. The sections were then rinsed in PBS (Phosphate buffer saline), treated with 0.5% H_2O_2 in methanol (to neutralize endogenous peroxidase), and with normal horse serum (1:20 in PBS) for 20 min each, at room temperature to block non-specific binding. Sections were then incubated with primary antibody (1:50 for MUC1, 1:40 for MUC2, 1:1,000 for MUC5AC and 1:20 for MUC6) overnight in a moisture chamber at room temperature. The tissues were rinsed with PBS and incubated with the corresponding biotinylated secondary antibody (1:300), and then with peroxidase-conjugated streptavidin (1:300) for 30 min each, at room temperature. Slides were rinsed with PBS and immerged in freshly prepared 0.05% DAB in 0.05 M Tris-HCl buffer, pH 7.6 containing 0.1% H_2O_2. Sections were then rinsed with tap water, counterstained with hematoxylin, dehydrated with stepwise increasing concentration of ethanol, cleared with xylene and mounted with permount. Slides were finally dried at ambient temperature

overnight. Slides omitted either primary antibody or biotinylated secondary antibody or HRP-streptavidin were carried as negative control. The positive control used was the strongly positive section of each mucin.

The expressions of mucin were scored as the positive percentage of stained tumor cells to all tumor cells: 0 = negative, 1-25% =1+, 25-50% = 2+ and >50% = 3+. Pattern of muicn expression was described as cytoplasmic, apical, luminal and stromal patterns.

3.2.2 Agarose Gel Electrophoresis and Immunobloting

3.2.2.1 Sample Preparation

Mucins in airway materials (tracheal lavage and healthy sputum) were extracted and solubilized with 6 M GuHCl reduction buffer (GuRB). Briefly, GuRB was added to dilute mucus samples (1:5) and then gently mixed overnight at 4°C. The extract was isolated by centrifuging at 3,000 rpm for 10 min and dialyzed in 6 M urea at 4°C, overnight prior to agarose gel electrophoresis. In case of serum, the extraction with GuRB was skipped.

Whole or Intact Mucin

Samples (10 µl) were diluted with 6 M urea (80 µl), mixed with 10x loading buffer (LB) up to 10% (v/v) (10 µl). The treated samples were spun for a few seconds to pellet the debris and 20 µl was loaded to agarose gel electrophoresis. GuHCl in the samples will be crystallized when it is in SDS-containing solution, therefore be sure that GuHCl is removed by dialyzing in 6 M urea.

Reduced Subunit Mucin

For standard reduction, samples (10 µl) diluted in 6 M urea (70 µl) were added with 10 µl of 10x dithiothreitol (DTT) solution (1.54 mg DTT in 100 µl 10x urea reduction buffer, pH 8.0), mixed and incubated at 37°C for at least 2 hours. In order to prevent re-forming of disulfide bonds the free thiol ends were alkylated by incubation with 25 mM (4.6 mg/ml) iodoacetamide (IAA) for at least 30 min in the dark (alkylation process). After reduction, reduced samples were added with 10 µl of 10X LB, spun for a few seconds and then loaded to gel (20 µl/well).

Alternative quick reduction was performed by incubating sample with reducing dye containing 10 mM DTT at 100°C for 10 min. The alkylation was carried out as described above. This procedure, however, is not suitable for serum samples because a number of

globular proteins in serum will be denatured and form gel-like material at high temperature (over 45°C).

3.2.2.2 Agarose Gel Electrophoresis

Mucins were separated in 0.7% (w/v) agarose gel electrophoresis (submarine). Agarose powder was weighed and completely melted in a running buffer containing 40 mM Tris-acetate, 1 mM EDTA, pH 8.0, and 0.1% (w/v) SDS. The gel was equilibrated in running buffer for 10 min prior to running. Electrophoresis was performed either short (60 V for 4 hours) or long run (25 V for 16 hours). Separated proteins in gel were then transferred to nitrocellulose membrane by vacuum-blotting in 4x SSC, pH 7.0, with constant suction pressure of 45 mbar for 90 min using a VacuGene XL Phamacia Biotech Blotter. For whole mucin blotting, the additional gel reduction was required by soaking the gel in 4x SSC, pH 8.0 containing 1.54 mg/ml DTT for 10-15 min prior to vacuum transfer.

3.2.2.3 Immunodetection of MUC5AC Mucin

The protocol for MUC5AC quantitation was as described by Thornton et al., 1989. The blotted membrane was blocked by incubation with 5% (w/v) non-fat dried milk in TBST at room temperature for 30 min and incubated with 1:10,000 MUC5AC antiserum diluted in TBST, at room temperature for 2 hours or at 4°C overnight if low amount of antigen was presented in the sample. After washing three times with TBST (5 min each), the membrane was incubated in 1:8,000 of goat anti-rabbit IgG conjugated with horseradish peroxidase for 1 hour, room temperature. The mucin was visualized by ECL detection system according to the protocol described by the manufacturer.

3.2.2.4 Quantitation of MUC5AC Mucin

The nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) detection system was used to quantitate the amount of MUC5AC mucin. Triplication of various dilutions of reduced serum mucins and MUC5AC standard (110 µg/ml) were electrophoresed on a 0.7% agarose gel, transferred to nitrocellulose membrane and probed with MUC5AC antibody (1:10,000). The blot was subsequently incubated with 1:5,000 secondary antibody conjugated to alkaline phosphatase at room temperature for 1 hour and washed three times (5 min each) with TBST. Blot was incubated with freshly prepared substrate solution containing NBT (0.404 mM) and BCIP (0.394 mM) in alkaline phosphatase buffer (100 mM Tris-HCl, 100 mM sodium chloride, 10 mM magnesium chloride, pH 9.0), at room temperature for 30 min. NBT/ BCIP substrate were catalyzed

by alkaline phosphatase developing dark blue precipitate (formazan and indigo dyes). The developed blot was then rinsed with water and dried with air stream using hair dryer. The yielded color was scanned and quantitated using image analysis machine and software (Biorad Laboratory, Richmond, CA).

3.2.2.5 Lectin Blotting

Lectin blotting was carried out to determine the sugar moieties of the samples: UEA-I (Fuc), WGA (GlcNAc), PNA (Gal), sWGA (GlcNAc), SJA (GalNAc), SBA (GalNAc) and LCA (Man). Partially purified serum mucins from gel filtration (see in 3.2.4) were reduced, electrophorosed on agarose gel and transferred to nitrocellulose membrane as described in 3.2.3. The membrane was blocked with 0.2% (v/v) Tween 20 in TBST for 30 min and incubated with 0.5 μg/ml biotinylated lectins in TBST for 30 min. After washing in TBST (3 times, 5 min each), the membrane was incubated in 1:8,000 HRP-conjugated straptavidin for 30 min at room temperature. The sugar-lectin complexes were visualized by ECL detection system. Each pattern of serum MUC5AC mucin detected by immunoblotting was used as the reference to localize mucin on lectin blots. The specificity of lectin signal was demonstrated by sugar inhibition, of which the lectin was incubated with 0.4 M of the specified sugar for 30 min before incubating with the membrane.

3.2.2.6 Membrane Stripping

Non-impregnated proteins on nitrocellulose membrane such as bound antibodies and blocked proteins can be stripped off and the blot can be re-detected with other probes. In brief, the developed membrane was washed three times (5 min each) in TBST and incubated in stripping buffer (0.2 M Glycine-HCl, pH 2.5 containing 0.05% Tween 20 and 0.1 M β-2-mercaptoethanol) at 60-70°C for 30-60 min. The stripped membrane was washed three times (5 min each) in TBST, followed by incubation with ECL reagent to check any remaining signal. The membrane was washed again three times in TBST, re-blocked with blocking reagent (5% non-fat dry milk in TBST) for 30-60 min and re-probed with the new antibody.

3.2.3 Gel Filtration Chromatography

In this study, Sepharose CL-2B (Sigma, Dorset, USA) was used for separation based on size ranged 70,000-40,000,000 Dalton. A column of 18 mm x 16 cm was manually packed with CL-2B bead by gravity according to manufacturer. The column was

run through with high quality water (Millipore water) at a flow rate of 0.2 ml/min overnight, and then equilibrated with two column volumes of 4 M GuHCl.

Serum (1 ml) was diluted in 4 M GuHCl (3 ml) and subjected to the Sephorose CL-2B column, at a flow rate of 0.2 ml/min. The eluent was monitored for absorbance at 280 nm and 1 ml fractions were collected using (AKTA Prime, Amersham Phamacia Biotech, UK). The fractions were analyzed for MUC5AC mucin using slot blot and the MUC5AC-containing fractions were pooled and kept at 4°C for further characterization.

3.2.4 Slot Blot

Samples were directly blotted onto a nitrocellulose membrane using slot blot apparatus (Minifold II 72 wells, Schleicher and Schuell, Germany) and be consequently performed either for PAS staining or Coomassie blue staining or immunodetection. In case of protein-rich sample such as serum, it was diluted 1:10 or more before loading. Filter paper and a nitrocellulose membrane (0.45 μm) were pre-wetted with water for a few seconds and assembled in a slot blot apparatus. Samples (10-200 μl) were blotted to the nitrocellulose membrane by gentle suction until samples were dried off. Blotted membrane was removed from the apparatus and rinsed in water to remove residual salts.

For PAS staining, the membrane was incubated in a freshly prepared hydrolysis solution of 1% (v/v) periodic acid in 3% (v/v) acetic acid at room temperature for 30 min. The membrane was rinsed twice with DW for 2 min each, and twice of 5 min each of freshly prepared 0.1% (w/v) sodium metabisulphite in 10 mM HCl. The blot was then incubated with Schiff's reagent for a 15 min, or until pink slots were clearly visible. The developed membrane was washed twice (5 min each) with 0.1% (w/v) sodium metabisulphite in 10 mM HCl, briefly rinsed with DW and then dried with warm air stream using hair dryer. The amount of glycoprotein was quantitated using Biorad image analysis machine and software (Biorad, CA, USA).

For MUC5AC detection, after rinsing with DW, the mucins on membrane were reduced by incubation with 1x URB containing 1.54 mg/ml DTT for 10 min at room temperature, with agitation. The membrane was then rinsed well with DW, washed twice (3 min each) with TBST and blocked with 5% non-fat dried milk in TBST for 20 min. The immunodetection for MUC5AC mucin was then accomplished using polyclonal MUC5AC antibody and ECL detection system as described in 3.2.3.3. Signal intensity of each slot represented amount of mucin was measured by a reflectance densitometer

(Biorad, CA, USA). A graph of intensity vs. fraction was plotted to display MUC5AC mucin content in each fraction.

3.2.5 Isopycnic Centrifugation

In brief, CsCl was added to the mucin samples (in 4 M GuHCl) to reach the density of 1.4 g/ml. The amount of CsCl added was calculated from the following equation.

$$x = v\,(1.347\rho - 0.0318M - 1.347)$$

where x is the weight of CsCl (g), v is the final volume (ml), M is molarity of GuHCl and ρ is density of CsCl (g/ml). The final weight of entire solution is the outcome of CsCl density (1.4 g/ml) multiply by the final volume (Mass = Density x Volume).

After adding CsCl, the mixture was subsequently adjusted to the final weight by adding 4 M GuHCl. CsCl crystals were completely dissolved by very gentle rolling for 30 min. The solutions were then filled into a quick-seal centrifuge tube (13 ml) (Beckman, Palo Alto, CA). Consequently, tubes were sealed, balanced and spun at 40K rpm, 15°C for 48 hours using ultracentrifuge machine (L8-80 ultracentrifuge, Beckman, CA) with swing-out rotor, SW55 (Beckman, Palo Alto, CA). Fractions of 500 µl were collected by emptying from the top. The density of each fraction was determined by weighing 100 µl of sample and calculated to density (g/ml). The factions were then analyzed for MUC5AC mucin using slot blot.

3.2.6 Anion-exchange Chromatography

A HPLC anion exchange chromatography, HR 5/5 (analytical scale) Mono-Q column (Amersham Pharmacia Biotech, UK) executed by AKTA Purifier (Amersham Pharmacia Biotech, UK) was used. The column was limited at 2 mg of loading protein. Partially purified serum mucins from 3.2.4 were firstly reduced with 10 mM DTT (at 37°C for 5 hours) and alkylated with 25 mM IAA (for 30 min in the dark at ambient temperature) as described in 3.2.3.1.2. The reduced samples were dialyzed against 6 M urea for 2 days, with 2-4 times of buffer changing for complete removal of GuHCl. The reduced mucin subunits were subsequently chromatographed on the Mono-Q column at a flow rate of 0.5 ml/min. The buffers used were- Buffer A: 6 M urea containing 10 mM piperazine, 0.05% (w/v) CHAPS, pH 5.0 and Buffer B: 6 M urea containing 10 mM piperazine, 0.05% (w/v) CHAPS, 400 mM lithium perchlorate, pH 5.0. The elution gradient buffer was 0-0.4 M $LiOCl_4$. After sample loading, column was run with Buffer A

(100% Buffer A, 0% Buffer B) for the first 10 min to wash out the unbound materials then the salt gradient was applied to elute the bound materials by linearly increasing the concentration of Buffer B from 0% to 100% (0-400 mM $LiOCl_4$). Fractions of 0.5 ml were collected and monitored for the absorbance at 280 nm and for MUC5AC mucin.

3.2.7 Rate-zonal Centrifugation

Rate-zonal centrifugation was performed to estimate mass of the partially purified intact and reduced subunit of MUC5AC mucins. A gradient of 6-8 M GuHCl was made by a gradient maker (Biorad Laboratory, Richmond, CA), which then whole or reduced mucins in 4 M GuHCl were layered on the top of the gradient. The tubes were centrifuged at 40K rpm, 15°C for either 3 hours for the whole mucin or 7 hours for the reduced mucin, using SW55 swing-out rotor (Beckman, Palo Alto, CA). Fractions (0.5 ml each) were collected by emptying the tubes from the top and detected for MUC5AC mucin using slot blot as described in 3.2.5.

3.2.8 High Performance Anion Exchange Chromatography - Pulsed Amperometric Detection (HPAEC-PAD)

For hydrolysis, samples (40-200 µg) were blotted on to PVDF membrane discs then hydrolyzed under nitrogen in 2 M trifluoroacetic acid (100°C, 5 hours). Excess acid was removed by evaporating in Speedy Vac machine. The monosaccharide pellet was afterward re-dissolved in appropriate volume (100 µl) of 0.1 M 6-deoxy glucose (6-DG) (an internal standard monosaccharide). Samples (20 µl) were further analyzed using HPAEC-PAD.

Samples containing 0.1 M 6-DG internal standard were chromatographed on a Carbopac PA1 column (4 mm x 250 mm, Dionex Crop., Sunnyvale, CA) accompanying with a PA1 guard column. The monosaccharides were eluted isocratically, at 0.1 ml/min, with 16 mM sodium hydroxide solution. Negatively charged sugars were detected by a pulsed amperometric electrochemical detector. Standard monosaccharides had to be run prior to the sample in order to provide a reference chromatogram. Each run took approximately 20 min, followed by washing column for 20 min with 100 mM sodium hydroxide (column regeneration) and 50 min with 16 mM sodium hydroxide to re-equilibrate the column. The relative amount of each monosaccharide was represented by integrated area under peak and the peak area of 6-GD standard was used for the data normalization.

3.2.9 Desialylation of Mucin

In order to elucidate how much sialic acid contributes to the migration of MUC5AC mucin on the agarose gel, sialic acids were cleaved by neuraminidase (sialidase) before subjecting to electrophoresis. Briefly, samples were dialyzed in 0.25 M Tris-acetate, pH 6.5 overnight and treated with 0.05 unit neuraminidase and 0.01 M $CaCl_2$ at 37°C for 10 min. Neuraminidase-treated and -untreated samples were then reduced, electrophoresed on agarose gel and transferred to nitrocellulose for immunodetection as described in 3.2.3.

3.2.10 Statistical Analysis

Statistical approach was employed in order to assess the clinical significance of obtained data in CCA patients. The parametric test was first priority of choosing. The non-parametric test was an alternative when the assumption of parametric test was violated. Statistical analysis was accomplished by STATA software (StataCorp, College Station, Texas). The significant level was considered at P value of less than 0.05. Charts were plotted using Microsoft Excel program. Statistical tests carried out in the present study were concisely described below.

3.2.10.1 Diagnostic Values

Diagnostic values: sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy were analyzed from 2 x 2 table to validate the potential of using serum MUC5AC mucin as tumor marker of CCA.

		Gold standard method (Histological proof)		
		Disease	*Disease-free*	
New test result (Serum MUC5AC)	*Positive*	TP (a)	FP (b)	a + b
	Negative	FN (c)	TN (d)	c + d
		a + c	b + d	a + b + c + d

Sensitivity = a / (a + c)

Specificity = d / (b + d)

PPV = a / (a + b)

NPV = d / (c + d)

Accuracy = (a + d) / (a + b + c + d)

3.2.10.2 Association Assessment

The association between two categorical variables (e.g. the presence of serum and tissue MUC5AC mucin) was analyzed by Chi square (χ^2) (parametric test) or Fisher's exact test (non-parametric test). The difference of continuous data between two independent groups was analyzed by either independent t-test (parametric test) or Mann Whitney test (non-parametric test).

3.2.10.3 Logistic Regression

Logistic regression was conducted in order to identify the clinical factors that influence the presence of MUC5AC in CCA sera. The presence of MUC5AC in serum was an outcome (or dependent) variable, whereas the clinical characteristics of CCA patients were predictors or independent variables or covariates and were used to investigate for crude odds ratios (OR) in univariate analysis. The p value of < 0.25 of crude OR and also evidence based of outcome onset were set as criteria for recruiting the candidate variables. Candidate predictors were subsequently put in the initial model and stepwise eliminated to obtain the best fit final model in the multivariate analysis. The model adequacy was assessed by test for goodness of fit. Ultimately, the magnitude of association (represented as adjusted OR) of predictors was obtained, which was controlled for the covariates.

3.2.10.3 Survival Analysis

The prognostic potentials of either serum MUC5AC, tissue MUC5AC or the MUC5AC patterns were assessed by survival analysis. Kaplan-Meier curve, plotted between the percentages of survival cases versus the specific time point, was estimated to compare the overall survival among investigating groups. Log-rank test was carried out to determine the difference of overall survival between groups based upon χ^2 - distribution.

The study designed the accrual time from January 1, 1998 to May 15, 2001. The dependent variable was survival time, which was computed from 30 days after surgery until the day that patients died. Subjects were followed up until event happened (failure cases) – cases with lost of follow up, missing or die due to other causes were censored cases. Cox's proportional hazard regression was analyzed. Model adequacy of final Cox's model was determined by assessing the proportional hazard assumption and the goodness of fit. The adjusted hazard ratio (HR) indicated the risk of outcome onset (death) of predictors, which was controlled for all considered covariates.

IV. RESULTS

4.1 Expression of Mucins in CCA Tissues and Clinical Significance

4.1.1 Apomucin profile expressed in CCA tissues

A panel of mucins: MUC1, MUC2, MUC5AC and MUC6 were investigated in CCA tissues using immunohistochemical analysis with antibodies specifically recognized core-protein portions. Histologically proved CCA sections (n = 58) were accomplished for each mucin. The patterns of apomucin immunohistostainings are shown in Table 4-1.

Table 4-1 The expression of apomucins in non-cancerous and CCA tissues using immunohistochemicalstainings

Apomucin type	Bile duct cells	
	Non-cancerous cells	CCA cells
MUC1	Lightly positive	Predominant apical and luminal staining
MUC2	Negative	Predominant cytoplasmic staining
MUC5AC	Negative	Predominant luminal and stromal expression
MUC6	Positive in peribiliary glands	Predominant cytoplasmic staining

MUC1 apomucin was the only mucin found in normal bile duct cells with low intensity (Figure 4-1). It was located principally on the apical cell surface and in the lumen (Figure 4-1B). MUC2, MUC5AC and MUC6 apomucins are gel-forming mucins, which had a similar staining pattern of luminal and cytoplasmic stainings with strongly positive in secretory vesicle (Figures 4-2, 4-3, and 4-4). MUC2 and MUC6 apomucins were weakly expressed in CCA tissue and not detected in non-cancerous biliary cells. The MUC6 apomucin however was intensively observed in peribiliary glands (Figures 4-4A and B). MUC2 was the only mucin that strongly expressed in the goblet cells (Figures 4-2D-E). MUC6 apomucin was intensively expressed in normal peribiliary glands but rather weak in CCA tissues (Figure 4-4). The stromal expression was not observed for MUC1, MUC2 and MUC6 apomucin stainings.

MUC5AC apomucin was not detected in normal biliary cells but it was vastly detected in cancerous cells (Figure 4-3). MUC5AC apomucin was principally located in

the duct lumen and secretory vesicles and were often in stroma with smear stained over the interstitial spaces (Figure 4-3E). As a result, MUC5AC apomucin was defined as a neoexpressed mucin in CCA. Correlation between MUC5AC apomucin grading and amount of MUC5AC transcript was revealed.

In the present study, MUC1 (74%) and MUC5AC (64%) apomucins were more frequently found in CCA tissues than MUC2 (26%) and MUC6 (28%) apomucins (Table 4-2). The intensity of MUC1, MUC2 and MUC6 apomucins was mostly low whereas MUC5AC intensity was high. The data suggested that CCA cells actively synthesized a gel-forming mucin, MUC5AC, rather than MUC2 and MUC6 heralding to investigate the mechanism of regulation and role of MUC5AC mucin in the biliary cancer.

Table 4-2 The intensity of apomucins expressed in CCA tissues

Grading	Apomucins stained in CCA tissues (n=58)			
	MUC1	MUC2	MUC5AC	MUC6
Negative	15 (26%)	43 (74%)	21 (36%)	42 (72%)
Positive	43 (74%)	15 (26%)	37 (64%)	16 (28%)
1+	23 (40%)	10 (17%)	10 (17%)	8 (14%)
2+	10 (17%)	3 (5%)	14 (24%)	7 (13%)
3+	10 (17%)	2 (4%)	13 (23%)	1 (1%)

4.1.2 Clinical Significance of Tissue Apomucins

The expression of apomucins in CCA tissues was classified into low and high expression for statistical analysis (Table 4-3). Apomucin gradings of 0 and 1+ were categorized as low expression and 2+ and 3+ as high expression, and were assessed for the relation to clinicopathological data of the patients including sex, age, histological grading, CCA type, staging, tumor mass, invasive routes as well as serum tumor markers, CEA, CA19-9 and ALP.

Table 4-3 Expressions of apomucins in CCA tissues according to the immunohistochemical grading

Expression	CCA tissues (n=58)			
	MUC1	MUC2	MUC5AC	MUC6
Low (0, 1+)	38 (66%)	53 (91%)	31 (53%)	50 (86%)
High (2+, 3+)	20 (34%)	5 (9%)	27 (47%)	8 (14%)

Table 4-4 The association of MUC1 apomucin expression and clinicopathological features of CCA patients

Clinicopathological Features	n	MUC1 Apomucin Expression		P value
		Low	High	
Age		38	20	0.739
- ≤ 55 years	22	15	7	
- > 55 years	36	23	13	
Gender		38	20	0.952
- Male	38	25	13	
- Female	20	13	7	
Histological grading		34	17	0.953
- Papillary	15	10	5	
- Well diff.	20	13	7	
- Moderate diff.	8	5	3	
- Poorly diff.	8	6	2	
Tumor type		37	20	0.563
- Central	2	2	0	
- Peripheral	49	31	18	
- Combined	6	4	2	
Tumor stage		37	20	0.930
- I-III	10	7	3	
- IVA	8	5	3	
IVB	39	25	14	

Table 4-5 The statistical association between MUC5AC apomucin expression and clinicopathological features of CCA patients (cont.)

Patient Characteristics	n	MUC5AC Apomucin Expression		P value
		Low	High	
Tumor mass		28	25	0.129
- ≤ 5 cm	14	10	4	
- > 5 cm	39	18	21	
Vascular invasion		27	30	0.396
- No	20	11	9	
- Yes	37	16	21	
Lymphatic invasion		28	30	0.181
- No	16	10	6	
- Yes	42	18	24	
Neural invasion		27	31	*0.034*
- No	30	18	12	
- Yes	28	9	19	
CEA		29	26	0.737
- Low (≤ 2.5 ng/ml)	16	9	7	
- High (> 2.5 ng/ml)	39	20	19	
CA19-9		30	26	0.186
- Low (≤ 100 U/ml)	29	18	11	
- High (> 100 U/ml)	27	12	15	
ALP		30	26	0.386
- Low (≤ 147 U/L)	25	15	10	
- High (> 147 U/L)	31	15	16	

diff: differentiation

The relation between patients' survival and expression levels of MUC1, MUC2, MUC5AC and MUC6 apomucins are summarized in Table 4-6. The median survival time of patients possessed high expression of MUC1 apomucin (145 days, 95% CI; 90.23-199.78) was about 2 fold shorter than those possessed low MUC1 apomucin production (312 days, 95% CI; 78.84-545.16) (Table 4-6). The Kaplan-Meier curve demonstrated that patients with high MUC1 apomucin expression had a significantly poorer outcome (P = 0.004) (Figure 4-5). Furthermore, multivariate approach using Cox proportional hazard regression confirmed that patients with high MUC1 apomucin expression had a 2.42-fold (95% CI, 1.08-5.41) risk of death higher than those with low MUC1 apomucin expression (Table 4-12). The survival analysis revealed a shorter survival of patients who possessed a high level of MUC5AC apomucin but this was not statistical significance (P = 0.141) (Figure 4-7). The Kaplan-Meier curves of MUC2 and MUC6 apomucins are shown in Figures 4-6 and 4-8, respectively.

Table 4-6 Median survival time and 95% CI of tissue apomucins

Tissue Mucin	No. of Subject	No. of Event	Median Survival Time (days) (95% CI)
1. MUC1	58	42	
a. Low	38	24	312 (78.84-545.16)
b. High	20	18	145 (90.23-199.78)
2. MUC2	57	41	
a. Low	52	38	224 (131.54-316.46)
b. High	5	3	189 (-38.59-416.59)
3. MUC5AC	58	42	
a. Low	31	21	329 (154.60-503.40)
b. High	27	21	190 (105.19-274.81)
4. MUC6	57	42	
a. Low	50	37	215 (157.31-272.69)
b. High	7	5	395 (-362.03-1152.03)

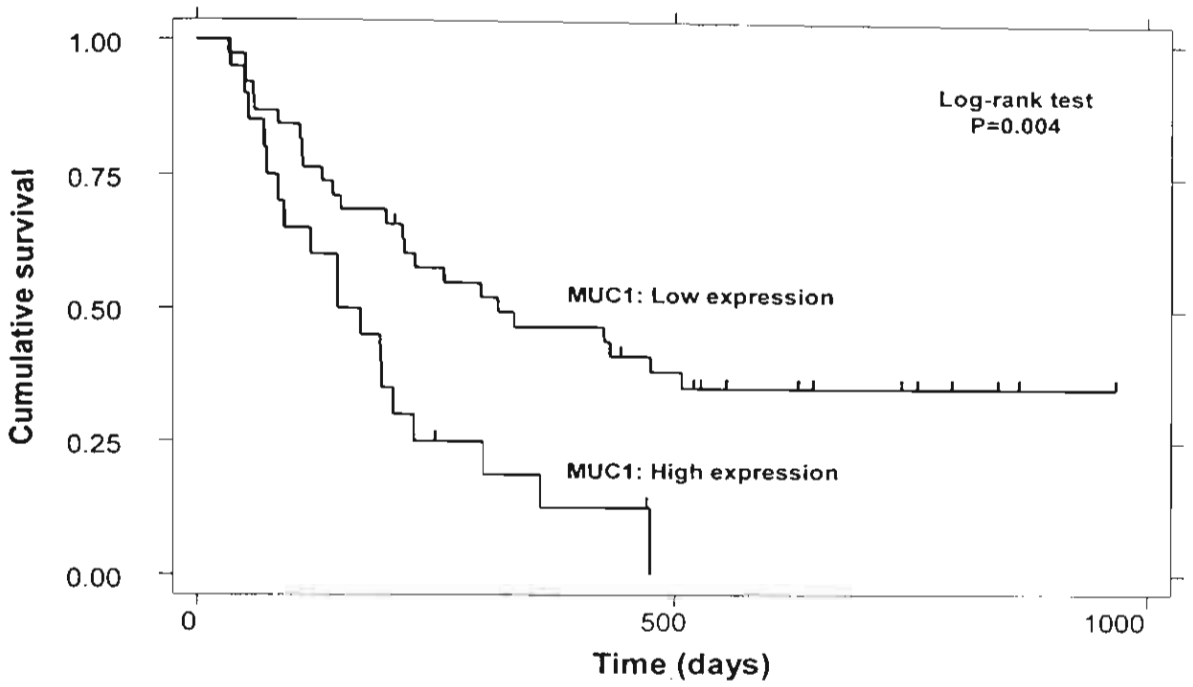


Figure 4-5 The Kaplan-Meier curve based on MUC1 apomucin expression in CCA tissues. Log-rank test revealed a statistical significance with P = 0.004.

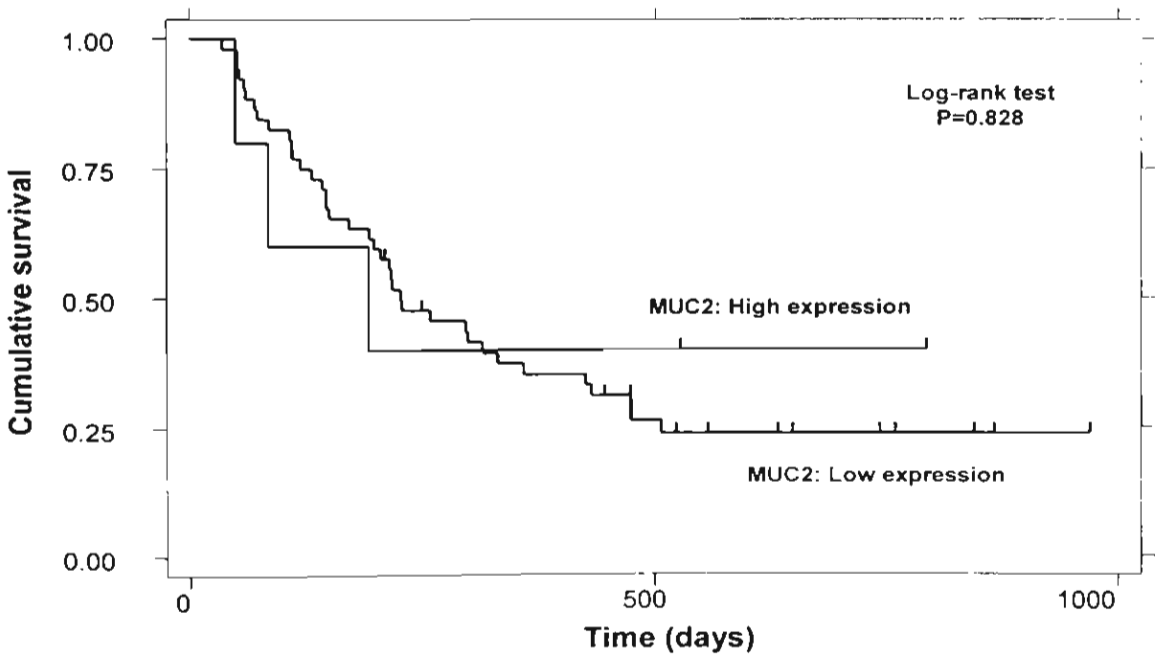


Figure 4-6 The Kaplan-Meier curve based on MUC2 apomucin expression in CCA tissues. Log-rank test revealed a non-statistical significance with P = 0.828.

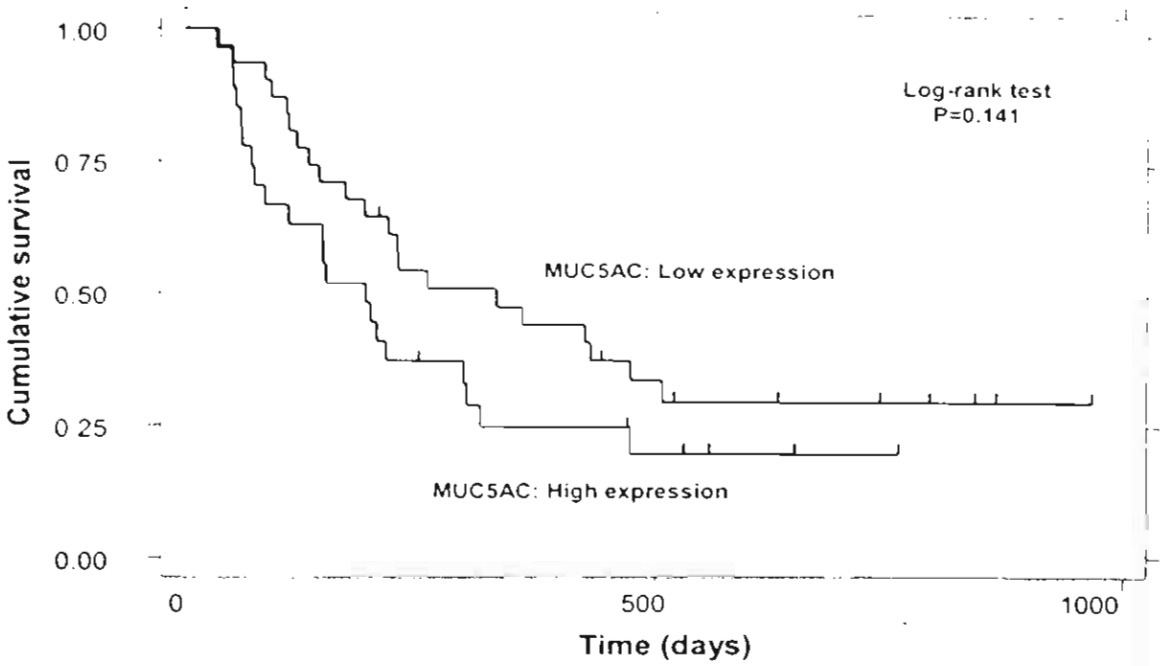


Figure 4-7 The Kaplan-Meier curve based on MUC5AC apomucin expression in CCA tissues. Log-rank test revealed a non-statistical significance with P = 0.141.

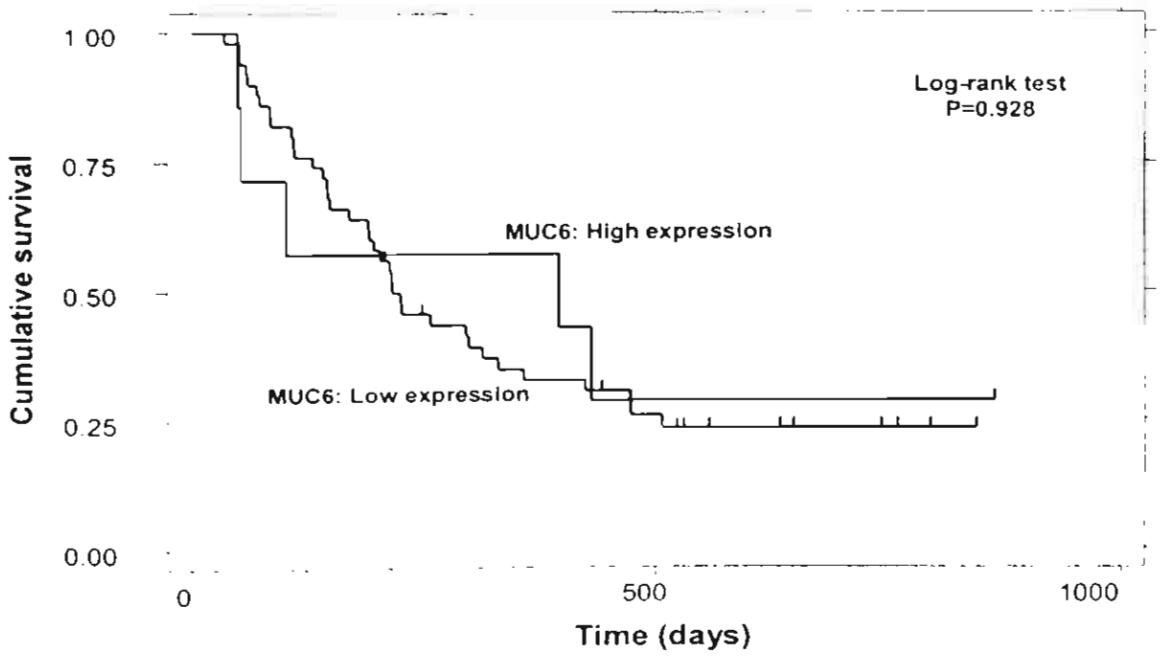


Figure 4-8 The Kaplan-Meier curve based on MUC6 apomucin expression in CCA tissues. Log-rank test revealed a non-statistical significance with P = 0.928.

4.2. Detection of MUC5AC mucin in serum samples

Intact and reduced MUC5AC mucins were determined in sera obtained from CCA patients (n = 179) and various control groups (n = 255). The electrophoretic patterns of intact MUC5AC mucin on agarose gel are shown in Figure 4-9. Various electrophoretic patterns and amount of serum MUC5AC (sMUC5AC) mucin were observed.

The frequency and percentage of positive cases for sMUC5AC mucin in each group are presented in Table 4-7. The highest positive case (62%, 112/179) was the CCA group. Only 2 of 61 (3%) were positive in benign biliary disease cases: one gall bladder stone and one common bile duct stone. Serum MUC5AC mucin was also detected in 6/60 (10%) of various gastrointestinal tract cancers: 2 each of esophageal cancer, pancreatic cancer and cancer of ampulla of vater. No positive case was found in the asymptomatic opisthorchiasis (0/60) and the healthy subjects (0/74).

4.2.1 The Diagnostic Values of sMUC5AC Mucin

The clinical value of serum MUC5AC was investigated using statistical approach. The diagnostic values, the relevance to clinicopathological feature and blood chemistry, the predicted magnitude of variables related to the expression of sMUC5AC and the prognostic value of sMUC5AC were analyzed.

The diagnostic values (sensitivity, specificity and positive and negative predictive values) were computed as shown in the 2 x 2 table (Table 4-8). Determination of sMUC5AC imparted sensitivity of 62.57% and specificity of 96.86%, positive predictive value of 92.56% and negative predictive value of 78.66%, respectively. The high PPV of sMUC5AC in this study indicated that the test was valuable to identify CCA subjects. The high accuracy of sMUC5AC determination (82.72%) indicated a good diagnostic tool of the test.

4.2.2 The Correlation of Serum MUC5AC to Tissue MUC5AC Apomucin, Clinicopathological Feature and Blood Chemistry Profile of CCA Patients

The presence of MUC5AC mucin in serum was significantly correlated to the immunohistostaining of MUC5AC apomucin in tumor tissue (Table 4-9). This suggested that serum MUC5AC mucin is originated from CCA burden. Characteristic parameters of CCA patients, clinicopathological features and blood profiles were tested for the association with sMUC5AC mucin as shown in Table 4-10. The results showed that expression of MUC5AC mucin in serum related to the progression of tumor- namely late state (particularly, stage IVB) (P = 0.092) and size (greater than 5 cm) of the CCA tumor

(P = 0.081). In addition, serum MUC5AC mucin was correlated to blood group B (P = 0.019), high level of serum direct bilirubin (P = 0.039) and high number of white blood cells (P = 0.021). However, the association effects of these three variables were not shown in the multivariate analysis using logistic regression (4.2.2.3) (data not shown).

4.2.3 Logistic Regression Model of Serum MUC5AC Mucin

The final logistic model of expression of sMUC5AC mucin in CCA patients adjusted for all covariates is shown in Table 4-11. The advanced stage of tumor (stage IVB) and expression of MUC5AC apomucin in tissue were independent predictors for MUC5AC mucin in CCA serum with adjusted OR of 15.61 (95% CI; 1.55-157.05) and 36.14 (95% CI; 3.88-336.79), respectively (P < 0.05) in which was controlled for age, gender, blood group and tumor type covariates. Hence, the independent factors to predict the presence of MUC5AC mucin in CCA serum were CCA stage IVB and the expression of tissue MUC5AC apomucin.

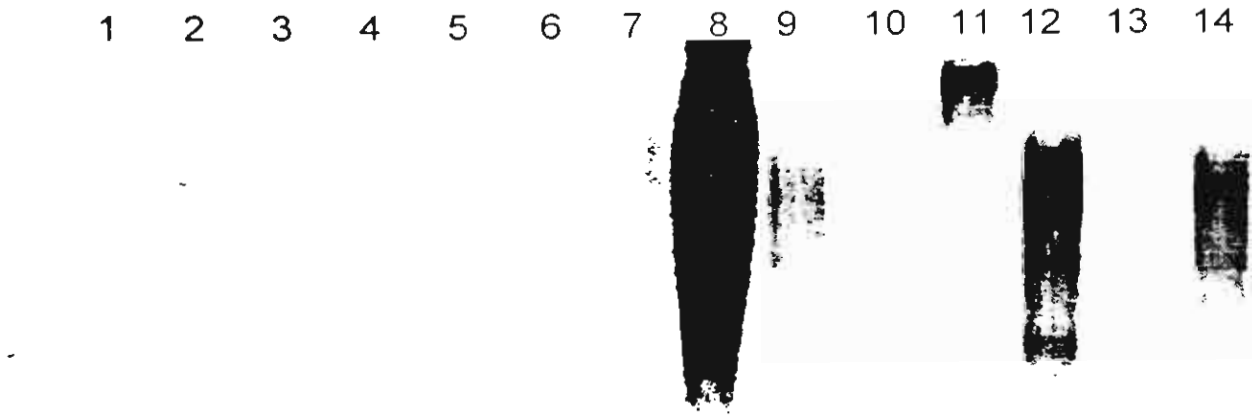


Figure 4-9 Electrophoretic patterns of intact sMUC5AC mucins in CCA sera. Serum samples were electrophoresed, probed with MUC5AC antibody and visualized by ECL detection as described in Materials and Methods. Lanes 1-14 are individual cases of CCA patients. Lanes 1, 4, 6 and 10 show negative sMUC5AC and the rest are positive with various amount and patterns of sMUC5AC mucin.

Table 4-7 MUC5AC mucin in serum from CCA patients and control groups

Subjects	No. of cases (n)	No. of positive cases (%)
1. Cholagiocarcinoma	179	112 (62%)
2. Benign biliary diseases	61	2 (3%)
- Gallbladder stone	37	1
- Cholecystitis and cholangitis	8	0
- Common bile duct stone	11	1
- Liver abscess	3	0
- Hepatitis	1	0
- Periductal fibrosis with fasioliasis	1	0
- Cholecystitis with opisthorchiasis	1	0
3. Hepato-pancreato-gastrointestinal tract cancers	60	6 (10%)
- Stomach cancer	12	0
- Ileum cancer	1	0
- Colon cancer	14	0
- Rectum cancer	6	0
- Ceacum cancer	1	0
- Gallbladder cancer	2	0
- Hepatoma	4	0
- Esophagus cancer	3	2
- Ampulla of Vater cancer	7	2
- Pancrease cancer	10	2
4. Asymptomatic opisthorchiasis	60	0
5. Healthy persons	74	0

Table 4-8 The diagnostic values of sMUC5AC determination for CCA

		Diseases		
		Non-CCA	**CCA**	**Total**
Serum MUC5AC Test	-	247	67	314
	+	8	112	121
	Total	255	179	434

Sensitivity	62.57 %
Specificity	96.86 %
Positive Predictive Value	92.56 %
Negative Predictive Value	78.66 %
Accuracy	82.72 %

Table 4-9 The correlation of MUC5AC mucin detected in CCA sera and tissues

Tissue MUC5AC	**n**	**Serum MUC5AC**		**P value**
		Negative	Positive	
Negative	19	14	5	*<0.001*
1+	10	3	7	
2+	14	3	11	
3+	12	0	12	

Table 4-10 The clinical relevance of sMUC5AC Mucin

Variables	n	Serum MUC5AC		P value
		Negative	Positive	
Age	58	21	37	0.822
- ≤ 55 years	21	8	13	
- > 55 years	37	13	24	
Gender	58	21	37	0.760
- Male	40	15	25	
- Female	18	6	12	
Blood group	58	21	37	*0.019*
- O	17	11	6	
- A	10	1	9	
- B	25	8	17	
- AB	6	1	5	
Histological grading	52	19	33	0.074
- Papillary	15	6	9	
- Well diff.	21	7	14	
- Moderate diff.	8	2	6	
- Poorly diff.	8	4	4	
Tumor type	58	21	37	0.167
- Central	3	0	3	
- Peripheral	49	17	32	
- Combined	6	4	2	
Tumor stage	58	21	37	0.092
- I-III	10	6	4	
- IVA	10	5	5	
- IVB	38	10	28	
Tumor size	53	20	33	0.081
- ≤ 5 cm	14	8	6	
- > 5 cm	39	12	27	
Jaundice	57	21	36	1.000
- No	50	19	31	
- Yes	7	2	5	
Total sialic acid	55	21	34	2.33
- Low (≤ 2.33 μmol/ml)	12	6	6	
- High (> 2.33 μmol/ml)	43	15	28	
Total protein	56	21	35	0.143
- Low (≤ 8.8 g/dl)	52	18	34	
- High (> 8.8 g/dl)	4	3	1	
Albumin	56	21	35	0.408
- Low (≤ 3.7 g/dl)	28	9	19	
- High (> 3.7 g/dl)	28	12	16	

Table 4-10 The clinical relevance of sMUC5AC Mucin (cont.)

Variables	n	Serum MUC5AC		P value
Globulin	56	21	35	0.077
- Low (≤ 3.6 g/dl)	16	3	13	
- High (> 3.6 g/dl)	40	18	22	
Total bilirubin	56	21	35	0.128
- Low (≤ 1.50 mg/dl)	41	18	23	
- High (>1.50 mg/dl)	15	3	12	
Direct bilirubin	56	21	35	*0.039*
- Low (≤ 0.25 mg/dl)	38	18	20	
- High (>0.25 mg/dl)	18	3	15	
ALT	56	21	35	0.521
- Low (≤ 36 U/L)	21	9	12	
- High (> 36 U/L)	36	12	23	
AST	55	20	35	0.333
- Low (≤ 32 U/L)	13	3	10	
- High (> 32 U/L)	42	17	25	
ALP	55	20	35	0.352
- Low (≤ 147 U/L)	23	10	13	
- High (> 147 U/L)	32	10	22	
WBC	55	20	35	*0.021*
- Low (≤ 10000 cells/ml)	30	15	15	
- High (> 10000 cells/ml)	25	5	20	
PMN	55	20	35	0.438
- Low (≤7500 total cells)	32	13	19	
- High (>7500 total cells)	23	7	16	
CEA	56	21	35	1.000
- Low (≤ 2.5 ng/ml)	16	6	10	
- High (> 2.5 ng/ml)	40	15	25	
CA19-9	57	21	36	0.977
- Low (≤ 100 U/ml)	30	8	19	
- High (> 100 U/ml)	27	13	17	

4.2.4 Survival Analysis for the Detection of Serum MUC5AC Mucin

The detection of MUC5AC in CCA serum was explored for the prognostic purpose using survival analysis for both univariate and multivariate approaches. The CCA patients who were positive for sMUC5AC mucin showed a median survival time of 144 days (95% CI; 90-195 days) whereas the median survival time of those who were negative for sMUC5AC mucin was of 507 days (95% CI; 225-967 days). Estimate overall survival using Kaplan-Meier curve analysis revealed that patients with presence of sMUC5AC

mucin had a significantly shorter survival than those with no sMUC5AC mucin (log-rank analysis, $P < 0.001$) (Figure 4-10).

To investigate whether sMUC5AC mucin can be used as an independent prognostic indicator, a multivariate analysis using Cox proportional hazards model was accomplished. The magnitude of risk of death was represented as hazard ratio (HR). Final Cox regression model of sMUC5AC mucin adjusted for all covariates is shown in Table 4-12.

Table 4-11 Multivariate analysis using logistic regression for the expression of sMUC5AC mucin in CCA patients

Variables	Crude OR	Adjusted OR (95% CI)	P value
1. Age			0.057
- ≤ 55 years	1	1	
- > 55 year	1.53	4.97 (0.87-28.44)	
2. Gender			0.668
- Male	1	1	
- Female	1.17	0.69 (0.11-4.20)	
3. Blood group			0.773
- O	1	1	
- A	10.00	1.40 (0.06-31.07)	
- B	2.70	1.38 (0.19-10.04)	
- AB	2.14	0.27 (0.01-11.43)	
4. Tumor type			0.114
- Central	1	1	
- Peripheral	0.88	0.86 (0.01-320.60)	
- Combined	0.25	0.08 (0.01-38.01)	
5. Tumor stage			*0.037*
- I-III	1	1	
- IVA	2.33	9.11 (0.44-189.78)	
- IVB	5.73	15.61 (1.55-157.05)	
6. Tissue MUC5AC Apomucin			*<0.001*
- Negative	1	1	
- Positive	16.07	36.14 (3.88-336.79)	

Table 4-12 Multivariate analysis of sMUC5AC mucin in CCA patients using Cox proportional hazards regression

Variable	Crude HR (95% CI)	Adjusted HR (95% CI)	P value
Tissue MUC1 apomucin			*0.037*
- Low	1	1	
- High	2.46 (1.30-4.64)	2.42 (1.08-5.41)	
Tissue MUC5AC apomucin			0.651
- Low	1	1	
- High	1.57(0.86-2.89)	0.80 (0.30-2.11)	
Serum MUC5AC mucin			*<0.001*
- Absence	1	1	
- Presence	4.02 (2.00-8.09)	7.80 (2.50-24.32)	
Age			0.082
- ≤ 55 years	1	1	
- > 55 years	1.22 (0.67-2.24)	2.11 (0.91-4.93)	
Histological grading			*<0.001*
- Papillary	1	1	
- Well diff.	2.77 (1.19-6.45)	5.08 (1.72-15.05)	
- Moderate diff.	4.31 (1.60-11.62)	4.85 (1.36-17.32)	
- Poorly diff.	1.72 (0.59-4.96)	4.90 (1.28-18.73)	
Tumor stage			0.052
- I-III	1	1	
- IVA	2.04 (0.65-6.44)	1.70 (0.32-8.97)	
- IVB	3.59 (1.31-8.64)	3.39 (1.07-10.81)	
Lymph node resection			*<0.001*
- Yes	1	1	
- No	2.58 (1.39-4.79)	4.31 (1.92-9.66)	

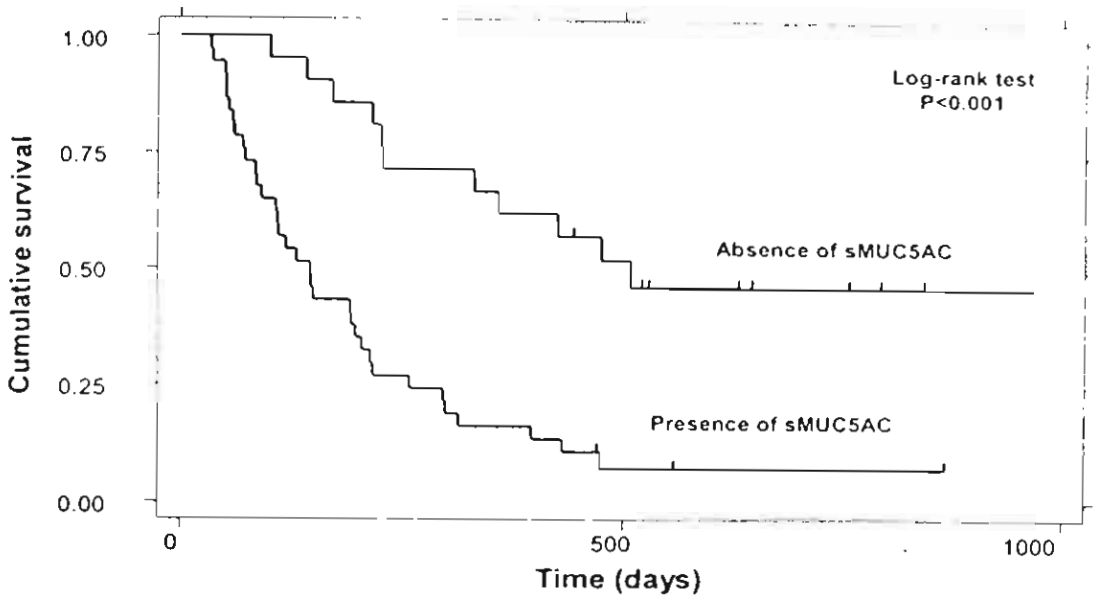


Figure 4-10 Estimated survival curve of sMUC5AC mucin for CCA patients. Serum MUC5AC mucin was determined as described in 3.4.3 and categorized into negative (absence) and positive (presence) sMUC5AC. Patients with presence of sMUC5AC mucin had significantly shorter survival time than those with absence of sMUC5AC mucin (log-rank analysis, $P < 0.001$).

The adjusted HR of MUC5AC mucin presented in serum was of 7.8 (95% CI; 2.50-24.32) which indicated that patients with positive for sMUC5AC mucin had a 7.8-fold risk of death greater than patients with negative for sMUC5AC mucin. Therefore, sMUC5AC mucin can be an independent prognostic marker for worse outcome of CCA patients. The Cox regression model (Table 4-12) also revealed that patients with well- or moderate- or poorly-differentiated histological gradings had significantly shorter disease-free time than those with papillary type ($P < 0.001$) (Table 4-12). In addition, CCA patients with tumor stage IVB had a 3.39-fold higher risk of death than those with early stage tumor. Moreover, CCA patients without lymph node resection had a shorter survival time than those with lymph node resection with adjusted HR of 4.31 (95% CI; 1.92-9.66).

4.2.5 Electrophoretic patterns of sMUC5AC mucin Found in CCA Patients

As shown in Figure 4-11, there three unique electrophoretic patterns of sMUC5AC mucin were identified and named according to their electrophoretic appearances as slow, smear and ladder patterns. The intact MUC5AC mucins found in serum could be reduced by DTT to reduced subunit, suggesting that circulating MUC5AC mucin existed in polymerized form and linked together by disulfide bonds. Each electrophoretic pattern of the intact form yielded a unique reduced form. Reduced slow MUC5AC pattern yielded two reduced subunits while smear and ladder MUC5AC mucins gave only one subunit.

The frequencies of slow, smear and ladder electrophoretic patterns of MUC5AC found in CCA sera were 9%, 46% and 45%, respectively (Table 4-13). The ladder and smear patterns were frequently found. However, there were eleven cases with unclassified pattern.

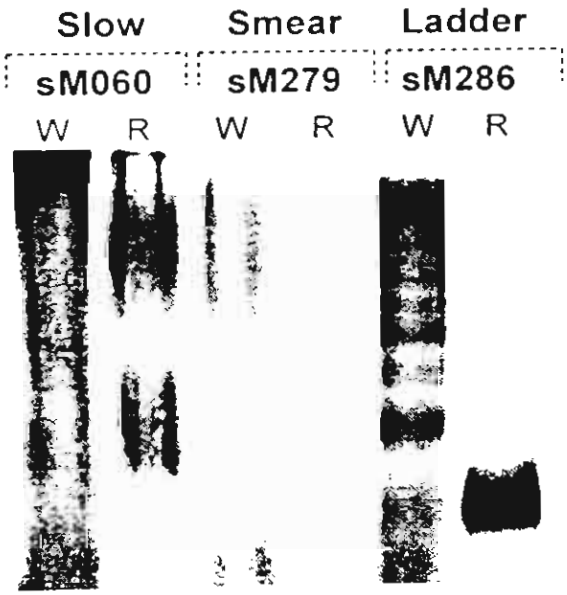


Figure 4-11 Electrophoretic patterns of sMUC5AC mucin from CCA patients. Sera obtained from three cases of CCA (M060, M279 and M286) were electrophoresed on 0.7% agarose gel. W represents ,whole or intact form and R represents reduced subunit form. The electrophoretic patterns were named according to the apparent migration patterns of intact forms to be slow, smear and ladder.

Table 4-13 Frequency of electrophoretic patterns detected in serum of CCA patients

Electrophoretic pattern	Frequency	%
1. Classified pattern	101/112	90
- Slow	9/101	9
- Smear	47/101	46
- Ladder	45/101	45
2. Unclassified pattern	11/112	10

4.2.6 Identification of Normal Electrophoretic Pattern of MUC5AC mucin

Since, MUC5AC mucin is a prominent mucin produced in airway tract and lung (Hovenberg et al., 1996, Reid et al., 1997, Buisine et al., 1999, Rubin, 2002, Voynow, 2002, Groneberg et al., 2002), therefore, the respiratory secretion such as sputum and tracheal lavage are appropriate sources to obtain normal MUC5AC mucin. The electrophoretic patterns of sputum from healthy persons and tracheal lavage from CCA patients were the ladder type (Figure 4-12). This suggested that the ladder type was a normal pattern of MUC5AC mucin. The MUC5AC electrophoretic patterns from serum and tracheal lavage of the same patient were also carried out. The result showed that the pattern of intact MUC5AC mucin in tracheal materials was always the ladder type whereas those from serum could be either ladder or slow or smear types (Figure 4-13).

4.2.7 The Clinical Significance of Electrophoretic Patterns of sMUC5AC Mucin

The electrophoretic patterns of serum MUC5AC mucin were categorized into normal (ladder type) and abnormal (slow and smear types) patterns. The normal type was found in 45% (45/101) whereas the abnormal type was found in 56% (56/101) of sMUC5AC positive patients. The association of electrophoretic patterns of sMUC5AC and clinicopathological characteristics of the patients was elucidated. As shown in Table 4-14, the abnormal form of sMUC5AC was correlated with gross type of tumor (central type, $P = 0.001$) and the presence of jaundice ($P < 0.001$).

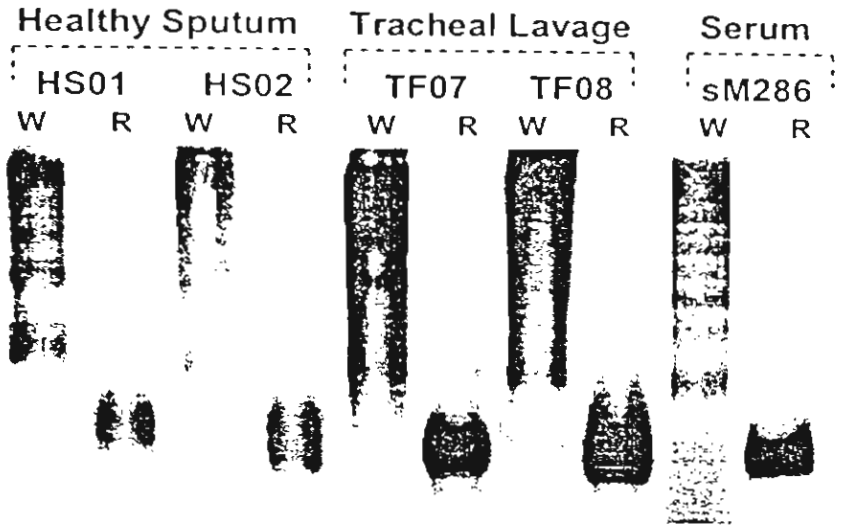


Figure 4-12 Comparison of intact and reduced subunits of MUC5AC mucins obtained from airway materials and serum. HS01 and HS02 represent sputum obtained from two healthy persons, TF07 and TF08 represent tracheal fluid from two CCA patients and sM286 represents serum from CCA patient. W = whole or intact mucin; R = reduced subunit mucin.

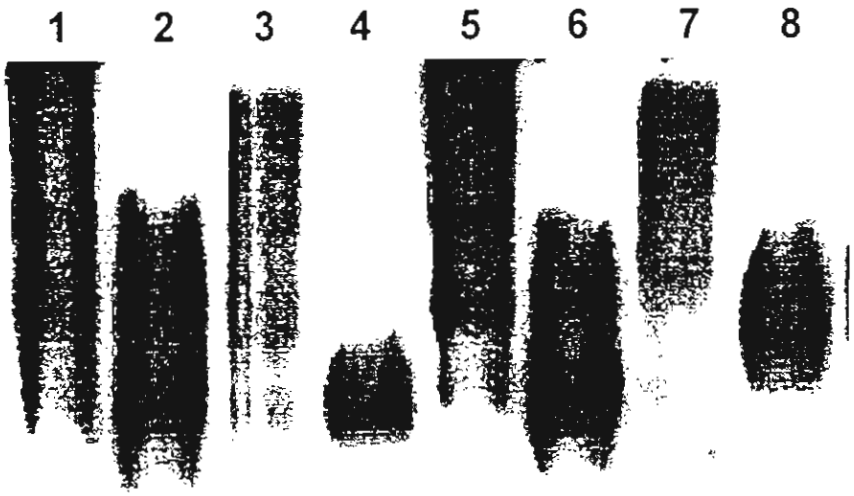


Figure 4-13 Electrophoretic patterns of tracheal lavage and its paired serum samples. Electrophoretic patterns of MUC5AC mucin from airway materials and the corresponding serum obtained from two CCA cases (1-4 and 5-8). Lanes 1, 3, 5 and 7 are intact mucins; lanes 2, 4, 6, and 8 are reduced subunit mucins. 1-2 and 5-6 are tracheal lavages; 3-4 and 7-8 are serum samples.

The associations of sMUC5AC electrophoretic patterns and patients' survival were estimated using Kaplan-Meier curve comparing between CCA patients who possessed normal and abnormal sMUC5AC mucin patterns. The data showed that there was no significant difference of the overall survival between these two groups of patients (P = 0.335) (data not shown) and their median survival times were of 145 days (95% CI; 83.82-206.18) and 148 days (95% CI; 92.61-203.39), respectively. As a result, the electrophoretic pattern of sMUC5AC mucin was not associated with the outcome of the patients.

Table 4-14 The clinical association of sMUC5AC pattern

Variables	n	Patterns of sMUC5AC		P value
		Normal	Abnormal	
1. Age	101	45	56	0.112
a. ≤ 56 years	56	21	35	
b. > 56 years	45	24	21	
2. Gender	101	45	56	0.161
a. Male	69	34	35	
b. Female	32	11	21	
3. Blood group	99	43	56	0.342
a. O	26	14	12	
b. A	20	7	13	
c. B	45	17	28	
d. AB	8	5	3	
4. Tumor type	100	45	55	*0.001*
a. Central	22	3	19	
b. Peripheral	51	31	20	
c. Combined	27	11	16	
5. Histological grading	72	31	41	0.384
a. Papillary	14	7	7	
b. Well diff.	44	16	28	
c. Moderate diff.	7	3	4	
d. Poorly diff.	7	5	2	

able 4-14 The clinical association of sMUC5AC pattern (cont.)

Variables	n	Patterns of sMUC5AC		P value
		Normal	Abnormal	
6. Tumor stage	100	45	55	0.791
a. I-III	7	3	4	
b. IVA	21	11	10	
c. IVB	72	31	41	
7. Tumor mass	34	18	16	1.000
a. ≤ 5 cm	7	4	3	
b. > 5 cm	27	14	13	
8. Jaundice status	98	45	53	*<0.001*
a. No	53	33	20	
b. Yes	45	12	33	
9. ALP	92	40	52	0.052
a. Low (≤ 147 U/L)	19	12	7	
b. High (> 147 U/L)	73	28	45	
10. CEA	96	45	51	0.423
a. Low (≤ 2.5 ng/ml)	25	10	15	
b. High (> 2.5 ng/ml)	71	35	36	
11. CA19-9	97	45	52	0.386
a. Low (≤ 100 U/ml)	45	23	22	
b. High (> 100 U/ml)	52	22	30	

4.3 Physical Characterization of Electrophoretic Patterns of sMUC5AC Mucins

As demonstrated in 4.2.7, three unique electrophoretic patterns of sMUC5AC mucin were observed in serum from CCA patients (Figure 4-11). The molecular characteristic of each sMUC5AC related to its electrophoretic patterns were analyzed.

Basically, charge size and shape are responsible for the movement of molecules in the agarose gel electrophoresis. Therefore, the physical features e.g., charge, size, density

and neutral monosaccharide composition of each sMUC5AC electrophoretic pattern were characterized, using anion-exchange chromatography, rate-zonal centrifugation, isopycnic centrifugation and HPAEC-PAD.

4.3.1 Partial Purification of MUC5AC Mucins from CCA Sera

Serum was chromatographed on Sepharose CL-2B column and eluted with 4 M GuHCl as described in Materials and Methods. The eluent was monitored for absorbance at 280 nm (Figure 4-14). Analysis of MUC5AC mucin by slot blot showed that sMUC5AC mucin was presented in the void fractions. The MUC5AC signals in fractions (29-40) after the void volume may be either non-specific binding proteins, or MUC5AC precursor, or partially glycosylated forms of MUC5AC, or complex material (from agarose electrophoresis, data not shown). To obtain only mature (fully glycosylated) form of MUC5AC mucin, void fractions (19-28) were pooled and kept for physical characterization.

4.3.2 Charge Distribution Analysis by Mono-Q column

Anion-exchange chromatography was performed in order to explore charge density of each electrophoretic pattern of sMUC5AC mucin. Void material obtained from Sepharose CL-2B gel filtration column was reduced and chromatographed on a strong anion resin (Mono-Q) column with linear gradient of 0-0.4 M $LiOCl_4$. Figure 4-15 demonstrated the different charge patterns obtained. The reduced subunit of the ladder type possessed the highest negative charge density which corresponded well with the farthest migration of this mucin in the agarose gel electrophoresis.

The slow sMUC5AC pattern yielded two charge peaks of reduced subunits which were compatible to the result of agarose gel electrophoresis (two reduced subunit bands were observed: S1, a slow band and S2, a fast band) (Figure 4-11). Agarose gel electrophoresis revealed that the first peak from the Mono-Q column was the slow migrated reduced subunit (S1), whereas the second peak (higher charge) was found in both, the slow (S1) and fast (S2) reduced subunits (data not shown).

The smear pattern yielded a peak of reduced subunit located between the first (low charge) and the second (high charge) peaks of the slow pattern. Electrophoresis revealed that the reduced subunits of smear sMUC5AC pattern (fractions 13-18) were gradually increased in charge density and migratory distance in the agarose gel. This may result in the smear appearance of the intact and reduced subunit found in agarose gel

electrophoresis.Comparisons of the chromatogram profiles obtained from Mono-Q anion-exchange column implied that the reduced subunit of sMUC5AC mucin from each electrophoretic pattern had different net negative charge: ladder > slow 2 (high charge form) > smear > slow 1 (low charge form).

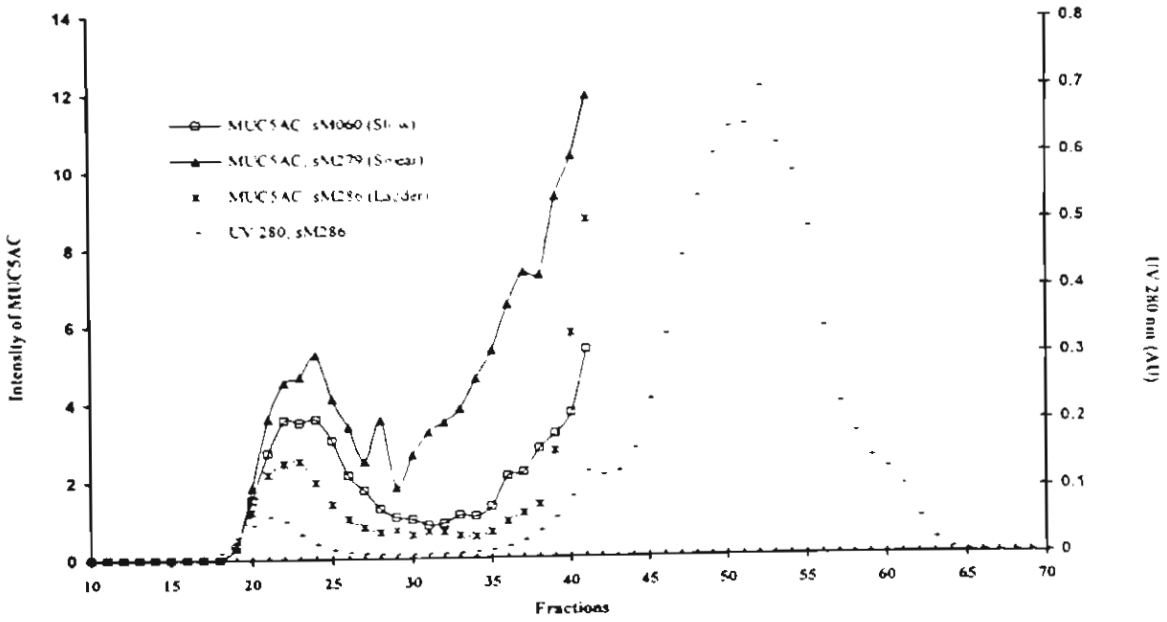


Figure 4-14 Chromatograms of sMUC5AC obtained from Sepharose CL-2B column. Crude serum obtained from CCA patients with slow (sM060), smear (sM279) and ladder (sM286) patterns was separately loaded to a Sepharose CL-2B column. The eluent was monitor for absorbance at 280 nm. Fractions 10-40 were screened for MUC5AC mucin by slot blot. MUC5AC-containing fractions (19-28) were pooled for further physical characterization.

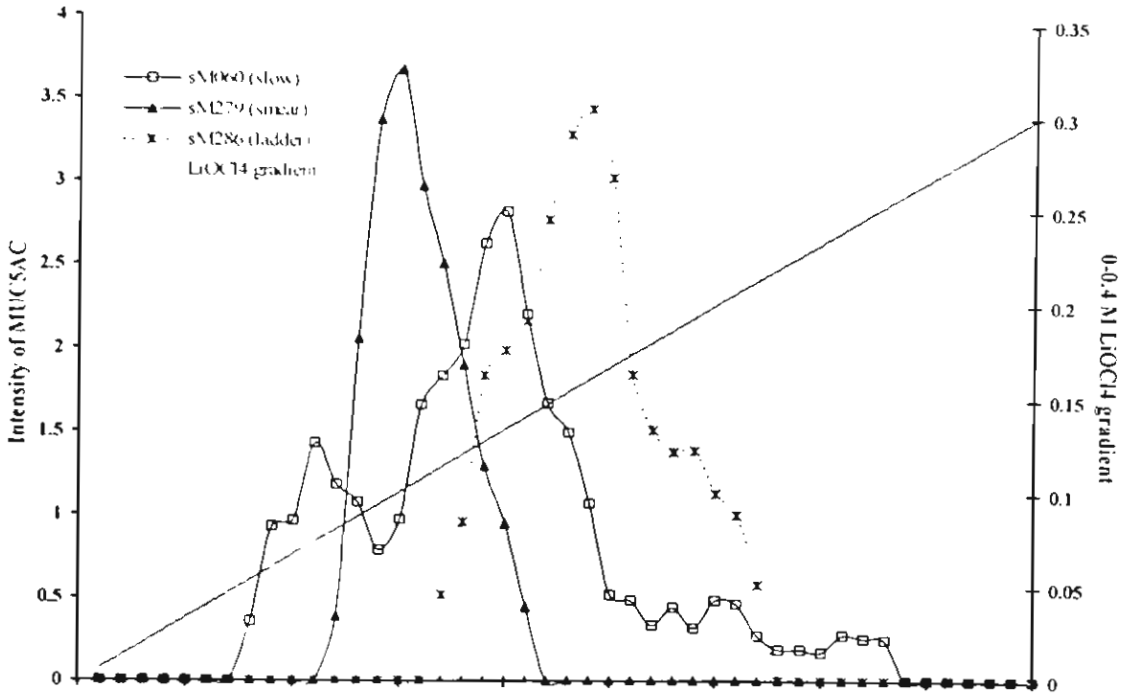
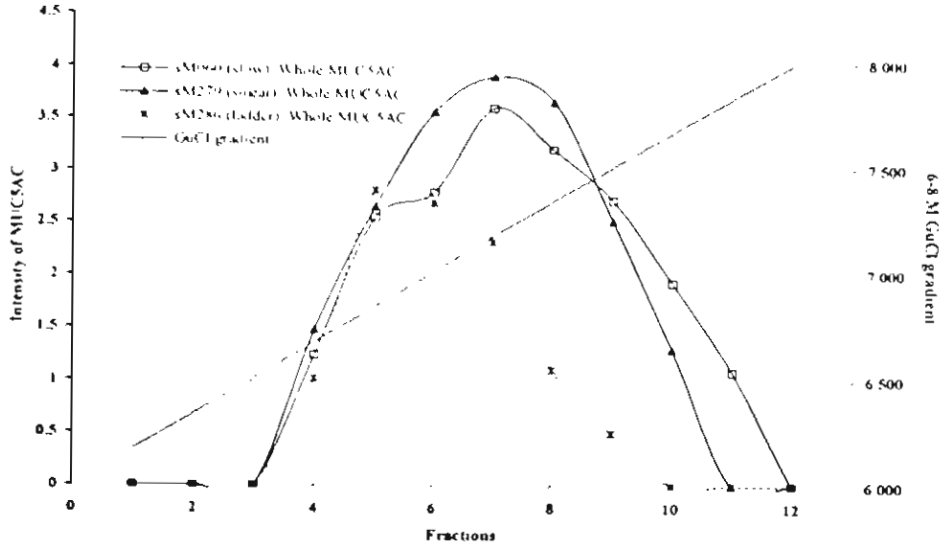


Figure 4-15 Chromatographic profiles of sMUC5AC mucin obtained from Mono-Q column. The void fractions from Sepharose CL-2B column were reduced and dialyzed against 6 M urea prior to applying to Mono-Q anion-exchange column. Bound material was eluted by a gradient of 0-0.4 M $LiOCl_4$ (shad line). Eluted fractions were slot blotted to nitrocellulose membrane and detected for MUC5AC mucin.

Zonal separation of MUC5AC molecules according to relative mass was carried out by rate-zonal centrifugation. Figures 4-16A and B compare size between the intact and reduced subunit forms of sMUC5AC. All intact mucins gave a broad peak. The intact form of the smear pattern provided a symmetric profile while the other showed irregular shapes. This may indicated the gradual sizes of mucin molecules in these intact mucins. Sizes of the intact sMUC5AC mucins slightly differed between slow and smear patterns but they were obviously bigger than the ladder type (slow > smear > ladder) (Figure 4-16A). Size of each sMUC5AC pattern was decreased after reduction. Relative size comparison between reduced subunit of sMUC5AC mucin revealed similar appearance to the intact mucin (slow > smear > ladder) (Figure 4-16B).

A; Intact mucin



B; Reduced subunit mucin

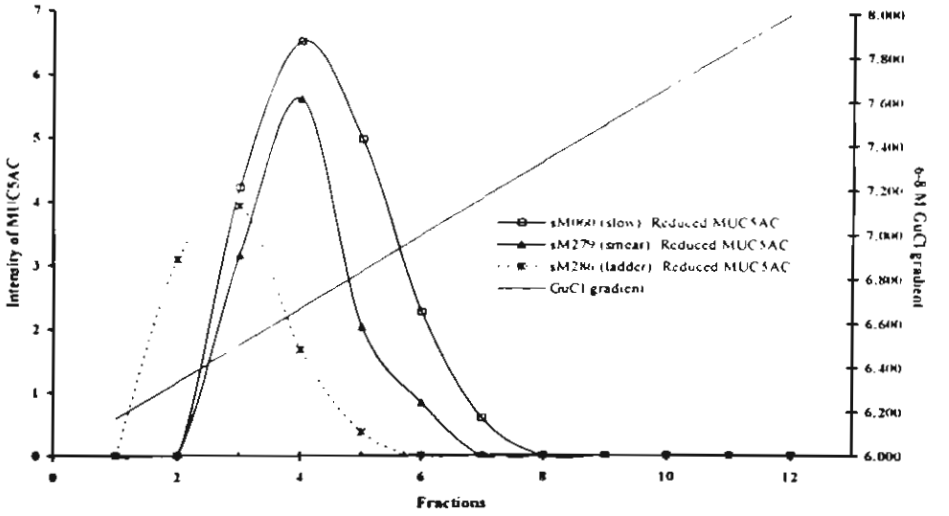


Figure 4-16 Size of intact and reduced subunits of sMUC5AC mucin determined by rate-zonal centrifugation. Intact and reduced void materials from Sepharose CL-2B column subjected to a gradient of 6-8M GuHCl as described in the Materials and Methods. A = intact mucin; B = reduced subunit mucin.

The resolution of size separation of the reduced subunits was enhanced by centrifuging sample at a longer period (7 hours). Under this condition, mucin of the reduced slow pattern showed two different sizes of reduced subunits (Figure 4-17).

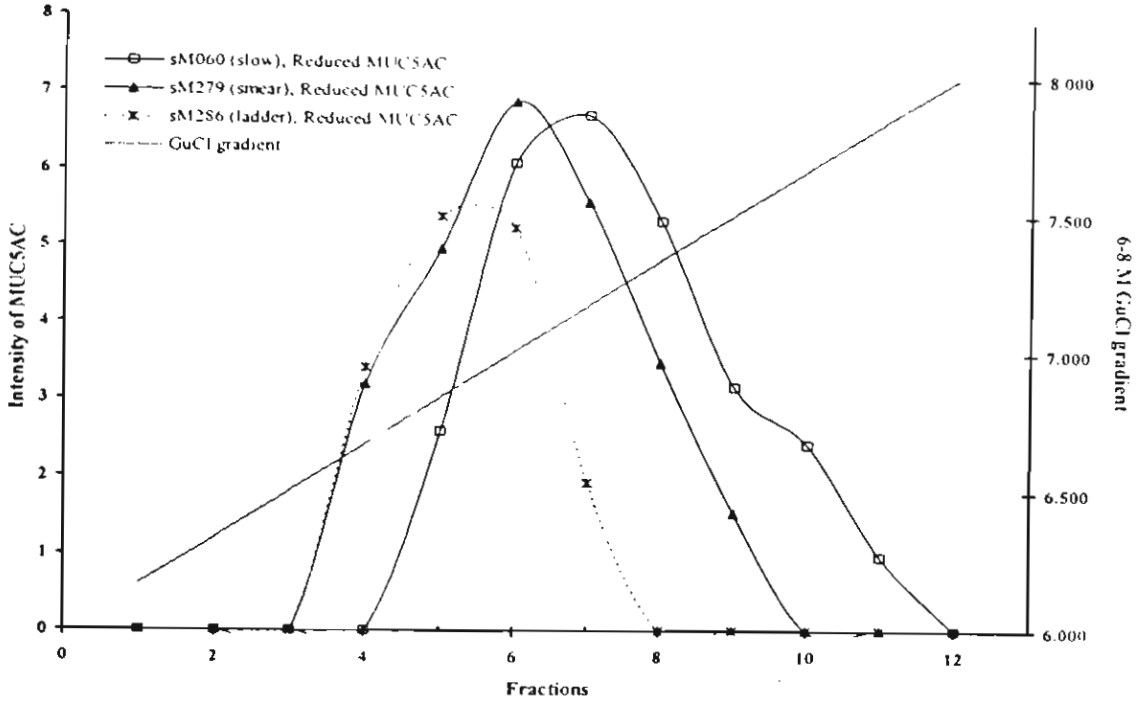


Figure 4-17 Size of reduced subunit of serum MUC5AC mucin determined by rate-zonal centrifugation. Void materials from Sepharose CL-2B column were reduced and subjected to rate-zonal centrifugation and centrifuged for 7 hours.

4.3.3 Density of sMUC5AC Mucin Determined by Isopycnic Centrifugation

CsCl density gradient centrifugation was employed for separation mucin regarding to density. The increasing of carbohydrate to protein ratio in glycoprotein causes the increasing of density, which means more sugar content gaining more density. In the present study, CsCl density gradient centrifugation was performed to estimate the density distribution of sMUC5AC mucin comparing between the three different patterns.

The result showed that ladder type sMUC5AC mucin had the lowest density comparing to those of slow and smear patterns, in both intact (Figure 4-18A) and reduced subunit states (Figure 4-18B). Since, the ratio carbohydrate to protein determines the density of glycoprotein therefore it suggested that comparing to other patterns, the ladder

sMUC5AC subunit had a less glycan attached. In contrast, the highest density of slow pattern sMUC5AC indicated a large molecule with copious oligosaccharides attached. The density of smear pattern was intermediate in between the slow and the ladder patterns.

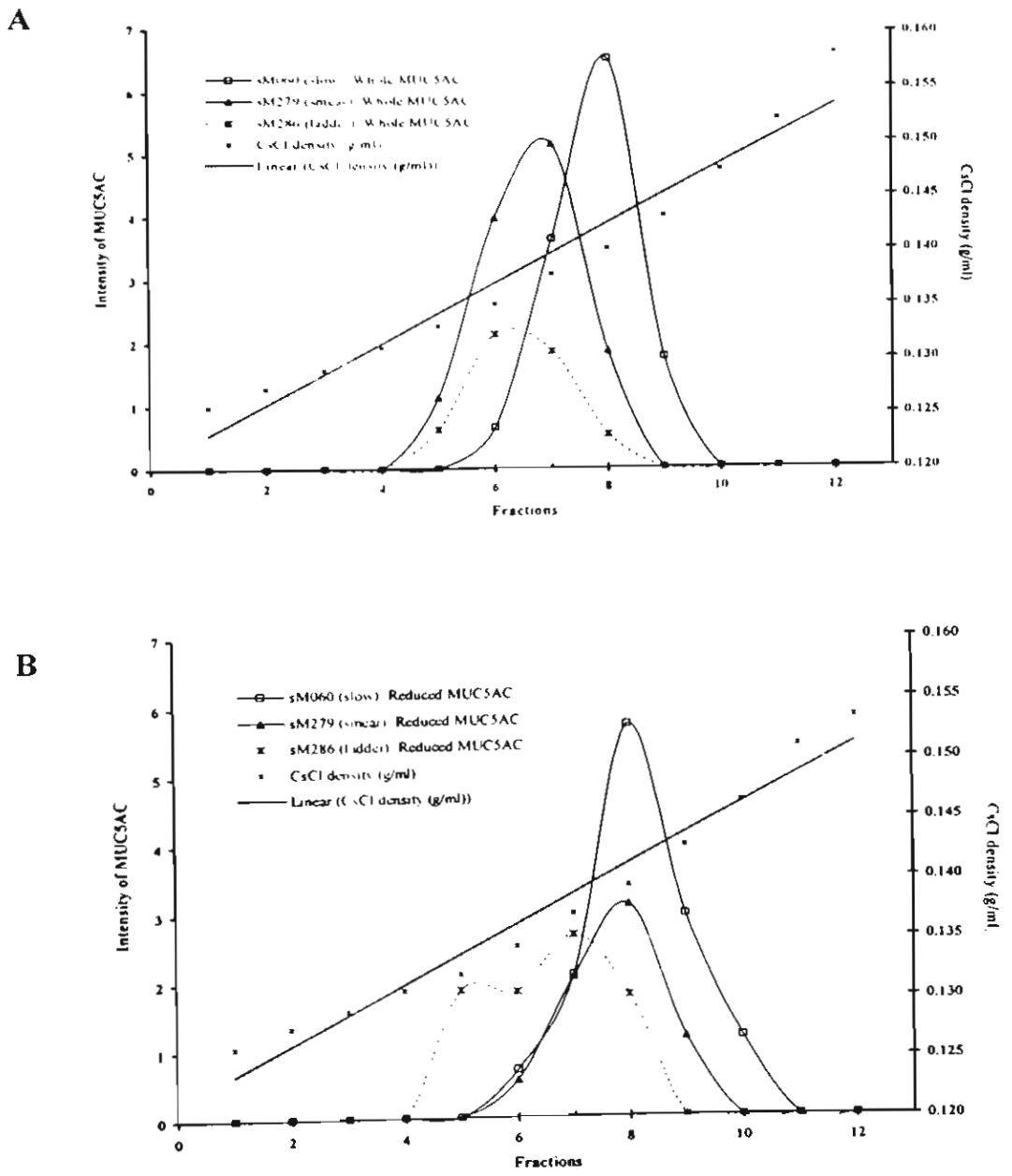


Figure 4-18 CsCl density gradient centrifugation of intact and reduced subunit of sMUC5AC mucin. Reduced mucins were mixed into CsCl solution as described in Materials and Methods. Intact mucin (A), reduced subunit mucin (B).

4.3.4 Composition of Neutral Monosaccharide in MUC5AC Mucind

Neutral monosaccharides (GalNAc, GlcNAc, Gal, Glc, Fuc, and Xyl/Man) composition of mucins was determined by high performance anion-exchange chromatography with pulsed amperometry detection (HPAEC-PAD) comparing between the different patterns of sMUC5AC mucins. Relative total amount and percentage of each neutral monosaccharide are reported in Table 4-15. The slow pattern sMUC5AC had the highest content of neutral sugars, and high ratios of GlcNAc and Gal to GalNAc. This suggested that slow sMUC5AC mucin had more sugar residues than smear and ladder types. The high amount of fucose found in this mucin indicated that the low charge density appearance of this mucin (Figure 4-15) may be due to this sugar. The ladder pattern of sMUC5AC possessed the lowest neutral monosaccharide content and low ratios of neutral monosaccharides to GalNAc. These suggested that the mucin composed of short (small) glycans.

The results indicated that three forms of sMUC5AC mucin differed in glycan part leading to the diversity of entire charge, size, density and probably shape. Basically, the negative charge of mucins was prominently contributed by sialic acid and sulfate residues. To explore whether the negative charge density of each sMUC5AC form was determined by sialic acid, the desialylation experiment was attained.

Table 4-15 Neutral monosaccharide compositions of the three sMUC5AC mucin patterns

Neutral Sugars	Slow sMUC5AC (sM060)		Smear sMUC5AC (sM279)		Ladder sMUC5AC (sM1286)	
	%Sugars	Ratio to GalNAc	%Sugars	Ratio to GalNAc	%Sugars	Ratio to GalNAc
Fucose	9.09	1.20	3.64	0.33	UD	UD
GalNAc	7.58	1.00	10.91	1.00	10.26	1.00
GlcNAc	34.85	4.60	27.27	2.50	28.21	2.75
Galactose	30.30	4.00	25.45	2.33	23.08	2.25
Glucose	13.64	1.80	25.45	2.33	30.77	3.00
Xyl/Man	4.55	0.60	7.27	0.67	7.69	0.75
Relative amount of neutral monosaccharides ($x 10^{-6}$)	690		466		398	

UD: Undetectable

4.3.5 Effect of Sialic Acid on the Migration of MUC5AC Mucins

In order to investigate the role of sialic acid on the migration of sMUC5AC mucin on agarose gel electrophoresis, sialic acid residues were removed from the mucin by neuraminidase prior to performing agarose gel electrophoresis. Figure 4-19 shows the electrophoretic pattern of each sMUC5AC form before and after neuraminidase treatment. The reduced subunits of all sMUC5AC patterns remained at the top of the gel after sialic acid residues were cleaved. The data suggested that the different migratory pattern of each type was principally influenced by s the negative charge of sialic acids. The farthest movement of reduced subunit of the ladder pattern implied the larger amount of sialic acid attached on the mucin comparing to other two patterns.

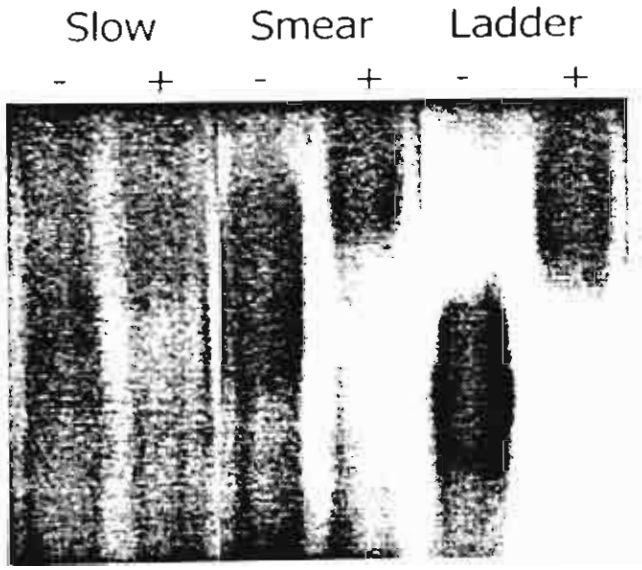


Figure 4-19 Electrophoretic patterns of reduced subunits of sMUC5AC mucins treated with neuraminidase. Partially purified mucins were treated with neuraminidase enzyme (37°C for 10 min) before the reduction. The reduced samples were then electrophoresed on 0.7% agarose gel, transferred to nitrocellulose and detected for MUC5AC mucin. (-) indicates without and (+) indicates with neuraminidase treatment.

4.3.6 Sugar Moieties of MUC5AC Mucins Determined by Lectin Blotting

The results of physical characterization (4.3.2-4.3.6) demonstrated that the difference of the three electrophoretic patterns of sMUC5AC mucins were due to the carbohydrate part in term of composition and/or content. In order to investigate the sugar moieties of these mucins, lectin blottings were accomplished. The lectin blottings are shown in Figure 4-20 and summary of the lectin-specific sugar determinants found for each pattern of sMUC5AC mucin is presented in Table 4-16. The data clearly showed the dissimilarity in sugar composition among the three forms of sMUC5AC mucins, except sugar recognized by SJA and SBA which were likely to be equally presented in all forms.

The summary table (Table 4-17) compares all physical characteristics among the three electrophoretic patterns of sMUC5AC mucin obtained from 4.3.2-4.3.7, and the molecular structure of each pattern was also postulated.

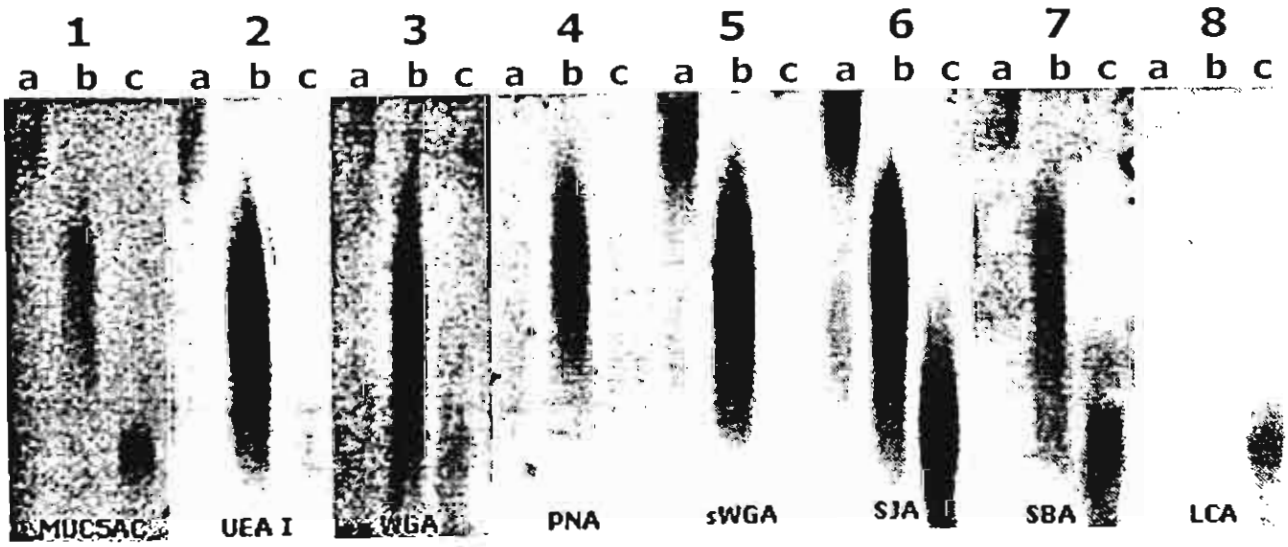


Figure 4-20 Lectin blottings of sMUC5AC mucin. Partially purified MUC5AC mucins from Sepharose CL-2B column were reduced and electrophoresed as described in 3.4.3. Reduced subunits of sMUC5AC from (a) slow, (b) smear and (c) ladder patterns were probed with MUC5AC antibody (1) or various lectins (2-8) and visualized by ECL detection.

Table 4-16 Lectin blottings of three different electrophoretic patterns of sMUC5AC mucin

Patterns of sMUC5AC mucin	Lectins						
	UEA-I	WGA	PNA	sWGA	SJA	SBA	LCA
Slow	+	+	UD	+++	+++	++	UD
Smear	+++	+++	+++	+++	+++	++	UD
Ladder	+/-	+/-	UD	UD	+++	++	+

UD: Undetectable, +/-: Weakly positive, +: Positive, ++: Moderately positive, +++: Strongly positive

Table 4-17 Summary of physical characteristics of sMUC5AC mucins

Physical characteristics	Electrophoretic patterns		
	Slow	**Smear**	**Ladder**
Charge density	2 charge forms (lowest and moderate)	Moderate	Highest
Size	Biggest	Moderate	Smallest
Density	Highest	Moderate	Lowest
Neutral oligosaccharide			
- Total content	Highest	Moderate	Lowest
- Fucose content	Highest	Moderate	UD
Neuraminidase treatment	Not move	Not move	Not move
Lectin- specific sugar moieties	Unique	Unique	Unique
Postulate molecular structure	large molecule linked with the huge low charge glycans	intermediate form between ladder and slow types	small molecule attached with the short-high charged glycans

V. DISCUSSION AND CONCLUSION

5.1 Expression of Apomucin in CCA Tissues

Immunohistochemistry of MUC1, MUC2, MUC5AC and MUC6 apomucin of CCA tissues revealed that MUC1 apomucin was over expressed. It was found at low amount in normal biliary cells but at greater amount in cancerous cells. It was in 74% of CCA cases. MUC1 apomucin was mainly localized at the apical surface and lumen of biliary duct suggesting the existence of both membrane-associated and secreted forms of MUC1 apomucin. This observation was also found in Japanese CCA cases (Sasaki et al., 1996, Higashi et al., 1999). However, serum MUC1 mucin could not be detected by the method used in this study. Up-regulation of MUC1 apomucin was reported in several cancers such as breast, lung, colorectal pancreas and cervical cancers (Ajioka et al., 1996, Nguyen et al., 1996, Apostolopoulos et al., 1999, Kim et al., 2002a) and seems to be a common feature of carcinoma

The role of MUC1 overproduction has been demonstrated to configure the binding properties of tumor cells, facilitating the metastatic spreading (McDermott et al., 2001). Hence, the overexpression of MUC1 found in CCA may concert the metastatic potential of this cancer. The association of MUC1 expression in CCA tissues and vascular invasion observed in this study supports this suggestion. It has been shown that up-regulation of MUC1 leaded to increasing the ability of cancer cell invasion (Suwa et al., 1998, Schroeder et al., 2003) and inducing cell detachment of prostate cancer cells (Huang et al., 2004). These observations suggest the dual roles (adhesion and anti-adhesion) of MUC1 mucin. In this study, therefore, overproduction of MUC1 apomucin may provide dual roles of an anti-adhesive effect mediated by either its length or charge repulsion by negatively charged sialylated O-linked glycans (Wesseling et al., 1996), and adhesive effect mediated via endothelial (E) -selectin ligands such as sialyl Le^x and sialyl Le^a to facilitate the transmigration through endothelial layer (McDermott et al., 2001, Fernandez-Rodriguez et al., 2001).

MUC2 apomucin has been shown to express focally in CCA tissues with more frequent in goblet cells presented in large bile ducts (Sasaki et al., 1996, Sasaki et al., 1998a; Sasaki et al., 1998b). The MUC2 expression profile found in this study agreed with the previous reports. MUC2 apomucin was not found in nonneoplastic bile duct cells

and infrequently expressed (26%) in CCA tissues with prominent staining in the secretory vesicles.

In biliary tract, MUC5AC apomucin was not expressed in nonneoplastic intrahepatic biliary tree and only focally, if present, in large bile ducts but frequently expressed in the large bile ducts of hepatolithiasis and CCA patients (Sasaki et al., 1998a; Sasaki et al., 1998b). Similar to the previous studies, MUC5AC apomucin was not observed in nonneoplastic bile ducts but dramatically expressed (64%) in CCA tissues. The neoexpression (de novo expression) of MUC5AC mucin was also observed in pancreatic cancer (Kim et al., 2002a). MUC6 apomucin was reported to be widely expressed in nonneoplastic biliary epithelial cells, particularly in intramural peribiliary glands and frequently expressed (80-100%) in the bile duct of cancerous area (Sasaki et al., 1998a; Sasaki et al., 1998b). This observation was contradicted to the present study, MUC6 apomucin was also found in peribiliary glands but was infrequently expressed (28%) in CCA tissues. Low expression of MUC6 may reflect a unique characteristic of CCA in Thai patients.

High level of MUC5AC apomucin in CCA tissues was found to relate to the progressive potential, advanced staging and invasion via neural fascicle. The direct evidence demonstrating the role of MUC5AC in progressiveness of cancer cell has not been established. However, the roles of MUC5AC mucin on tumor progression can be proposed as follow. Since, MUC5AC is a gel-forming mucin which can form a mucus layer covering the cells, MUC5AC-producing tumor cells therefore may use the mucus layer to prevent the attacks from harmful substances as well as from host immune cells. The loss of function of adhesion molecules: E-cadherin and the E-cadherin independent cell-cell interaction in HT-29 5M21 cell line, a highly-MUC5AC secreting cell have been demonstrated (Truant et al., 2003). This might support the anti-adhesive role of MUC5AC-producing cells to promote the detachment of cancer cells from tumor mass. The related study showed that HT-29 MTX, which mainly produced MUC5AC mucin and had a reduction of sialyl Le^x and sialyl Le^a ligands, had a lower adhesive capacity to E-selectin comparing to its parent, HT-29 cell line (Kitamura et al., 1996). This may suggest a less vital role of MUC5AC mucin in intravasation and extravasation processes but again emphasize the anti-adhesive role of MUC5AC mucin in this cell line. The association of MUC5AC-producing CCA cells and neural invasion found in the present study suggests the presence of adhesion ligand(s) on MUC5AC mucin that can bind to adhesion

molecules of neural cells which promotes tumor establishment and migration along the neural bundle. The evidence to support this speculation however has not been demonstrated.

MUC1 apomucin is an independent marker for poor prognosis of patients with CCA. The prognostic value for MUC2, MUC5AC and MUC6 was not revealed in the present study. The prognostic potential of MUC1 apomucin expression was suggested with hazard ratio of 2.42 adjusted for covariates. This finding agrees well with other studies. High expression of MUC1 apomucin has been reported to relate to the worse outcome of patients with intrahepatic (Higashi et al., 1999) and extrahepatic CCA (Takao et al., 1999). The prognostic value of MUC1 apomucin observed in the present study can be extrapolated to all types of CCA except distal extrahepatic type.

5.2 Determination of sMUC5AC mucin in CCA patients

Detection of MUC5AC mucin in human serum using agarose gel electrophoresis and immunoblotting was first reported in this study. MUC5AC was detected mainly in sera from CCA patients with 96.8% specificity and 62.5% sensitivity. It was detected in 3% of non-CCA subjects and none of opithorchiasis and healthy persons. The result suggests the high power of sMUC5AC mucin in discrimination of CCA. Serum MUC5AC mucin was detected in one from 37 of gall bladder stone patients and one from 11 of common bile duct stone. The overproduction of MUC5AC found in these conditions may be the consequence of chronic irritation and inflammation due to stone which lead to the overproduction of inflammatory mediators and a vast production of mucins (Shoda et al., 1999, Borchers et al., 1999). The increased expression of MUC5AC mRNA has been reported in dysplastic cells of stone-containing intrahepatic bile ducts compared with normal bile duct controls (Lee and Liu, 2001). Furthermore, hepatolithiasis or intrahepatic bile duct stone has been known as one of predisposing factors of CCA. The presence of sMUC5AC mucin might therefore indicate the progression of transformation from stone-irritating biliary cells to neoplastic cells. Serum MUC5AC mucin was also found in 2/3 of esophagus, 2/7 of ampulla of Vater and 2/10 of pancreatic cancers. MUC5AC is not a constitutive mucin in esophagus but it has been found to strongly express in precancerous situation, Barrett's esophagus (specialized intestinal metaplasia) (Arul et al., 2000, Warson et al., 2002) and squamous cell carcinoma of esophagus (Labouvie et al., 1999). Therefore, the sMUC5AC mucin found in esophagus cancer patients might be originated

from either precancerous metaplastic lesion or the tumor burden. In pancreatic tumor, it was likely possible that sMUC5AC mucin might be shaded by tumor tissue, since several articles demonstrated the neoexpression of MUC5AC in pancreatic tissues (Balague et al., 1995, Yonezawa et al., 2002, Luttges et al., 2002, Terris et al., 2002). There is no report of MUC5AC expression in cancer of ampulla of Vater, so far. However, the cells of ampulla of Vater and pancreas are originated from the same cell linage therefore their apomucin expressions might be similar in term of vast production of MUC5AC mucin in the tissue.

Several serum markers have been identified and evaluated for diagnosis of CCA. CA19-9 (cut-off = 37 U/mL) provided the sensitivity and specificity of 56.7% and 64.4%, respectively whereas IL-6 (cut-off = 0.18 ng/mL) provided sensitivity and specificity of 71.1% and 26.7%, respectively (Tangkijvanich et al., 2004). The low specificities of these two markers reduced the value of the test in differentiating CCA from other liver cancers, in contrast to sMUC5AC mucin in the present test, no positive test was found in hapatoma cases. CA19-9 is the most frequent marker reported for CCA. However, the diagnostic value of this test is controversial Recent study suggested that serum CA19-9 determination was a useful additional test for differential diagnosis of CCA with sensitivity and specificity of 77.14% and 84.78%, respectively (Qin et al., 2004). The other independent study found that biliary CA125, CEA and CA19-9 yielded specificities of below 80% (75.7%, 33.3% and 60%, respectively) (Chen et al., 2002). In contrast, the unreliability of $CA_{19\text{-}9}$ and CEA used for identifying CCA patients with primary sclerosing cholangitis was stated (Bjornsson et al., 1999). Other marker, such as serum biliary alkaline phosphatase, was suggested to differentiate non-jaundiced CCA patients from other non-jaundiced carcinoma patients with the sensitivity, specificity, PPV and NPV of 85%, 79% and 81% and 83%, respectively (Bhudhisawasdi et al., 2003). Detection of sMUC5AC mucin therefore is the most reliable diagnostic marker for CCA comparing with other markers documented so far.

The multivariate analysis using logistic regression showed that the status of tissue MUC5AC apomucin and staging of tumor (stage IVB) possessed a significant magnitude of relation to the presence of sMUC5AC mucin. The correlation between the expression of MUC5AC mucin in tissue and serum suggested that sMUC5AC mucin was originated from CCA mass. The correlation of sMUC5AC mucin and the late stage of tumor implied that MUC5AC mucin may play a role in the progression of CCA. This observation agrees well with the result obtained from tissue MUC5AC discussed in 5.1. Using Cox

proportional hazard model indicated that sMUC5AC was an independent prognostic marker for CCA. The association of MUC5AC mucin expression and the poor outcome of patients may be based on the site of tumor. The MUC5AC-negative tumors related to poor clinicopathological parameters and worse survival was found in colorectal carcinoma (Kocer et al., 2002) and the reduction of tissue MUC5AC apomucin was suggested as an independent marker for worse outcome in patients with gastric carcinoma (Baldus et al., 2002). This discrepancy remained to be elucidated what the actual role of MUC5AC mucin in these cancers.

Various electrophoretic patterns of sMUC5AC mucin were first observed in this study. The ladder electrophoretic pattern of MUC5AC mucin has been demonstrated in normal and asthmatic respiratory secretions (Thornton et al., 1996) and human intestinal cell line HT-29 (Sheehan et al., 2000). Each ladder band represented a different-sized oligomer and the proper multimerization of gel-forming mucins (Sheehan et al., 2000). MUC5AC mucin detected in healthy sputum and tracheal lavage of CCA patients was also shown to be the ladder pattern. The smear and slow type sMUC5AC mucins found in the present study therefore were considered as extraordinary (abnormal) forms. The molecular basis of this different migratory pattern was explored. A number of studies have been reported the aberrant glycosylation of mucin found in cancer and this may be responsible for the bizarre electrophoretic patterns found in CCA-associated sMUC5AC mucin.

Even the original source of sMUC5AC mucin was not definitely proven in this study, however, several evidences indicated that MUC5AC found in serum of CCA patients was originated from tumor tissues. First, no MUC5AC mucin was detected in serum of healthy persons suggested that in general no constitutively-produced MUC5AC mucin secreted into blood circulation. Second, sMUC5AC mucin detected in serum of CCA patients was significantly correlated with MUC5AC expressed in CCA tissues. The overproduction of MUC5AC mucin from tumor may leak into blood circulation resembling the existence of serum MUC1 in breast cancer patients (Murray et al., 1995). The leaking of tumor secretion into bloodstream has been suggested to be due to the depolarization of cancer cells which causes the basolateral secretion of cell products (MUC5AC mucin) and easily get into bloodstream. Alternatively, metastasizing cancer cells existing in the blood may secrete mucin directly to the serum. The secreted mucins produced by cancer cells may be useful for either cytoprotecting in an extremely unfriendly condition or facilitating the metastasis such as to escape the immune

surveillance or providing the ligands for cell-cell interaction in metastatic process (Hollingsworth and Swanson, 2004).

Serum MUC5AC mucin from CCA patients exhibited three different electrophoretic patterns, of which the smear and slow patterns were identified as abnormal patterns. These two abnormal patterns of sMUC5AC mucin were significantly related to hilar type CCA and the presence of jaundice. Most of CCA originated at perihilar bile duct are jaundice, thus, the abnormal electrophoretic pattern of sMUC5AC mucin may relate to bile duct obstruction. This suggestion may be supported by the observation that high level of serum ALP, which is a biliary tract obstruction marker was found (45/52, 87%) in patients with abnormal sMUC5Ac pattern. It is evident that bile acids can stimulate mucin secretion in human gallbladder-derived biliary cells (Chignard et al., 2001), which may be a common protection mechanism of the epithelia against the detergent action of bile salts (Klinkspoor et al., 1999). The bile acid-induced mucin production was also observed in colon cancer cell lines (Shekels et al., 1996). Bile acids not only stimulate the mucin production but also alter the glycosylation process. Alteration of the expression of glycosyltransferases, particularly sialyltransferase by bile acids has been documented (Li et al., 1998). Thereby, the over-flooded of bile components in jaundice CCA may somehow activate MUC5AC synthesis and alter the glyco-synthesis of MUC5AC mucin generating the abnormal form in electrophoresis.

5.3 Physical Characteristics of sMUC5AC Mucins

Physical characteristics that affected the migration of mucin in electrophoresis - namely charge, size, density and monosaccharide content- were investigated by anion-exchange chromatography, rate-zonal centrifugation, isopycnic centrifugation and HPAEC-PAD. Comparing the data of each sMUC5AC form, the ladder sMUC5AC possessed the highest charge content, smallest size, lowest density and lowest neutral oligosaccharide content but *vice versa* for slow pattern sMUC5AC. Based on the assumption that core protein of MUC5AC mucin was not altered in carcinogenesis process, the high density of glycoprotein will suggests the high ratio of glycan to protein, a copious carbohydrate linked to core protein. The data therefore suggested that the ladder sMUC5AC type was the smallest molecule with short chain but highly-charged glycans whereas the slow sMUC5AC type was a huge molecule with long chain but low-charged glycans. The smear sMUC5AC mucin was an intermediate molecule of those two mucin

types. The proposed molecular components of these mucins were agrees well with the migration of mucins in the agarose gel electrophoresis. The reduced subunit of the ladder sMUC5AC type moved farthest distance while the reduced subunits of smear and slow types moved slower.

Fucose was detected in sMUC5AC mucins with slow and smear patterns but not in ladder pattern suggesting the fucosylation was gained in the abnormal types. So far, this observation has not been reported in other cancers, however the increasing of fucosylation has been documented in hepatoma (Noda et al., 1998). Desialylation of all mucin types indicated the difference of charge density among sMUC5AC patterns, and this may be prominently contributed by sialic acid. The result also suggested that the ladder type had higher sialylation compared to the smear and slow types. In addition, data from the lectin blotting demonstrated the different lectin-specific sugar epitopes among three electrophoretic types of sMUC5AC mucin. This may be due to the differences in their glycosylation pattern.

The specific sugar moiety of SJA and SBA is GalNAc residue, which is the first sugar of mucin-type O-linked glycans. The linkage of GalNAc to Ser/Thr-apomucin forms a Tn antigen which is believed to be a hallmark antigen of mucin and this antigen was dramatically expressed due to incomplete glycosylation (Itzkowitz et al., 1991; Itzkowitz et al., 1989, Therkildsen et al., 1993, Schmitt et al., 1995, Babino et al., 2000). In the present study, SJA- and SBA-specific epitopes were found in all three types of sMUC5AC mucin reflecting the occurrence of aberrant glycosylation in these mucins. This observation suggests an alternative assay for sMUC5AC mucin. A sandwich ELISA using antibody to MUC5AC and SJA/ SBA lectin will provide a specific detection of MUC5AC mucin in serum.

Since, 50-80% of the mucin mass is carbohydrate, the alteration in glycosylation of mucin therefore directly affects the entire charge, size and density of the mucin. Sialic acid and fucose residues are known as terminal sugars. Capping of sialic acid but not fucose determines the charge density of the molecule. Altered glycosylations of mucins, particularly sialylation and fucosylation have been reported in various cancers (Brockhausen et al., 1995), such as colon cancer (Bresalier et al., 1996) and hepatoma (Noda et al., 1998). The present study demonstrated clearly that the three forms of sMUC5AC differed in their glycoparts both sugar content and composition, particularly

the capping process. Comparing to the normal ladder form, the variant glycoforms, slow and smear sMUC5AC mucins exhibited lower charge, greater size and denser density due to an increase in glycosylation (especially fucosylation) and decrease sialylation. However, the process of which the glycofom of sMUC5AC mucin be altered and the biological role of these abnormal glycosylated mucins are remained to be elucidated. The prognostic value of the abnormal sMUC5AC in CCA patients was not shown in this study.

5.4 Conclusion

The aims of the present study were to explore the expression profile of apomucins (MUC1, MUC2, MUC5AC and MUC6) in CCA tissues, identify the CCA-associated mucin and its clinical significance.

MUC1 was overexpressed and MUC5AC was neoexpressed in CCA tumor tissues and hence were considered as CCA-associated mucins. Tissue MUC1 apomucin was found to associate with vascular invasion and proved to be an independent indicator for poor prognosis. MUC5AC apomucin was shown to be related to neural invasion but its prognostic potential was not found. MUC5AC mucin but not MUC1 could be detected in serum samples using agarose gel electrophoresis and immunoblotting. The diagnostic value of determination of sMUC5AC in discriminating CCA patients was first demonstrated in this study. Detection of sMUC5AC mucin provided the sensitivity, specificity, PPV and NPV of 62.57%, 96.86%, 92.56% and 78.66%, respectively. The presence of sMUC5AC mucin was significantly related to the expression of MUC5AC apomucin in CCA tissues and the late stage of tumor. The correlation between MUC5AC mucin expressed in CCA tissue and serum suggested that sMUC5AC mucin was secreted from CCA burden. In addition, this is the first report for sMUC5AC mucin in providing a prognostic value to independently predict the worse outcome in patients with CCA.

Three electrophoretic patterns of sMUC5AC mucin were identified in the present study: ladder, smear and slow patterns. The ladder pattern was proved to be a normal pattern of MUC5AC mucin whereas the smear and slow patterns were abnormal types. The expression of abnormal type MUC5AC mucin was associated with jaundice status of the patients suggesting the relation between the accumulation of bile and MUC5AC production. Bile components may activate the biosynthesis of MUC5AC mucin and alter the glycosylation.

Physical characterization of these three electrophoretic patterns demonstrated that these mucins were different in their carbohydrate content, composition as well as

glycosylation pattern. The abnormal type sMUC5AC mucins (smear and slow) had higher glycan content but lower charge density than the normal type (ladder). The molecular structures of these sMUC5AC mucins were postulated: the ladder sMUC5AC was a small molecule attached with the short-high charged glycans. The slow sMUC5AC was a giant molecule linked with the huge low charge glycans, whereas the smear sMUC5AC was an intermediate form between ladder and slow types.

5.5 Future Direction

The outcome of this study is the finding that MUC1 and MUC5AC mucins were CCA-associated mucins. Determination of MUC1 expression in tissue and MUC5AC in serum can be either diagnostic or prognostic markers for patients with bile duct cancer. Basically, MUC5AC mucin is a secreted mucin which forms a gel layer covering the cell surface and acts as a physical shield. In this study, the vast production of MUC5AC mucin was observed in CCA. The mechanism by which MUC5AC gene is regulated and the association of this mucin in tumor progression/ metastasis are raised for further investigation.

The biological roles of mucins in cancer concerting in invasion, metastasis and protection have been suggested (Hollingsworth and Swanson, 2004). MUC5AC carrying carbohydrate epitopes for *Helicobacter Pylori* binding has been demonstrated and proposed to participate in cell-cell recognition (Linden et al., 2002). Role of MUC5AC mucin on cell-cell and cell-matrix interactions leading to cancer progression remain to be explored. Moreover, how MUC5AC mucin provides a protective function for cancer cells during metastasis, particularly in blood circulation is also a challenging field. The abnormal glycosylation of sMUC5AC mucin was found to associate with jaundice status of the patients, thus it is interesting to investigate how the bile components can affect the expression of glycosyltransferases in these cancer cells. The definite mechanism by which MUC5AC mucin released to blood circulation is not proved in this study, whether the MUC5AC mcuin can be expressed and secreted by metastasized cells that circulated in blood is also remained to be elucidated. Since, the detection of sMUC5AC mucin provided a high specific diagnostic tool for CCA, alternative method to detect MUC5AC mucin using a more convenient assay such as ELISA should be developed.

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มหาวิทยาลัยขอนแก่น

หนังสือฉบับนี้ให้ไว้ เพื่อแสดงว่า

โครงการวิจัยเรื่อง: การศึกษามิวซินของมะเร็งท่อน้ำดีที่สัมพันธ์กับการติดพยาธิใบไม้ตับ (Characterization of mucin expressed in liver fluke associated cholangiocarcinoma)

ผู้วิจัย: รองศาสตราจารย์โสพิศ วงศ์คำ และคณะฯ

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คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น

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Serum MUC5AC mucin as a potential marker for cholangiocarcinoma

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Abstract

Aberrant expression of MUC5AC mucin is obvious in cholangiocarcinoma tissues, however, this mucin has never been detected in the serum. Using immunoblotting marked with antibody *vs.* MUC5AC core protein, we could detect MUC5AC mucin in the serum of 112 from 179 cholangiocarcinoma patients (62.6% sensitivity), two of the 62 with benign hepatobiliary diseases, six of the 60 with hepato-gastrointestinal cancer, and none in either the 60 active opisthorchiasis or 74 healthy persons. Detection of serum mucin in the serum of cholangiocarcinoma patients corresponded well to the MUC5AC expressed in individual tissues. Serum MUC5AC may be used to enhance the diagnostic accuracy of cholangiocarcinoma.

Keywords: Cholangiocarcinoma; Mucin; MUC5AC; Tumor marker; Bile duct

1. Introduction

Cholangiocarcinoma (CCA), bile duct epithelial cancer, is a relatively rare cancer that almost always presents with an extremely poor prognosis [1]. Whereas CCA is rare in Western countries, it is found frequently in Southeast Asia, especially North east Thailand where the liver fluke *Opisthorchi. viverrini* is endemic [2]. The relationship between infection with the liver fluke and CCA is strongly supported by both epidemiological [3,4] and experimental evidence [5,6].

Mucins are high molecular weight glycoproteins, which can be divided into secreted and membrane-bound forms. In many human carcinomas, the

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expression profile of mucins is altered, with certain mucins being up-regulated while others are down-regulated [7,8]. The majority of CCA in humans are adenocarcinoma and mucin producing [9]. Aberrant and altered mucin expressions in CCA are obvious. Extensively expressed MUC1 apomucin, focally expressed MUC2 apomucin and frequently expressed MUC3 apomucin in the central CCA types have been reported [10]. Also documented are the frequent and aberrant expression of the 'gastric type', MUC5/6 apomucin, in biliary epithelial cells with dysplasia [11] and non-invasive CCA [9,10,12,13]. Immunohistochemical studies have revealed that MUC5AC mucins are associated with the type and histological grading of the cancer [11] and the cirrhotic condition of the patient [9].

Our study provides the first evidence that MUC5AC apomucin, frequently and aberrantly expressed in CCA, can be detected with high sensitivity and specificity in the serum of CCA patients as compared to those with benign hepatobiliary diseases and hepato-gastrointestinal cancers. Moreover, the apomucin was not detected in the serum of healthy persons. The presence of serum MUC5AC corresponded well with the immunohistochemistry of individual CCA subjects.

2. Materials and methods

2.1. Subjects

Patients undergoing surgical resection of hepato-gastrointestinal cancer at the Department of Surgery, Faculty of Medicine, Khon Kaen University, between 1998 and 1999, were asked to volunteer in the study. Informed consent was obtained from each subject. The Ethics of Human Research Committee, Khon Kaen University, approved details of the study. Preoperative serum was obtained from 179 patients with CCA, 60 with hepato-pancreato-gastrointestinal cancer, and 62 with benign hepatobiliary diseases (Table 1). Serum samples from 74 healthy persons and 60 with active opisthorchiasis were included as controls. The UICC TNM classification and staging were used for tumor assessment. Cancer diagnosis was verified by histology whereas the diagnosis of benign disease was based on clinical and histological findings. Opisthorchiasis was defined for asymptomatic persons as the detection of *O. viverrini* eggs in the feces. Serum samples were stored at −20 °C until analysis. Surgical specimens including cancerous and non-cancerous hepatic portions from patients with CCA ($n = 72$) were obtained, fixed in 10% neutral buffered formalin, and embedded in paraffin.

2.2. Chemicals

MAN-5ACI was a polyclonal anti-sera raised against the synthetic peptide coupled to the keyhole limpet hemocyanin from the specific sequence within the MUC5AC mucin [14]. Goat anti-rabbit IgG-peroxidase produced by Zymed (San Francisco, USA) was used. The chemiluminescence reagent kit, Hyperfilm and Hybond nitrocellulose membrane were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

2.3. Agarose gel electrophoresis and immunoblotting

The presence of MUC5AC mucin was detected by peptide antibody to a sequence in the C-terminus of the core protein employed after Western blotting following an agarose electrophoresis step.

Serum (20 μl) treated with loading buffer to a final concentration of 40 mM Tris–acetate (pH 8.0), 5 mM EDTA, 0.1% SDS, 0.6 M urea and bromphenol blue was subjected to 0.7% agarose gel electrophoresis and vacuum-transferred to a nitrocellulose membrane [15]. After blocking with 0.05% Tween 20 and 5% non-fat dry milk in buffer, the membrane was incubated with 1:10,000 MAN-5ACI antisera and 1:8000 goat anti-rabbit IgG-peroxidase. The membrane was incubated with Chemiluminescence Reagent Plus and exposed to the Hyperfilm with an intensifying screen. The specificity of the MUC5AC antibody was confirmed when no immunoreactivity was observed when the antibody was omitted from the detection system.

Serum treated with 6 M urea and 10 mM DTT at 37 °C for 2 h and with 25 mM iodoacetamide in the dark for 30 min was used as reduced mucin. The treated mucin was mixed with loading buffer and subjected to electrophoresis as mentioned above.

Table 1
Characterization of subjects in the study of serum MUC5AC detection

Subject	No. of cases	No. of +ve cases (%)
Cholangiocarcinoma	179	112 (62.6)
Benign biliary diseases	62	2 (3.2)
Gallbladder stones	37	1
Cholecystitis and cholangitis	8	0
Common bile duct stones	11	1
Liver abscess	3	0
Hepatitis	1	0
Periductal fibrosis with fasiolıasis	1	0
Cholecystitis with opisthorchiasis	1	0
Hepato-pancreato-gastrointestinal tract cancers	60	6 (10)
Cancer of the		
Stomach	12	0
Ileum	1	0
Colon	14	0
Rectum	6	0
Ceacum	1	0
Gallbladder	2	0
Liver	4	0
Esophagus	3	2
Ampulla of Vater	7	2
Pancreas	10	2
Asymptomatic opisthorchiasis	60	0
Healthy persons	74	0

2.4. Immunohistochemical detection of MUC5AC mucin

Sections 4 μm thick were immunostained using avidin-biotin complex staining [16]. The sections were incubated with 1:1000 MUC-5ACI antisera followed by 1:300 biotinylated goat anti-rabbit immunoglobulin G (Vector Laboratories, Burlingame, CA). After washing, the sections were incubated with 1:300 of Streptavidin-peroxidase (Vector) and reacted with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) and 0.1% H_2O_2 in 50 mM Tris (pH 7.8). The positive staining was abolished when PBS was applied instead of the primary antibody.

The intensity of MUC5AC expression was semi-quantitatively classified into four groups on the basis of the percentage of positive tumor cells: 0%, negative; 1–25%, +1; 26–50%, +2; >50%, +3.

2.5. Statistical analysis

Statistics were performed using STATA software (StataCorp, College Station, TX). Sensitivity and specificity were calculated for the study population. The statistical significance was set for $P < 0.05$.

3. Results

Gel-forming mucins are responsible for the viscoelastic properties of the mucus gels found on the epithelial surfaces of the body. These macromolecules have an oligomeric structure stabilized by disulphide bonds and can be depolymerized by treatment with a reducing agent [17]. Agarose gel electrophoresis of unreduced MUC5AC mucins exhibited a major band just entering the gel with evidence of a 'ladder' of faster-migrating minor bands (Fig. 1, lane 2). Reduction of MUC5AC mucin with DTT yielded a faster band representing a monosubunit of the mucin (Fig. 1, lane 1).

In our study, 49.2% of the CCA cases were peripheral or intrahepatic CCA, 29.1% were central or extrahepatic CCA and 20.7% were a combination of both types. Of the 179 patients examined, 71.5% were

Fig. 1. Agarose gel electrophoresis of reduced and non-reduced MUC5AC mucins in serum of a CCA patient. (1) Reduced MUC5AC; (2) non-reduced MUC5AC.

men. The ratio of males ($n = 128$) to females ($n = 51$) was 2.5:1. Most of the tumors (88%) were advanced (pTNM IVA and IVB). The median age of the CCA patients was 56 years (range 33–84 years).

3.1. Diagnostic values of serum MUC5AC

Using immunoblotting, MUC5AC mucin was first demonstrated in the human serum (Fig. 1). MUC5AC was detected frequently in the serum of 112 of the 179 patients with CCA (62.6%). By contrast, only two of the 62 patients with benign hepatobiliary diseases (3.2%), and six of the 60 with hepato-gastrointestinal cancers (10.0%) were positive for serum MUC5AC (Table 1). No MUC5AC was detected in the serum of healthy persons or those with opisthorchiasis. The frequency of detection of MUC5AC apomucin in the serum of CCA patients was significantly higher than for the control groups ($P < 0.001$). According to the population studied, detection of serum MUC5AC gave a sensitivity of 62.6%, a specificity of 96.9%, and negative and positive predictive values of 78.7% and 93.3%, respectively.

The distinction between the CCA patients and those with other pathological disorders with similar clinical presentations, such as benign hepatobiliary diseases and those with hepato-gastrointestinal cancers, is of clinical importance. The presence of serum MUC5AC was identified in 63% of patients with CCA and excluded 100% of normal adults and those with opisthorchiasis, 96.7% with benign hepatobiliary diseases and 90% with hepato-gastrointestinal cancers.

3.2. Tissue MUC5AC and serum MUC5AC

The immunohistochemical localizations of polyclonal anti-MUC5AC antibody binding to MUC5AC apomucins expressed in CCA (a total of 72 primary CCA sections) were examined. MUC5AC was frequently expressed in CCA tumor tissues (48 of 72, 66.6%). The expression of MUC5AC was localized in the biliary epithelial cells of the tumor tissues but not the normal biliary cells. Stainings in the cytoplasmic, apical surface, lumen of the tumor gland, and nearby stromal tissues, were evident (data not shown). In all cases, the intensities were prominent only in the tumor cells located at the invasive front but not those located within the central tumor mass.

Detection of MUC5AC mucin from individual serum corresponded well with the expression of MUC5AC mucin in the tissues ($P < 0.001$) (Table 2). Twenty of the 24 CCA cases (83.3%) with negative MUC5AC in the tumor tissues were also negative for serum MUC5AC, and 38 of the 48 cases (79.2%) that exhibited MUC5AC in the tumor tissues were also positive for serum MUC5AC. In addition, detection of serum MUC5AC was associated with the degree of MUC5AC expressed in the tumor tissue. Almost all of the CCA of the patients (18/19, 94.7%) whose tissues possessed a high percentage of positive MUC5AC (+3) were positive for serum MUC5AC, whereas 62% of the patients with lower percentages of tissue MUC5AC (+1) were positive for serum MUC5AC (Table 2). These results imply the tumor

Table 2
Detection of serum and tissue MUC5AC in CCA patients[a]

Tissue MUC5AC	Serum MUC5AC		
	Negative	Positive	Total
0	20 (83.3)	4 (16.7)	24
+1	6 (37.5)	10 (62.5)	16
+2	3 (23.1)	10 (76.9)	13
+3	1 (5.3)	18 (94.7)	19
Total	30	42	72

[a] χ^2, $P < 0.001$. Numbers in parentheses are percentages.

origin of MUC5AC detected in the serum of CCA patients.

4. Discussion

Biliary epithelial cells in the intrahepatic large bile ducts constantly expressed MUC3, MUC6 and MUC5B apomucin, whereas MUC5AC, a secretory mucin found in abundance in gastric mucosa, is rarely expressed [11,18,19]. In CCA, extensively expressed MUC1 apomucin and focally expressed MUC2 apomucin were documented [11]. MUC5/6 apomucin and MUC5AC apomucin were more frequently expressed in 70.9% [10] and 57.1% [20] of CCA tumor tissues, respectively. Our MUC5AC immunohistochemistry data revealed aberrant expression of MUC5AC in 66.7% of tumor tissues, which agrees with the other reports [10,20]. The frequent and aberrant expression of MUC5AC apomucin in CCA suggests that biliary epithelial cells gain a gastric apomucin phenotype during carcinogenesis.

Not only was the aberrant expression of mucin found when cells turned cancerous, the missed transport of mucin and over-expression of a particular mucin were also observed in several cancers. Once the mucins gain access to the interstitial space, they are taken up into the blood stream where they can be detected. To our knowledge, only MUC1, MUC2, MUC5B and MUC6 have been detected in human serum [21]. So, ours is the first report of MUC5AC being detected in human serum. It may be that CCA has a particular propensity to secrete MUC5AC mucin into the blood because of mechanical blockage of the bile duct, loss of polarity of biliary cells or early blood vessel invasion.

A significant expression of MUC5AC was clearly demonstrated in the serum and bile duct epithelia of our series of CCA patients. Detection of MUC5AC mucins in CCA bile duct epithelia and not in non-malignant bile duct or liver parenchyma, revealed by immunohistochemical staining, provided strong evidence that circulating MUC5AC mucins originated in the CCA tissues. This conclusion was corroborated by a strong correlation between the level of MUC5AC expressed in the tumor tissues and the detection of MUC5AC in the serum.

Unlike CCA, several studies have reported decreased expression of MUC5AC in gastric carcinoma [22–24]. The expression of MUC5AC mucin in gastric tumor is associated with tumor type and tumor location [22,24–26]. We did not detect MUC5AC mucin in the serum of any stomach cancer either because of the low expression of MUC5AC in this type of cancer or because the mucin produced from the stomach is secreted through the intestinal tract. The latter was evidenced by the absence of serum MUC5AC detected in the serum obtained from any of the healthy persons who had high gastric mucins production (Table 1).

In our study, MUC5AC mucin was also detected in the serum of the patients (one of each) with gallbladder stones and common bile duct stones. The unusual detection of serum MUC5AC mucin in these two patients may be due to the inflammation caused by the stones. Evidently, the inflammatory cytokines produced by inflammatory cells (i.e. IL1 and TNFα) stimulate mucin gene expression and mucin release in the cancer cell line [27].

The clinical values of serum MUC5AC were clarified by determining the frequency of MUC5AC in the serum of CCA patients and the control groups. According to the population studied, MUC5AC apomucin was detected in the serum of CCA patients with 62.6% sensitivity and 96.9% specificity. The clinical utility of using serum MUC5AC for distinguishing between CCA patients and those with benign hepatobiliary diseases and cancer of the hepato-gastrointestinal tract was better than using the general serum assay CA19-9 for several reasons. (1) Serum MUC5AC had a good correlation with MUC5AC expressed in the tumor tissue in contrast to patients with large amounts of CA19-9 in the tumor tissue with little in the blood, and vice versa [28]. (2) MUC5AC was not detected in the serum of patients with benign or chronic inflammation of the bile duct (cholecystitis, cholelithiasis and opisthorchiasis), whereas marked elevation of serum CA19-9 has been noted in benign patients with cirrhosis and acute cholangitis [29]. (3) Determination of serum MUC5AC was more specific in diagnosing a particular cancer. It can be found in the serum of 62% of patients with CCA, but none with gastric cancer ($n = 12$), colon cancer ($n = 14$) and hepatoma ($n = 4$). In contrast, CA19-9 has been used to diagnose adenocarcinoma arising from various sites

in the gastrointestinal tract (i.e. cancer of bile duct, pancreas, gastric, colon, esophagus and hepatoma) with varied sensitivity [30,31]. (4) MUC5AC was not detected in the serum of normal healthy persons.

The high sensitivity and specificity of serum MUC5AC in the determination of CCA patients makes serum MUC5AC a potential serological marker for CCA. The determination of serum MUC5AC in conjunction with a scanning test such as ultrasound or CT scanning, or other biochemical tests, would enhance the diagnostic accuracy of CCA in the investigation of patients with a known liver mass or cyst, and for monitoring after resection or chemotherapy for CCA.

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logic factors were subjected to univariate and multivariate analyses using the Cox proportional-hazard regression model. The relative risk of death was compared using the assessment of hazard ratio. Differences were considered significant at $P < 0.05$.

RESULTS

Of 179 patients with CCA who were examined, 71.5% were men, and the ratio of males ($n = 128$) to females ($n = 51$) was 2.5:1.0. The median patient age was 56 years (range, 33–84 years). Peripheral or intrahepatic CCA was the major tumor type (49.7%), 29.4% of tumors were central or extrahepatic CCA, and 20.9% of tumors were a combination of both types. Most tumors (89%) were advanced-stage lesions (Stage IVA or IVB).

Correlation between Serum MUC5AC and Clinicopathologic Features

Using univariate analysis, the association of serum MUC5AC in patients with CCA and blood analysis was determined. The expression of serum MUC5AC had no association with age, gender, body mass index, blood group, serum markers of liver functions, parameters of complete blood count, or clinicopathologic findings (data not shown). Two variables, tumor stage and tumor size, were identified as statistically significant (Table 1). The proportion of patients with positive serum MUC5AC status was significantly higher in patients who had Stage IVB disease compared with patients who had disease in other stages ($P = 0.009$). The detection of serum MUC5AC apomucin was more frequent in patients who had larger tumors (> 5 cm; $P = 0.01$).

To identify the independent variables that were related to the expression of serum MUC5AC in patients with CCA, logistic regression analysis was performed. Factors that were identified at a significance level of $P < 0.25$ in the univariate analysis were included in the logistic regression analysis. Table 2 shows that blood group Type A and Stage IVB tumors were related independently to the expression of MUC5AC in serum samples from patients with CCA, with adjusted odds ratios of 3.24 and 3.20, respectively.

Correlation between Serum MUC5AC and Cumulative Survival Rate

Cumulative survival was compared in patients with primary CCA between patients who had positive serum MUC5AC status and patients who had negative serum MUC5AC status. Patients who survived for < 30 days were considered to have died perioperatively and thus were excluded from the analysis. Patients with positive serum MUC5AC status had a significantly poorer prognosis (median survival, 127 days; 95% confidence interval [CI], 107–180 days) compared with patients who had negative serum MUC5AC status (median survival, 329 days; 95% CI, 199–458 days; $P < 0.001$) (Fig. 1A).

Nonetheless, a bias may have been introduced into the current analysis by considering the different stages of disease together. Comparisons of the cumulative survival curves according to tumor stage demonstrated that patients who had Stage IVB CCA had a significantly poorer prognosis compared with patients who had Stage IVA or lower CCA ($P < 0.001$). To eliminate such a bias, cumulative survival rates were compared according to serum MUC5AC status in patients with CCA tumors in the same stage. Among patients with Stage IVA tumors, the median survival for patients with positive serum MUC5AC status was 172 days, compared with 336 days for patients with negative serum MUC5AC status. However, no statistically significant difference was demonstrated between

TABLE 1
Correlation between Expression of Serum MUC5AC and Clinicopathologic Features of Patients with Cholangiocarcinoma

Variable	No. of patients	sMUC5AC		P value
		Negative	Positive	
Age (yrs)				
≤56	99	40	59	0.360
>56	80	27	53	—
Gender				
Male	128	51	77	0.290
Female	51	16	35	—
Blood group				
O	52	23	29	0.356
A	29	7	22	—
B	79	29	50	—
AB	13	5	8	—
Tumor type				
Central	52	25	27	0.110
Penpheral	88	31	57	—
Combined	37	10	27	—
Histologic grading				
Papillary	28	13	15	0.852
Well-differentiated	78	30	48	—
Moderately differentiated	13	5	8	—
Poorly differentiated	12	4	8	—
TNM stage				
I–III	20	12	8	0.009
IVA	46	22	24	—
IVB	112	33	79	—
Tumor size (cm)				
≤5	22	14	8	0.010
>5	43	13	30	—

sMUC5AC: serum MUC5AC (secretory mucin of the gastric mucosa).

TABLE 2
Multivariate Analysis Using the Logistic Regression Model for the Detection of Serum MUC5AC in Patients with Cholangiocarcinoma

Variable	Adjusted OR	95% CI	*P* value
Age (yrs)			
≤56	1.00	—	0.135
>56	1.68	0.85–3.34	—
Gender			
Male	1.00	—	0.532
Female	1.27	0.60–2.65	—
Blood group			
O	1.00	—	0.007
A	3.24	1.03–9.73	—
B	1.38	0.61–2.97	—
AB	1.08	0.29–4.05	—
Tumor type			
Central	1.00	—	0.361
Peripheral	1.52	0.69–3.37	—
Combined	1.20	0.74–5.40	—
TNM stage			
I–III	1.00	—	0.039
IVA	1.58	0.51–4.92	—
IVB	3.20	1.12–9.15	—

OR: odds ratio; 95% CI: 95% confidence interval.

the two groups ($P = 0.143$). Among patients with Stage IVB tumors, patients who had positive serum MUC5AC status had a significantly poorer prognosis compared with patients with negative serum MUC5AC status, with a median survival of 116 days versus 329 days, respectively ($P < 0.0003$) (Fig. 1B).

Multivariate Analysis of Prognostic Factors

The Cox proportional-hazards regression model was used to assess the effects of different variables on patient survival. The 18 prognostic factors and serum MUC5AC were analyzed. All factors with $P < 0.25$ were put in the initial Cox model using backward stepwise selection. Finally, six independent variables (i.e., serum MUC5AC, age, gender, histologic grade, tumor stage, and lymph node resection) were identified as significant for the prediction of survival and were included in the final Cox regression analysis (Table 3). Multivariate analysis with adjustment for all covariates showed that patients who had positive serum MUC5AC status had a 2.5-fold higher risk of death compared with patients who had negative serum MUC5AC status ($P < 0.001$).

DISCUSSION

CCA is an uncommon malignancy for which no specific tumor marker has been found to date. Our previous study showed that MUC5AC mucin, an aberrant mucin produced by bile duct tumors, was detected in

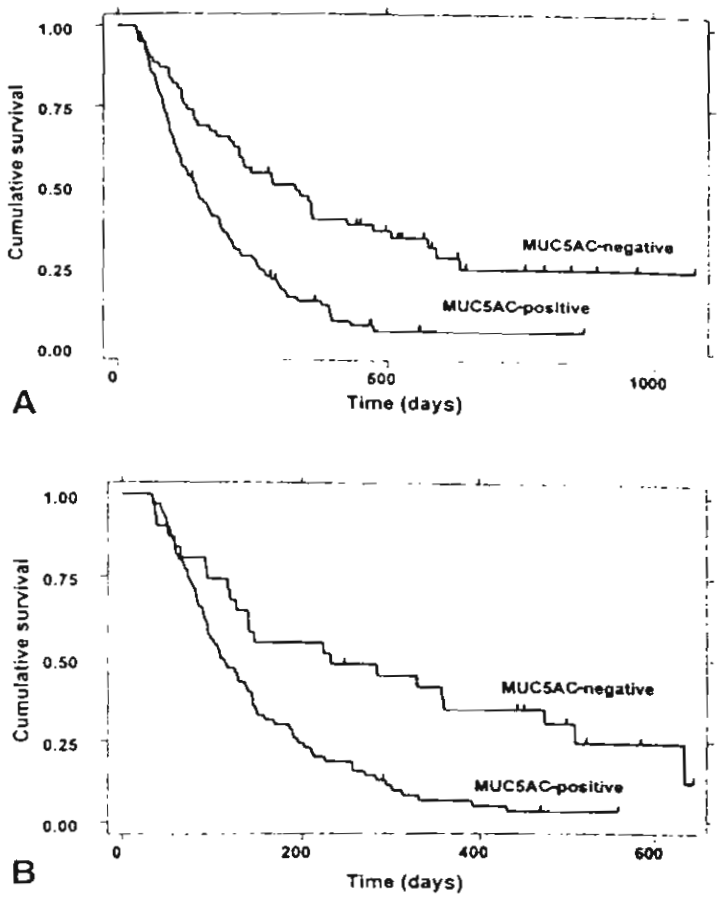

FIGURE 1. Survival curves generated using the Kaplan–Meier method. (A) Survival curves showed that patients with cholangiocarcinoma who were positive for serum MUC5AC had a less favorable prognosis compared with patients who were negative for MUC5AC ($P < 0.001$). (B) In the subgroup of patients with Stage IVB cholangiocarcinoma, positive serum MUC5AC status was correlated with a lower survival probability compared with negative serum MUC5AC status ($P = 0.0003$). Tick marks indicate censored patients.

the serum of patients with CCA with high specificity and sensitivity.[19] In the current study, we demonstrated that MUC5AC apomucin was detected in the sera of patients with CCA regardless of age, gender, blood group, tumor location, histologic tumor type, or clinical features of the patients.

In contrast, the relation between serum MUC5AC and tumor burden was characterized. Patients who had positive serum MUC5AC status seemed to harbor larger tumors compared with patients who had negative serum MUC5AC status. In addition, when the proportion of patients with positive serum MUC5AC status was assessed in accordance with the comprehensive staging, it was found that the rates increased gradually with the stage. Among patients with Stage II or lower CCA, the positive rates were 40%, but the rates increased significantly to 52% for patients with Stage IVA CCA and to 70% for patients with Stage IVB

TABLE 3
Results of Multivariate Analysis Using a Cox Proportional Hazards Regression Model

Variable	Adjusted HR	95% CI	*P* value
sMUC5AC			
Negative	1.00	—	<0.001
Positive	2.50	1.50–4.16	—
Age (yrs)			
≤56	—	—	0.771
>56	1.07	0.69–1.66	—
Gender			
Male	—	—	0.213
Female	0.74	0.46–1.20	—
Histologic grading			
Papillary	—	—	<0.001
Well-differentiated	2.03	1.06–3.87	—
Moderately differentiated	1.63	0.70–3.77	—
Poorly differentiated	2.41	0.98–5.92	—
TNM stage			
I–III	—	—	0.009
IVA	1.55	0.66–3.64	—
IVB	2.6	1.24–5.74	—
Lymph node resection			
Yes	—	—	<0.001
No	1.63	0.99–2.68	—

HR: hazard ratio; 95% CI: 95% confidence interval; sMUC5AC: serum MUC5AC (secretory mucin of the gastric mucosa).

CCA ($P = 0.009$). The univariate and multivariate analyses using logistic regression also revealed a different frequency of expression for MUC5AC in patients with Stage IVB CCA tumors. This observation suggested the possible role of MUC5AC in the development of tumor metastasis.

Blood group isoantigens of the ABH systems are represented by a variety of glycoproteins and glycolipids, the antigenic specificity of which is determined by variation in their constituent carbohydrate chains.[23] Multivariate analysis using the logistic regression in the current study showed significant relation of blood group Type A and the detection of MUC5AC in serum compared with other blood groups when blood group Type O was used as the reference group. The association of blood group and MUC5AC mucin expression has not been reported to date. However, differences in the cumulative survival rates of patients with CCA in different blood groups were not observed in this study ($P = 0.69$). Further investigation will be needed to explain the possible molecular and genetic basis for this correlation.

Mucins are the main components of mucus and were believed to have the unique function of protecting and lubricating the epithelial surfaces. The study of mucin structure and the correlation between structure and function show that mucins have an important role in growth; fetal development; epithelial renewal, differentiation, and integrity; carcinogenesis; and metastasis.

A number of studies have reported the importance of mucin as a prognostic factor for several types of malignant disease.[24–28] In the current study, the expression of MUC5AC was an independent prognostic variable for survival in patients with CCA. Patients who had positive serum MUC5AC status had a significantly poorer survival prognosis compared with patients who had negative serum MUC5AC status ($P < 0.001$). This poor outcome may not have been due to the high proportion of patients with advanced-stage disease in the MUC5AC positive group, because a significant difference in the survival rate between the two groups still was demonstrated in patients with Stage IVB CCA, even when the patients were stratified according to disease stage. The presence of MUC5AC in serum was correlated with tumor size and metastatic features, suggesting that these variables may be responsible for the poor outcome of patients with CCA who have positive serum MUC5AC status.

The link between MUC5AC expression and malignant progression is significant in two ways. First, MUC5AC is negatively-charged, and cells that express high levels may repel each other and enhance cell migration. Second, the highly viscous gel formed by MUC5AC surrounds the tumor emboli and may protect the tumor from proteolysis and limit the escape of immunogenic cells. Results from experimental data may support this possibility.

The high sensitivity and specificity of serum MUC5AC in the determination of CCA[19] and the association of serum MUC5AC with tumor burden, regardless of the histologic grade and tumor type, allow the potential use of serum MUC5AC as a serologic marker for all types of CCA. The determination of serum MUC5AC levels in conjunction with a imaging tests, such as ultrasound or computed tomography scanning, or other biochemical tests (i.e., CA19-9 or carcinoembryonic antigen), may be used to enhance the diagnostic accuracy of CCA in the investigation of patients with a known liver mass or cyst and for monitoring after patients undergo resection or receive chemotherapy for CCA. In addition, serum MUC5AC may be useful in predicting patient outcome and in selecting appropriate treatment options.

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9 mol/l phosphoric acid, followed 20 min after by 0.5 ml of a mixture containing 0.8 mol/l sodium arsenite, 0.5 mol/l sodium sulfuric and 0.05 mol/l sulfuric acid. When the yellow-brown color had disappeared, 1.5 ml of 0.04 mol/l thiobarbituric acid in 0.5 mol/l sodium sulfuric was added and the solution was heated in a boiling water bath for 15 min. After cooling, 2.15 ml of cyclohexanone was added, the content was vortexed and centrifuged. The absorbance of the cyclohexanone layer was then measured spectrophotometrically at 549 nm. The assay gave good reproducibility with a 4.5% coefficient of variation (CV). *N*-acetylneuraminic acid between 0.4 and 4 mmol/l was used to calibrate the curve. Duplicate measurements of sialic acid were performed for each sample. A serum TSA concentration higher than 2.33 mmol/l was considered abnormal [7].

2.4. Blood analysis

Results of the complete blood count and blood serum analysis for liver function (i.e., total protein, serum albumin, serum globulin, total bilirubin, direct bilirubin, alkaline phosphatase, aminotransferases) were obtained from the clinical records. Serum MUC5AC mucin was determined according to Sheehan et al. [20]. Briefly, 10 μl of serum was treated with 80 μl of urea buffer containing 6 mol/l urea in 0.1 mol/l Tris–HCl, 5 mmol/l EDTA, pH 8 and 10 μl loading buffer, containing 40 mmol/l Tris-base, 20 mmol/l acetic acid, 1 mmol/l EDTA, 30% glycerol, 1% SDS and bromphenol blue. The mixture was subjected to 0.7% agarose gel electrophoresis and transferred to nitrocellulose membrane. After blocking with 0.05% Tween 20 and 5% nonfat dry milk in buffer, the membrane was incubated with 1:10,000 MAN-5ACI antisera and 1:8000 goat anti-rabbit IgG-peroxidase. The membrane was incubated with chemiluminescent reagent and exposed to an X-ray film. The tumor markers AFP, CEA and CA19-9 were measured using an immunochemistry analyzer (Elecsys 2010, Roche) based on a sandwich electrochemiluminescence immunoassay. Tumor markers were determined on the requests of the clinicians. AFP was analyzed in 86 patients to differentiate hepatoma from non-jaundiced CCA cases, whereas

Table 2
Blood parameters according to increased and normal serum concentrations of TSA

	Serum TSA-normal ($n=44$)		Serum TSA-increased ($n=128$)		
	Median	Range	Median	Range	p^a
Blood chemistry					
TSA (mmol/l)	2.03	0.92	3.04	2.53	<0.001
TSA (μmol/mg protein)	27.3	59.1	41.23	41.19	<0.001
Total protein (6.5–8.8 g/dl)	7.5	6.5	7.5	4.6	0.634
Total bilirubin (0.25–1.5 mg/dl)	0.95	51.8	1.75	47	1.251
ALT (4–36 U/l)	42	1340	52	348	0.312
AST (12–32 U/l)	67	318	68	559	0.593
ALP (37–147 U/l)	201	318	281	9997	0.033
Total blood cells					
White blood cells (cells/ml)	9100	11,000	11,450	28,000	<0.001
PMN (cells/ml)	5862	6367	8232	28,732	<0.001
Eosinophil (cells/ml)	334	1848	299	3450	0.436
Tumor markers					
AFP[b] (0–10 U/ml)	2.1	25	2.5	220	0.079
CEA (0–25 ng/ml)	3.9	4500	5.2	27,049	0.624
CA19-9 (0–37 U/ml)	110	72,600	100	9366	0.699

Numbers in parentheses are normal values.
Corr. coeff.=correlation coefficient.
[a] Mann–Whitney Rank Sum test.
[b] In AFP determination, there were 18 cases with serum TSA-normal and 68 cases with serum TSA-increased.

CEA and CA19-9 were measured to confirm diagnosis of CCA.

2.5. Statistic analysis

Data were analyzed with respect to clinical, laboratory and histopathological findings and survival. Statistical analysis was performed using SigmaStat statistical software (Jandel Scientific, Corte Madera, CA). Statistical comparisons were made using a two-tailed Student's *t*-test or the Mann-Whitney rank sum test. The χ^2 test was used for qualitative data. The relationships between serum TSA and blood chemistry, blood cells and tumor markers were assessed using the Spearman Rank Order correlation or the Pearson product moment correlation. A linear regression was used to measure the strength of the correlation. Survival rates were calculated by the Kaplan–Meier method using STATA's log rank test (Stata, College Station, TX). Significance was set at $p < 0.05$.

3. Results

3.1. Serum TSA and TSA content in tumor tissue

Serum TSA and TSA content in primary tumor tissues were determined in 25 CCA patients, of which, 18 were intrahepatic, 2 were extrahepatic and 5 were combined-CCA. The sialic acid content in the tumor tissue (median, 59.4 nmol/mg protein; range, 26.3–171.6) was significantly higher than in the serum (34.8 nmol/mg serum protein) ($p < 0.001$). The tumor tissue TSA content was not related to the serum TSA concentrations, tumor location, tumor staging or the distance metastasized (data not shown).

3.2. Serum TSA and clinicopathologic features

Serum TSA concentrations were increased in 130 of the 172 subjects (75.6%). The increase of serum TSA was not significantly related to age, sex, body mass index, blood group, tumor location, tumor stage, metastatic condition, histological types or histological grading (data not shown). However, the relationships were evaluated between circulating serum TSA concentrations and serum ALP, white blood cell count and polymorphonuclear leukocytes (PMN) ($p < 0.001$) (Table 1). We also observed the association of increased serum TSA and serum MUC5AC mucin. CCA patients with positive serum MUC5AC mucin had a significantly higher serum TSA concentration (2.96 ± 0.69 mmol/l) than those with negative serum MUC5AC (2.72 ± 0.63 mmol/l) ($p < 0.05$).

To investigate the possible cause of increased serum TSA in CCA patients, the subjects were divided according to their serum sialic acid concentrations into a normal TSA group (≤ 2.33 mmol/l) and an increased TSA group (>2.33 mmol/l). With regard to patient characteristics and clinicopathologic features, the differences between the proportions of

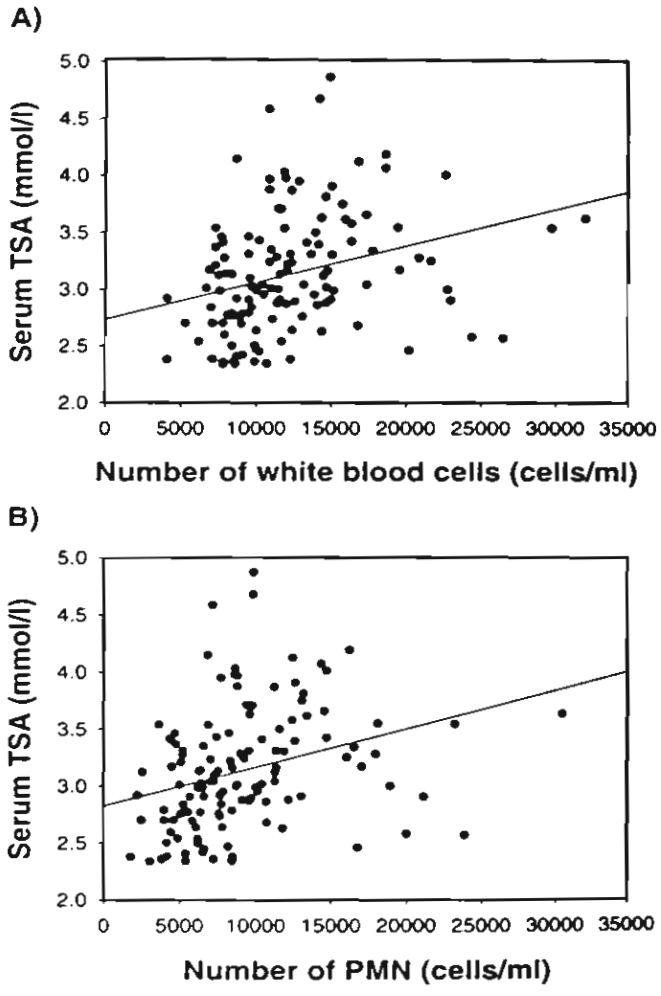

Fig. 1. Serum TSA concentrations correlate with blood parameters of cholangiocarcinoma patients. (A) The white blood cell count ($r = 0.297$, $p < 0.001$); (B) PMN ($r = 0.3$, $p < 0.001$).

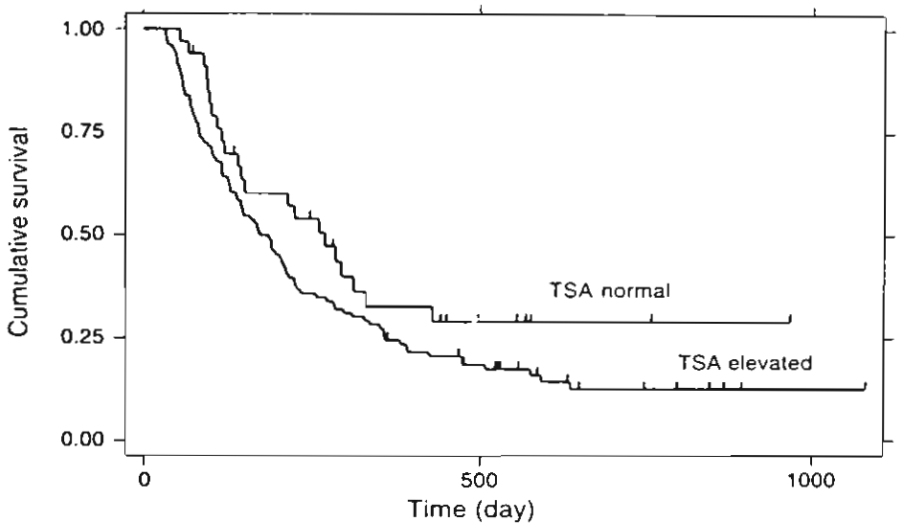

Fig. 2. Cumulative survival curves according to serum TSA concentration. Patients with increased serum TSA had a poorer prognosis than patients with normal concentrations, though not statistically significant. Ticks indicate the censored cases.

subjects with normal ($n = 44$) vs. increased ($n = 128$) serum TSA were not significant. However, serum sialic acid per milligram of serum protein, serum ALP, white blood cell count and the number of PMN were significantly higher in the increased TSA group ($p < 0.001$) (Table 2).

Direct associations between the concentration of serum sialic acid and serum ALP, white blood cell count, PMN ($p < 0.001$) and serum CA19-9 ($p < 0.05$) were observed in the increased TSA group (Table 3) (Fig. 1), but not in the subjects with normal serum TSA.

3.3. Serum TSA and survival rate

Cumulative survival was compared among patients with primary CCA with increased and normal serum TSA concentrations; however, most (80%) of the CCA patients had died by the time we were preparing this manuscript. Notwithstanding, the CCA patients with high concentrations of serum sialic acid had a shorter median survival (172 vs. 270 days) (Fig. 2), though not statistically different from those with normal concentrations.

Nine prognostic factors, including serum TSA, serum TSA/mg protein, age, tumor stage, tumor resection, lymph node resection, serum protein, white blood cell count and serum CEA concentration, were entered into a multivariate analysis using a Cox regression. Between patients with normal vs. high serum TSA, no significant differences were observed in the crude or the adjusted hazard ratios.

Table 3
Correlation between concentrations of serum TSA and blood parameters

	Serum TSA-normal ($n = 44$)		Serum TSA-increased ($n = 128$)	
	Corr. coeff.	p	Corr. coeff.	p
Blood chemistry				
Total protein	0.090	0.599	− 0.051	0.566
Total bilirubin	0.160	0.305	0.036	0.688
ALT	0.009	0.952	0.070	0.432
AST	− 0.007	0.960	0.076	0.394
ALP	0.074	0.627	0.341	< 0.001
Blood cell count				
WBC	0.199	0.194	0.383	< 0.001
PMN	0.173	0.259	0.424	< 0.001
Eosinophil	0.014	0.924	− 0.145	0.101
Tumor markers				
AFP[a]	0.120	0.626	− 0.210	0.085
CEA	0.097	0.524	− 0.066	0.461
CA19-9	− 0.311	0.040	0.206	0.022

Correlation coefficient were analyzed by Spearman Rank Order correlation

Corr. coeff. = correlation coefficient

[a] In AFP determination, there were 18 cases with serum TSA-normal and 68 cases with serum TSA-increased.

Table 4
Correlation between serum CA19-9 concentrations and the numbers of white blood cells

		Serum TSA-normal		Serum TSA-increased	
		Corr. coeff.	p	Corr coeff.	p
CA19-9	vs. WBC	0.055	0.720	0.182	0.043
	vs. PMN	0.108	0.484	0.201	0.025

Correlation coefficient was analyzed by Spearman Rank Order correlation.
Corr. coeff. = correlation coefficient.

3.4. Correlation between serum TSA, CA19-9 and PMN

Serum ALP, CA19-9, white blood cell count and PMN were significantly higher in the group with increased serum TSA and all showed a positive correlation with serum TSA (Table 3). We further investigated any possible relationship among these parameters in the serum normal TSA vs. increased TSA groups. Only serum CA19-9 in the TSA-increased group correlated with the white blood cell count and PMN in the blood (Table 4).

4. Discussion

In recent years, several studies on serum sialic acid concentrations in cancer patients have been published. We recently reported a significant elevation of serum TSA in CCA patients with a cutoff value of 2.33 mmol/l, a sensitivity of 72% and a specificity of 82% [15]. In this study, we aimed to determine the clinical values of the increased serum sialic acid concentrations found in CCA patients and its possible cause by examination of any correlation that might exist between serum TSA and TSA content of the tumor tissue, clinicopathologic features, surgical findings and blood parameters.

Very little data has been collected concerning sialic acid content in tumor tissue and observations are sometimes contradictory. Increased tumor sialic acid content in relation to normal tissue has been reported in human pancreatic cancer, skin squamous cell carcinoma, melanoma [21], lung cancer [10] and prostate cancer [22]. Conversely, a decreasing sialic content in cancerous tissue, as compared to normal tissue, was reported for cervical tissue [23], endometrial tissue and colon cancer [3,24–26].

A study on sialic acid content in human CCA tissues has so far not been reported. We have not compared sialic acid content in tumor vs. non-tumor tissues since concurrent normal bile duct tissues were unavailable. Tissue sialic acid content may vary from tissue to tissue. For example, the sialic acid content of CCA tumor tissue in our study was distinctly higher (59.4 nmol/mg of tissue protein) than the values obtained for lung cancers (i.e., 5.18–8.74 nmol/mg of tissue protein) [27]. Sialic acid content of CCA tissue showed no significant differences between tumor location, staging or metastatic condition of the tumor as similarly observed in a study on endometrial cancer [12]. We found no correlation between serum and tumor sialic acid concentrations in CCA patients, just as none was observed in colorectal cancers [3].

The contribution of tumor tissue to the concentration of serum TSA was investigated by comparing serum TSA before and after tumor tissue removal. We recorded no significant reduction in serum TSA concentration after surgery. However, this negative observation does not disprove the contribution of tumor tissue to the concentration of serum TSA. Elevation of serum TSA could also be due to inflammation and acute phase reaction [28], which may explain why the serum TSA was maintained even after surgery.

To date, studies in which sialic acid concentrations were simultaneously evaluated in the serum and tissue of patients are rare. In our study, the ratio tumor TSA to milligrams of tissue protein was significantly higher than serum TSA to milligrams of serum protein ($p < 0.001$). This observation supports the hypothesis of an enhancement in tumor sialoglycoconjugate biosynthesis being the cause of an increase in serum sialic acid content. Possibly, the newly synthesized sialoglycoconjugates are released from the tumor into the blood. The correlation of serum TSA and concentrations of three secreted sialoglycoconjugates—viz. serum CA19-9, MUC5AC mucin and ALP—in CCA patients support this hypothesis. Other explanations for the higher serum sialic acid content in CCA patients cannot be excluded, such as an increase, not only in the concentration of serum glycoprotiens and glycolipids, but also in the degree of sialylation of these substances. In fact, an increased activity of

sialyltransferase in the serum from several cancers, such as in the colon, has been shown [3,29,30]. A decrease in serum sialoglycoconjugate degradation and/or an altered clearing of these glycoconjugates by the liver could also account for the elevation in serum sialic acid. Some authors have suggested that increased serum sialic acid concentrations in patients with cancer reflects an inflammation reaction to the tumor, leading to increased output of acute phase proteins from the liver [3,18]. We observed increases in the numbers of inflammatory cells (white blood cells and PMN) in the group with increased TSA in the serum.

Serum ALP is a glycoprotein, synthesized mainly by liver and bile duct epithelia. Increased serum ALP is a common clinical feature found in CCA patients. Serum ALP with increased serum TSA was significantly higher in our CCA patients than it was in patients with normal serum TSA. The correlation of serum ALP and serum TSA concentrations was observed only in the group with increased serum TSA, suggesting a possible role of serum ALP in the elevation of serum TSA in CCA patients.

MUC5AC is a secretory gel-forming, highly glycosylated mucin. The overexpression of this mucin in CCA tissue has been documented [31–34]. Currently, we have detected MUC5AC mucin in the sera from CCA patients with 62.6% sensitivity and 96.9% specificity [35]. Increasing the concentration of MUC5AC mucin found in CCA serum may be related to the increased number of inflammatory cells found in the serum of the TSA-increased group, since it is evident that inflammatory cytokines produced by inflammatory cells (i.e., IL1 and TNFα) could stimulate mucin gene expression and mucin release in the cancer cell line [36]. These observations support the contribution of serum MUC5AC mucin in the elevation of TSA in the sera of CCA patients.

The serum antigen CA19-9 is an epitope on a complex oligosaccharide, a sialylated lacto-*N*-fucopentose II, related to the Lewis blood group antigens. Several investigators have noted increased CA19-9 serum concentrations in CCA patients [37]; however, the significant role of this antigen in cancer is unclear. In our study, increased serum CA19-9 was observed in CCA patients with both normal and increased serum TSA, but a positive correlation between serum CA19-9 and serum TSA was significant only in patients with increased serum TSA. This observation supports an important role of serum CA19-9 in the elevation of TSA in the serum of CCA patients.

We observed no differences in the clinicopathologic features among CCA patients with normal and increased serum TSA, except higher concentrations of serum ALP, CA19-9 and white blood cell counts, particularly PMN in the CCA patients with increased serum TSA. Statistical analysis showed that the white blood cell count and PMN were related to the concentrations of serum CA19-9, but only in CCA patients with increased serum TSA. The relationship between serum TSA, CA19-9 and the number of PMN in the serum of TSA-increased CCA patients suggests a possible connection between these factors. The chemotactic activity of CA19-9 for neutrophils has been demonstrated [38]. Serum CA19-9 may contribute to the high white blood cell counts, particularly the PMN in the blood, and stimulate the function of immune cells. Active secretion of cytokines and glycoproteins from these immune cells may partly cause the elevation of TSA in the serum of CCA patients.

In conclusion, our data suggest that the sialic acid content in tumor tissues and serum TSA are not related to the burden of the disease. Patients with increased serum TSA showed no difference in cumulative survival or the hazard ratio from those with normal serum TSA. The serum sialic acid concentration was directly correlated to tumor products such as ALP, MUC5AC mucin and CA19-9. A significant correlation between serum TSA, serum CA19-9 and number of PMN was documented. These factors together with other possible factors mentioned may be responsible for the elevation of TSA in the serum of CCA patients.

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