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CA-S27: A novel Lewis A associated carbohydrate epitope is diagnostic and prognostic for cholangiocarcinoma

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Keywords: Monoclonal antibody; FUT3; tumor marker; mucin; metastasis

List of Abbreviations: CA-S27 = carbohydrate antigen-S27; CCA = cholangiocarcinoma; mAb = monoclonal antibody; Le^a = Lewis a; FBS = fetal bovine serum; HRP = horseradish peroxidase; DAB = diaminobenzidine tetrahydrochloride; TMB = 3,3′,5,5′-tetramethyl benzidine; OD = optical density; FUT3 = fucosyltransferase III; GAPDH = Glyceraldehyde-3-phosphatedehydrogenase; reversed transcriptase-PCR = RT-PCR; ROC = Receiver operating characteristic; ST3Gal = β-galactoside-α-2,3 sialyl transferase; ECM = extracellular matrix; FN = fibronectin; IHC = immunohistochemistry; ROC = Receiver operating characteristic

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Abstract

Early and specific diagnosis is critical for treatment of malignant cancers. Increased expression of tumor-associated markers has been shown to be highly predictive for cholangiocarcinoma (CCA). In this study, a S27 monoclonal antibody (mAb) which recognizes a cancer-associated modification of serum MUC5AC with Lewis-a (Le^a) associated epitopes was established. The carbohydrate antigen was designated as carbohydrate antigen-S27 (CA-S27). A SBA-S27 mAb sandwich ELISA to determine CA-S27 in serum was successfully developed. High level of CA-S27 was detected in serum of CCA patients and could differentiate the patients of CCA from those of gastro-intestinal cancers, hepatomas, benign hepatobiliary diseases and healthy subjects with high sensitivity (87.5%) and high negative predictive value (90.4%). CA-S27 detected in serum of CCA patients was of tumor origin as CA-S27 was detected in CCA tissues and the level of CA-S27 in serum was dramatically reduced after the tumor removal. A prognostic value for serum CA-S27 was revealed, because patients with high serum CA-S27 had significantly shorter survival periods than those with low serum CA-S27 regardless of serum MUC5AC levels. Fucosyltransferase-III (FUT3) was shown to be a regulator of CA-S27 expression. Suppression of CA-S27 expression with siRNA-FUT3 or CA-S27 neutralization with S27 mAb significantly reduced growth, adhesion, invasion and migration potentials of CCA cells *in vitro*. A similar treatment had no effect on the CCA cell line without FUT3 expression. ***Conclusion***: This study is the first to demonstrate that serum CA-S27, a novel carbohydrate antigen, has potential as diagnostic and prognostic markers for CCA patients. CA-S27 has a functional role in promoting cell growth, adhesion, migration and invasion of CCA cells.

Introduction

Glycosylation is one of the posttranslational modifications of proteins which can alter protein conformation and may consequently modulate the function of the protein as well as protein–protein interactions. Alteration of cellular glycosylation profiles during carcinogenesis and the correlation of glycosylation profiles with tumor progression have been shown in many studies(1-4). These proteins are mostly found extra-cellularly on the plasma membrane and appear as secreted proteins in body fluid which are readily accessible for diagnostic and therapeutic purpose. Therefore many glycoproteins are used as biomarkers for cancer prognosis and diagnosis.

Cholangiocarcinoma (CCA), the second most common liver cancer, has the highest global incidence in the northeast of Thailand(5) . While CCA was rare worldwide in the past, the mortality-incidence of CCA increased globally in the recent 2-3 decades(6, 7). CCA is a slow growing tumor with no early warning symptoms, resulting in a late diagnosis(8), where curative surgery is seldom possible(9). Combinations of the present tumor serum markers for CCA allow the diagnosis of CCA in general; however, these markers have limited value for the determining if a CCA has a higher grade and if it is invasive or metastatic. As therapeutic options increase, determining the malignant potential of a CCA may become important to match the appropriate therapy to the patient. Also, early detection of CCA could possibly to allow for surgery before the tumor has metastasized. Hence, there is a great clinical need to improve the biomarkers for the detection and prediction of CCA.

In this study, we have employed a S27 monoclonal antibody (S27 mAb) which recognizes Lewis-a (Le^a) associated epitope designated as carbohydrate antigen-S27 (CA-S27), and

evaluated the possibility of using CA-S27 as diagnostic and prognostic markers for CCA. In addition, the functional role of CA-S27 in tumor progression of CCA is first reported.

Materials and Methods

Patient samples: Sera and paraffin-embedded tissues were almost similarly collected and stored as the previous study(4). Informed consent was obtained from each subject and the study protocol was approved by the Ethics Committee for Human Research, Khon Kaen University.

Cell lines and cell culture: Human CCA cell lines (KKU-M139, M156, M213, M214, and KKU100) were established from primary CCA tumor tissues(10). An immortalized cholangiocyte cell line (MMNK1), a representative of normal cell, was a gift from Dr. Kobayashi N.(11). All cell lines were cultured in DMEM (Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS), 1% L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin at 37^{o}C and 5% CO_2.

S27 mAb: The S27 mAb was obtained from the hybridoma established and screened as previously described(4).

Glycoconjugate microarray: The S27-specific sugar was analyzed by a glycocojugate microarray consisting of 98 known sugar compounds as previously described(12, 13).

Immmunohistochemistry (IHC) of CCA tissues: CA-S27 was determined in paraffin-embedded CCA tissue sections by the standard indirect immunoperoxidase method as previously described(4). In brief, the sections were incubated with 5 µg/mL of S27-mAb, at

room temperature, overnight and incubated with 1:500 horseradish peroxidase (HRP) conjugated goat anti-mouse IgM (Southern Biotechnology, Birmingham, AL) for 1 h. The immunoreactivity was developed and scored as previously described(4). Sections incubated without S27-mAb were used as negative controls. Anti-MUC1 antibody (Invitrogen, Carlsbad, CA) was used as the protein specific antibody control. The immunohistochemisty of MUC5AC were determined using anti-MUC5AC antibody (CLH2, Santa cruz, CA)(14).

Characterization and identification of specific antigen recognized by S27-mAb: The antigen specific to S27 mAb were demonstrated as described previously(4). Briefly, an apparent molecular weight was determined by gel filtration chromatography, SDS-polyacrylamide gel electrophoresis and western blotting. The characteristic of specific epitope of S27 mAb was identified by periodate oxidation and proteinase K/tryptic digestion, glycoconjugate microarray and LC/MS/ MS analysis.

SBA-S27 sandwich ELISA: Level of serum CA-S27 was quantitated using soybean agglutinin (SBA) captured S27 mAb EISA. The protocol was similar to that reported for SBA-S121 SBA sandwich ELISA(4). Briefly, 50 µl of 40 µg/mL of SBA (Sigma, St. Louis, MO) was coated overnight on an ELISA plate at 4^{o}C. After blocking, 1:10 serum was added following with 1 µg/mL S27 mAb and 1:4,000 HRP-conjugated goat anti-mouse-IgM. The signal was developed with 3,3′,5,5′-tetramethyl benzidine (TMB; Sigma) substrate solution. The reaction was stopped with 2N sulfuric acid and the optical density (OD) was read at 450 nm. All serum samples were tested in duplicate.

Sandwich ELISA for MUC5AC: MUC5AC in serum was determined using MUC5AC-SBA sandwich ELISA(15). Level of serum MUC5AC with OD ≤ 0.074 and OD > 0.074 was

considered as low and high MUC5AC(15), respectively. The sandwich ELISA using anti-MUC5AC mAb (clone 22C5) coupled with S27-mAb was performed as described previously(4).

Cell ELISA: KKU-M213 and MMNK1 (3 x 10^3 cells/well) were seeded on a 96 well plate and cultured for 24 h. Cells were fixed with 4% paraformaldehyde at room temperature for 15 min and the non-specific binding was blocked with 3% BSA in PBS-Tween for 1 h. After incubated with S27 mAb (1 µg/ml, 100 µL) for 1 h, 100 µL of HRP-conjugated goat anti-mouse IgM (1:2,000) was added. The reactivity was detected using TMB substrate as mentioned above.

RNA extraction and PCR: Total RNA from cultured cells were extracted using RNeasy kit (Qiagen, Valencia, CA) and converted to cDNA using SuperScript™ II Reverse Transciptase Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cDNA converted from 125 ng of total RNA were used for semi-quantitative PCR analysis of fucosyltransferase III (FUT3). Glyceraldehyde-3-phosphatedehydrogenase (GAPDH) was used as an internal control. The primer sequences were: 5′-ACAGGTCCAAGTTCAAGCC-3′ (forward) and 5′-CAGCAGCAATTTCCCTCA-3′ (reverse) for FUT3(16); 5′-ATCCCATCACCATCTTCCAGG-3′ (forward) and 5′-TGGTGGTGCAGGAGGCATTGC-3′ (reverse) for GAPDH. The amplification reaction was 95°C 5 min with 25 cycles of 95°C 30 sec, 55°C 30 sec, 72°C 30 sec; and 72°C for 5 min. PCR reaction (5 µl) was electrophoresed on a 2% agarose gel in 22.5 mM Tris/borate/EDTA buffer, pH 8.0 containing 0.5 µg/ml ethidium bromide. Gel was visualized under UV light (ImageQuant 400 image analyzer) and the expression of FUT3 was analyzed using ImageQuant™ TL analysis software (GE healthcare).

Glycosyltransferases profiling: The expression profiles of 186 glycosyltransferases of KKU-M213 and MMNK1 were determined using quantitative RT-PCR (17).

CA-S27-Immunocytofluorescent staining: Cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton-X100 in PBS. After blocking with 5% normal horse serum in PBS for 20 min, 2 μg/mL of S27 mAb was added for 1 h followed by 1:500 of Alexa Fluor®488-conjugated goat anti mouse-IgM (Invitrogen, Carlsbad, CA) and 1:2,000 of Hoechst-33342 (Molecular Probes, Eugene, OR). The fluorescent signals were observed under a microscope.

Transient knockdown of FUT3: KKU-M139 (5 x 10^4 cells) were transfected with 100 pM of siFUT3 (Eurogentec, San Diego, CA) or siControl (Silencer Negative Control siRNA #1, Ambion, Austin, TX) using Lipofectamin-2000 (Invitrogen, Carlsbad, CA). The sequences of siRNA specific for FUT3 (siFUT3) were 5′-UCCAUUUAAGAUACUCUGATT-3′ (sense); and 5′-UCAGAGUAUCUUAAAUGGATT-3′ (anti-sense). The siControl used in the study have no significant sequence similarity to any of mouse, rat, or human transcript sequences. After 72 h of siRNA transfection, the expression of FUT3 was monitored by semi-quantitative PCR and the expression of Le^a was determined by immunocytofluorescent staining.

Surface CA-S27 neutralization: CA-S27 epitopes on the cell surfaces were neutralized by incubating cells (2 x 10^4 cells) with 10 µg S27-mAb in Opti-MEM (Gibco, city, country) at 37°C for 2 h. Mouse IgM (Sigma) was used as non-reactive IgM control.

Invasion and migration assays: The invasion and migration assays were performed according to the manufacture's instruction using BioCoat™ Matrigel™ Invasion Chambers and Falcon™ Cell culture inserts (8 µm pore-size, BD Biosciences, Bedford, MA). After 72 h of siRNA treatment or two hour of antibody neutralization, 4 x 10^4 treated cells in Opti-MEM were subjected to the upper chamber which the lower compartment contained 10% FBS-DMEM. Cells were allowed to migrate or invade for 20 h or 24 h, respectively. After incubation, migrated or invaded cells were fixed with 25% methanol, stained with 0.5% crystal violet and count under a microscope using low-power field (100 x magnification)..

Adhesion assay: A 96 well plate was pre-coated with 50 µL of 50 µg/mL Matrigel™ (represented for extracellular matrix; ECM) or 10 µg/mL fibronectin (FN; Sigma) in Opti-MEM. After washing and blocked with 2% BSA in PBS for 30 min, 2 x 10^4 cells/100 µL were seeded and allowed to adhere for two hour. Non adhered cells were washed out and the adhered cells were fixed with 30% methanol, 30 min, stained with 0.5% crystal violet and count under a microscope.

Cell proliferation assay: Cells (3,000 cells) were seeded in a 96 well plate and cultured in the complete media. After 24 h, the culture medium was replaced with 1% FBS in DMEM containing with 1 µg of S27 mAb or IgM control. The cell number was measured at 0, 24,

48, and 72 h using CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (MTS; Promega, Madison, WI) according to the instruction of manufacture.

Statistical Analysis: Statistical analysis was performed using SPSS 16.0 software (SPSS, Chicago, IL) and SigmaStat 3.1 (Systat Software, San Jose, CA). All statistic analysis was as described previously(4). A *P*-value < 0.05 was considered statistically significant.

Results

Establishment of a S27 monoclonal antibody which recognizes CA-S27 in tumor tissues and serum of CCA patients

We have established mAbs using pooled tumor tissues from CCA patients as immunization antigen source(4). SBA-captured ELISA and cell-ELISA were used for hybridoma screening with serum glycoproteins and cell surface molecules, respectively. A S27 mAb was selected as it exhibited higher reactivity with pooled CCA serum than those from healthy subjects as shown by SBA-captured ELISA (Fig. 1A) and showed strong reaction with CCA cells (KKU-M213) than normal bile duct epithelium cells (MMNK1) as shown by cell ELISA (Fig. 1A) and S27-immunocytochemical fluorescent staining (Fig. 1B).

To characterize the S27 epitope, tumor tissues and serum from CCA patients were pre-treated with $NaIO_4$ or protease enzymes prior to react with S27 mAb. Antibody specifically reacted with MUC1-core protein was used as the positive control for protease treatment. S27 mAb failed to react with the $NaIO_4$-treated serum (Fig. 1C-a) and tissue (Fig. 1C-b) while the protease treated samples remained actively reacted with S27 mAb. These results indicated that S27 mAb recognizes the glycan as the antigen epitope. Using glycoconjugate-microarray, the glycan epitope was further analyzed to be Le^a associated glycan (Fig.1D) without cross reactivity to any members of Lewis antigens-sLe^a, Le^b, *etc*. CA19-9, the

antibody specifically reacted to sialyl-Le^a, was used as positive control of the microarray analysis (Supplementary Fig.1). Since Le^a polymers were immobilized on the glycoconjugate-microarray(12) therefore S27 mAb is more likely to react with the complex of Le^a polymer rather than the Le^a monomer. To verify this, S27 mAb was used to detect Le^a blood group on the referent red cells according to the standard saline and two-stage enzyme techniques(18). S27 mAb failed to react with Le^a positive red cells (data not shown) and hence the epitope was designated as carbohydrate antigen-S27 (CA-S27) in the subsequent studies.

MUC5AC mucin was the major source of CA-S27 detected in serum

To determine the core protein of CA-S27 found in serum, pooled serum from CCA patients was subjected to a sepharose-6B chromatography column, SDS-polyacrylamide gel electrophoresis and western blotting. Results obtained from the sepharose-6B column (Fig. 2A-a) and western blot (Fig. 2A-b) indicated a high molecular weight of CA-S27 associated glycoprotein. LC/MS/MS analysis of the bound fraction from S27-affinity chromatography column demonstrated MUC5AC mucin as the CA-S27 associated glycoprotein found in serum (Fig. 2B). The result was confirmed by the positive reactivity of serum proteins from sandwich ELISA using S27-mAb coupled with anti-MUC5AC (Fig. 2C).

CCA tissues exhibited high reactivity of CA-S27

To investigate whether CA-S27 was the bile duct epithelium origin, we performed IHC of CCA patients' tissues using S27-mAb as primary antibody. No CA-S27 reactivity was found in hepatocytes but the high signal was detected in the bile duct epithelial cells of non tumorous liver tissue (Fig. 3A-a) and bile duct tumor cells (Figs. 3A-b, 3A-c), suggesting the biliary epithelium origin of the CA-S27 found in serum. IHC of 45 histological proven

CCA cases revealed that almost all CCA tissues (43 of 45, 95.5%) expressed high reactivity of CA-S27, and hence no analysis of the relationship between CA-S27 expression and clinico-pathological findings of the patients can be assessed. As MUC5AC mucin is rarely expressed in normal bile duct epithelia, we questioned whether CA-S27 detected in normal bile duct epithelium was conjugated to MUC5AC mucin. The IHC using S27 mAb and MUC5AC antibody performed on the serial sections of normal liver tissues obtained from cadaveric donors, fetus, CCA and hepatoma patients showed that all normal bile duct epithelia of the tested samples were positive for CA-S27 but not MUC5AC mucin (Fig 3B). These results implied that the positive signal of CA-S27 found in normal bile duct epithelia was unlikely to be conjugated on MUC5AC mucin.

Serum CA-S27 as a diagnostic indicator for CCA

SBA-S27 mAb sandwich ELISA was used to determine the level of CA-S27 in serum. Of 96 histological proven CCA patients and 192 controls (52 active *Opisthorchis viverrini* infection, 42 benign biliary diseases, 47 gastro-intestinal carcinoma and 51 healthy subjects), the serum CA-S27 of CCA patients (mean ± SD = 0.206 ± 0.234) was significantly higher than those of control groups (0.029 ± 0.029) ($P < 0.001$; Fig. 4A). Receiver operating characteristic (ROC) analysis revealed the significance of serum CA-S27 level in distinguishing CCA patients from the control groups ($P < 0.001$) with the area under curve of 0.822 (Fig. 4B). The cut-off value obtained from the ROC curve was 0.0268 which provided 87.5% (84/96) sensitivity, 58.8% (113/192) specificity, 51.5% (84/163) positive predictive value, and 90.4% (113/125) negative predictive value. In addition, serum CA-S27 levels of post-operative sera from 17 CCA patients were significantly decreased comparing with those of corresponded pre-operative sera ($P < 0.05$; Fig 4C), suggesting the tumor origin of CA-S27 found in serum.

High level of serum CA-S27 indicated the poor survival of CCA patients

To signify the prognostic relevance of serum CA-S27, level of serum CA-S27 from CCA patients were categorized into low (N = 64) and high (N = 32) CA-S27 according to the mean value of CCA group (OD450 nm = 0.206). Survival analysis using Kaplan-Meier plot and Log-Rank test revealed that CCA patients with low serum CA-S27 had a better survival than those with high serum CA-S27 (P < 0.001; Fig. 4D). The median survival time of patients with low *vs*. high serum CA-S27 were 256 days (95%CI; 185-326 days) *vs.* 145 days (95%CI; 115-174 days), respectively. In order to investigate the influence of CA-S27 on the overall survival of patients in relation to MUC5AC, Log Rank analysis was used to compare the survival rates of patients with low *vs*. high CA-S27 in the low MUC5AC (Fig. 4E) and high MUC5AC groups (Fig. 4F). Regardless to serum MUC5AC level, patients with high serum CA-S27 had a significantly shorter survival than those with low serum CA-S27.

The association of serum CA-S27 and the clinical features of the patients was further analyzed using univariate analysis. Gender, histopathology, and tumor staging had no influence on the level of serum CA-S27. Only serum MUC5AC was found to be significantly correlated with level of serum CA-S27 ($P < 0.001$; Table 1). In addition, serum CA-S27 together with age, histopathology and staging of tumor were independent indicators for poor survival of CCA patients as shown by multivariate analysis using Cox-proportional hazard ($P < 0.001$; Table 2).

CA-S27 expression was associated with FUT3 expression

To evaluate the functional role of CA-S27 in CCA cell lines, we first determined the glycosyltransferase profile in KKU-M213 in comparison to MMNK1 cell line using the

quantitative reversed transcriptase-PCR (RT-PCR) array (17). Of 98 glycosyltransferases tested, FUT3 (the key enzyme for synthesis of Le family) was highly expressed in KKU-M213 with 461 times higher than that of the MMNK1 (Supplementary Table 1).

The expression levels of FUT3 and CA-S27 were determined in four CCA cell lines, KKU-M139, KKU-M156, KKU-M214, and KKU100. As determined by RT-PCR, FUT3 was differentially expressed in CCA cell lines in the order of KKU-M156> KKU-M139 and KKU-M214 whereas FUT3 was undetectable in KKU100 (Fig. 5Aa). The immunocytofluorescent staining of CA-S27 indicated the corresponding of positive CA-S27 staining and the level of FUT3 expression in CCA cell lines (Fig. 5Ab).

Suppression CA-S27 significantly decreased the metastatic potential of CCA cell lines

To address the functional importance of CA-S27, we employed RNAi to deplete the expression of FUT3 in KKU-M139 which moderately expressed FUT3. GAPDH was detected to assess the specificity of siFUT3 and used as an internal control. Treated KKU-M139 with siFUT3 significantly suppressed FUT3 expression to 45% of the siControl cells and reduced the CA-S27 expression as demonstrated by S27 immunofluorescent-staining (Fig. 5B). Suppression of CA-S27 expression by siFUT3 consequently decreased as compared to the controls in the number of cell invaded and migrated (Fig. 5C). Given control as 100%, the relative numbers of invaded (33 ± 18%) and migrated cells (61 ± 20%) in the siFUT3 treated group were lower than those of the controls ($P < 0.05$). A similar observation was made from the adhesion assay. The number of adhered cells for siFUT3 treated cells were 40 ± 18% for ECM adhesion assay and 59 ± 20% for FN adhesion assay as compared to the siControl, respectively ($P < 0.05$).

The roles of CA-S27 on cell invasion, migration and adhesion were further addressed using four CCA cell lines which differentially expressed CA-S27. Neutralizing CA-S27 expression with S27 mAb significantly reduced the numbers of cells invaded, migrated (Fig. 5D) and adhered of KKU-M139, KKU-M156 and KKU-M214 (Fig. 5E) to approximately 40% of the isotype-matched (IgM) controls. In contrast, the similar treatment did not alter the above functions of KKU-100 that had no CA-S27 expression.

Suppression of CA-S27 significantly decreased cell proliferation

The role of CA-S27 on cell proliferation of CCA cell lines with various expression levels of CA-S27 was evaluated. The proliferation of CA-S27 expressing cells was suppressed 2-6 folds when cells were treated with S27 mAb as compared with the cells treated with IgM control ($P < 0.05$; Fig. 6). However, the similar treatment did not suppress growth of KKU100 with negative CA-S27 expression.

Discussion

Alteration of glycosylation resulting in variation of carbohydrate expression is closely related to certain diseases and to the progression of neoplasms(19). Variation of glycoconjugates is accepted to represent a pivotal step for oncogenic transformation. It facilitates tumor cell migration and metastasis by affecting cell-cell and cell-matrix interactions(19-21). In the present study, a S27 mAb recognizing CA-S27 was established. We demonstrated for the first time that CA-S27 is a novel CCA associated marker. First, high levels of CA-S27 were detected in serum from CCA patients as compared to the controls, suggesting the diagnostic value of serum CA-S27 for CCA. Second, serum CA-S27 was shown to be of tumor origin and associated with poor patient outcome which underscored the prognostic value of serum

CA-S27. Third, CA-S27 significantly contributed to the metastatic activity and growth of CCA cells.

CA-S27 detected in serum of CCA patients was the tumor origin. Thus, CA-S27 was detected in CCA tissues and its level in serum was dramatically reduced after tumor removal. The LC/MS/MS analyses indicated MUC5AC mucin as a source of CA-S27 detected in serum. This finding was supported by the fact that positive signals of CA-S27 in CCA tissues and the level of CA-S27 in serum were associated with the expression of MUC5AC in tissues and level of MUC5AC in serum. Aberrant expression of MUC5AC mucin in tissues and serum from CCA patients have been reported in many studies(4, 15, 22, 23).

Normal bile duct epithelia also expressed CA-S27, however CA-S27 detected in normal biliary cells is probably attached to different core glycoproteins than MUC5AC mucin. This notion is supported by the fact that normal bile duct epithelia of liver tissues obtained from CCA patients and non CCA related subjects (cadaveric donors, fetuses and hepatoma subjects) were positive for CA-S27 but not for MUC5AC mucin. In addition, we rarely detected CA-S27 in serum of healthy subjects and other control groups.

In this study, we successfully developed SBA-S27 mAb sandwich ELISA to determine CA-S27 in serum. The levels of serum CA-S27 of CCA patients were significantly higher than those of the control groups, allowing one to effectively differentiate CCA patients from the patients with gastro-intestinal cancers, hepatomas, benign hepatobiliary diseases and healthy subjects. Our analysis had high sensitivity (87.5%) and high negative predictive value (90.4%).

The expression of Le^a is controlled by the Le loci, the gene coding for FUT3 (the key enzyme in the synthesis of Le family). Several polymorphisms of FUT3 are found in different ethnic populations. A study of Le genotype in Northeastern Thai population using PCR-SSP and multiplex-PCR revealed that the allele frequencies of the Le (FUT3) genes between the CCA patients (n = 220) and healthy controls (n =170) were not significantly different(24). CCA patients exhibited 73.64% Le^a positive and 26.36% Le^a negative comparing to healthy persons with 81.76% Le^a positive and 26.36% Le^a negative, respectively. With regard to this figure, one would expect to detect CA-S27 in approximately 75-85% of CCA patients from the Northeastern Thai. Our ELISA system could detect CA-S27 in serum of 87.5% (84/96) of CCA patients; therefore the patients with low level of serum CA-S27 are likely to be the patients who had Le negative genotype.

The level of serum CA-S27 was significantly associated with survival of CCA patients. Since high level of serum MUC5AC has been shown to be associated with poor survival of CCA patients(15, 22) and CA-S27 detected in serum from CCA patients was identified to be conjugated to MUC5AC mucin, therefore we further investigated whether CA-S27 or MUC5AC mucin had more influence on the survival of CCA patients. It is shown in the present study that patients with high serum CA-S27 had a shorter survival than those with low serum CA-S27 regardless of serum MUC5AC levels (Figs. 4E-F). Moreover, the level of serum CA-S27 was found to be an independent factor that influenced the patients' survival. Patients with high serum CA-S27 had 2.3 times higher risk of death than those with low serum CA-S27 (Table 2). This information reveals for the first time the prognostic value of serum CA-S27 levels in CCA.

CA-S27, the glycan moiety recognized by S27 mAb is related but not identical to Le^a, since it reacted with Le^a polymer in the glycoconjugate-microarray but failed to recognize the Le^a positive blood cells. Le^a is a trisaccharide of Gal-β1,3(Fuc-α1,4)-GlcNAc which is expressed in epithelia of several gastro-intestinal organs including stomach, small intestine, colon, and pancreas, etc., with unidentified function. Our study demonstrated, for the first time, the clinical significance of CA-S27, a Le^a associated epitope. Silencing of FUT3 expression using siRNA targeting to FUT3 significantly suppressed the expression of CA-S27 in KKU-M139 and resulted in the significant decrease in cell proliferation, invasion, migration, and adhesion of KKU-M139 to ECM and FN.

The role of other Lewis antigens (e.g., sLe^a and sLe^x), but not of Le^a in human cancer has been demonstrated by others. Suppression of FUT3 expression resulted in the reduction of Lewis antigen synthesis and consequently decreased tumor growth and metastasis in many cancers(25-27). Suppression of FUT3 expression by anti-sense for FUT3 significantly reduced the selectin binding and *in vivo* liver metastasis of colon cancer cells(26) and peritoneal colonization of human pancreatic cancer cells(25, 27).

Suppression of FUT3 not only affects the expression of Le^a but also other Lewis antigens such as sLe^a and sLe^x (25-27), therefore neutralizing CA-S27 with mAb specifically to CA-S27 is a more precise approach to determine the possible role of CA-S27. The significant role of CA-S27 in tumor progression was demonstrated by us in four CCA cell lines with various degree of CA-S27 expression. Neutralization of CA-S27 expression using S27 mAb significantly reduced cell proliferation, invasion, migration and adhesion of KKU-M139, KKU-M156 and KKU-M214 but not those of KKU-100 which had no CA-S27 expression. The functional role of CA-S27 in promoting cancer progression suggests a function for CA-

S27 in cellular and molecular signaling and a potential application of using CA-S27 for therapeutic target. The mechanism by which CA-S27 is involved in tumor progression is not yet clear and more experiments are needed to address this point.

Conclusion

This study reports the clinical value of CA-S27 in serum as a critical diagnostic and prognostic marker for CCA. High level of serum CA-S27 was detected in CCA patients and was associated with unfavorable outcome. The finding that the specific appearance of CA-S27 is associated with enhanced tumor growth and tumor invasiveness suggests a role for CA-S27 in cellular/molecular signaling and raises a possibility that CA-S27 may represent an attractive therapeutic target in CCA.

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Table 1 Correlation of serum CA-S27 and clinical data of CCA patients

Variable	No. of patients	Serum CA-S27		*P*
		Low (OD ≤ 0.206)	High (OD > 0.206)	
Age				
≤ 56 years	52	33	19	0.469
> 56 years	44	31	13	
Gender				
Male	69	47	22	0.632
Female	27	17	10	
Histopathology				
PAP	21	15	6	0.600
Non-PAP	75	49	26	
Tumor stage				
I-III	14	12	2	0.102
IVA and IVB	82	52	30	
Serum MUC5AC				
Low	54	49	5	< 0.001
High	42	15	27	

Table 2 Multivariate analysis of serum CA-S27 in CCA patients

Variable	N	Adjusted HR	95% CI	*P*
Serum CA-S27				
Low (OD ≤ 0.206)	64	1	1.229-4.419	**0.009**
High (OD > 0.206)	32	2.271		
Age				
≤ 56 years	52	1	1.072-2.567	0.023
> 56 years	44	1.659		
Gender				
Male	69	1	0.496-1.251	0.313
Female	27	0.788		
Histopathology				
PAP	21	1	1.477-4.709	0.001
Non-PAP	75	2.638		
Tumor stage				
I-III	14	1	1.287-4.515	0.006
IVA and IVB	82	2.411		

Supplementary

Table 1 Glycosyltransferase expression profile of KKU-M213 and MMNK1

GENE	KKU-M213	MMNK1	M213/MMNK1 ratio
FUT1	4090.17	762.24	5.37
FUT2	23.25	23.25	1.00
FUT3	**948.73**	**2.06**	**461.16**
FUT4	5609.82	1684.86	3.33
FUT5	28.37	88.61	0.32
FUT6	29.42	36.45	0.81
FUT7	4.99	1.31	3.80
FUT9	15.40	68.24	0.23
FUT8	27746.43	19577.60	1.42
FUT10	1301.66	1151.93	1.13
FUT11	1671.26	3742.11	0.45
ST3GAL1	25050.94	7793.27	3.21
ST3GAL2	3719.12	12329.74	0.30
ST3GAL3	1648.57	4506.37	0.37
ST3GAL4	704.70	988.77	0.71
ST3GAL5	613.82	2752.41	0.22
ST3GAL6	271.08	336.36	0.81

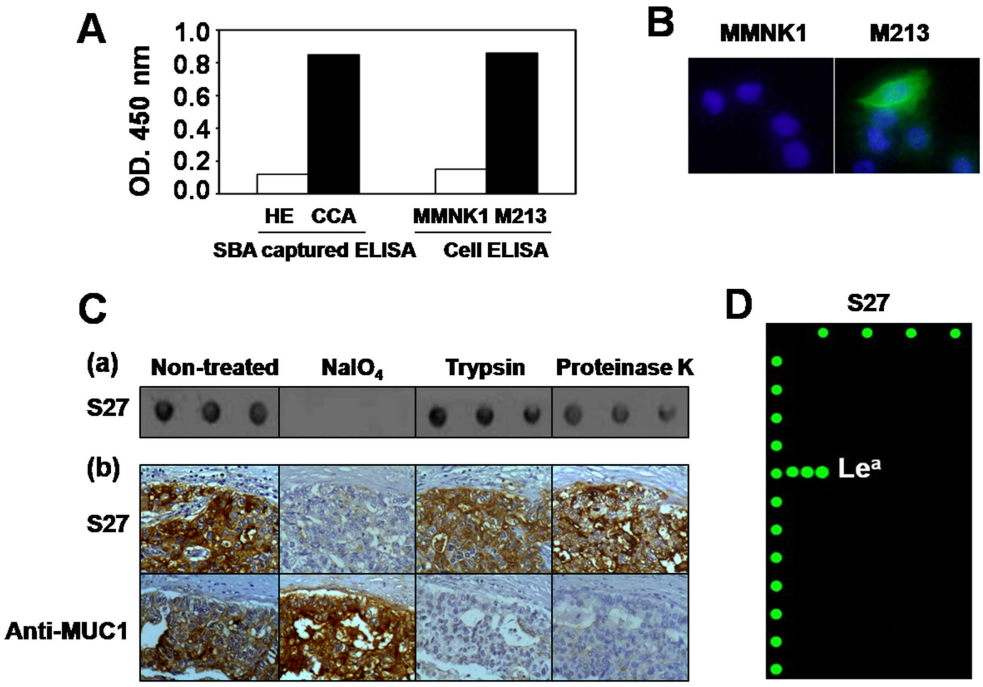

Figure 1 S27 monoclonal antibody recognizes carbohydrate antigen related to Lea. Using SBA-S27 sandwich ELISA, S27 mAb exhibited higher reactivity with pooled CCA serum than those from healthy subjects (HE) (A); cell ELISA (A) and immunofluorescent staining (B) of CCA cell line (KKU-M213) and immortal normal bile duct epithelium cells (MMNK1) using S27 mAb. CCA serum (C-a) and tissues (C-b) were pre-treated with NaIO4, trypsin or proteinase K prior reacted with S27 mAb. Of 98 known sugar moieties contained in the glycoconjugate microarray, only Lea epitope was recognized by S27 mAb (D). Anti-CA19-9 specifically reacted with sialyl Lea was used as the positive control for microarray system (Supplementary Fig. 1).
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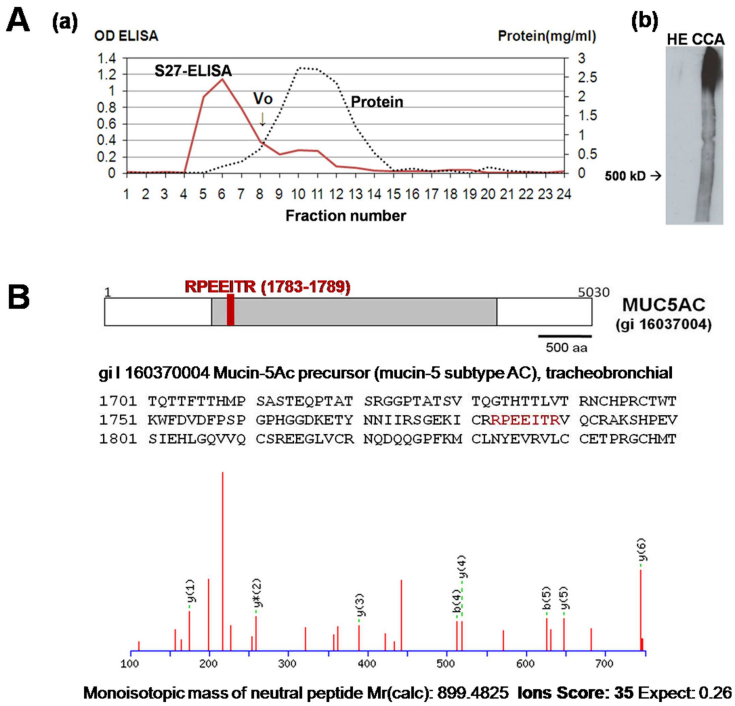

Detectors	HRP conjugated anti-IgM	-	+	+	-	+	+	+
	HRP conjugated SBA	+	-	-	+	-	-	-
Primary Ab	S27 mAb	-	-	-	-	+	+	+
Antigens	CCA pooled serum	-	-	-	+	+	+	-
	$NaIO_4$ treated CCA pooled serum	-	-	-	-	-	-	+
Captures	SBA	-	+	-	-	+	-	-
	Anti-MUC5AC (22C5)	+	-	+	+	-	+	+

Figure 2 MUC5AC mucin was the major source of CA-S27 detected in serum. Gel filtration using sepharose 6B chromatography (A-a) and western blotting of CCA serum (A-b) revealed that CA-S27 was an epitope of a large molecular weight protein. The S27-immunopurification followed by LC/MS/MS analysis revealed that a purified protein contained a peptide (RPEEIRTV, red) mapped on MUC5AC sequence with a MASCOT score of 35 (B). The result was confirmed by the sandwich ELISA using anti-MUC5AC and S27-mAb which revealed the positive signal similar to SBA-captured ELISA using S27 mAb (C).
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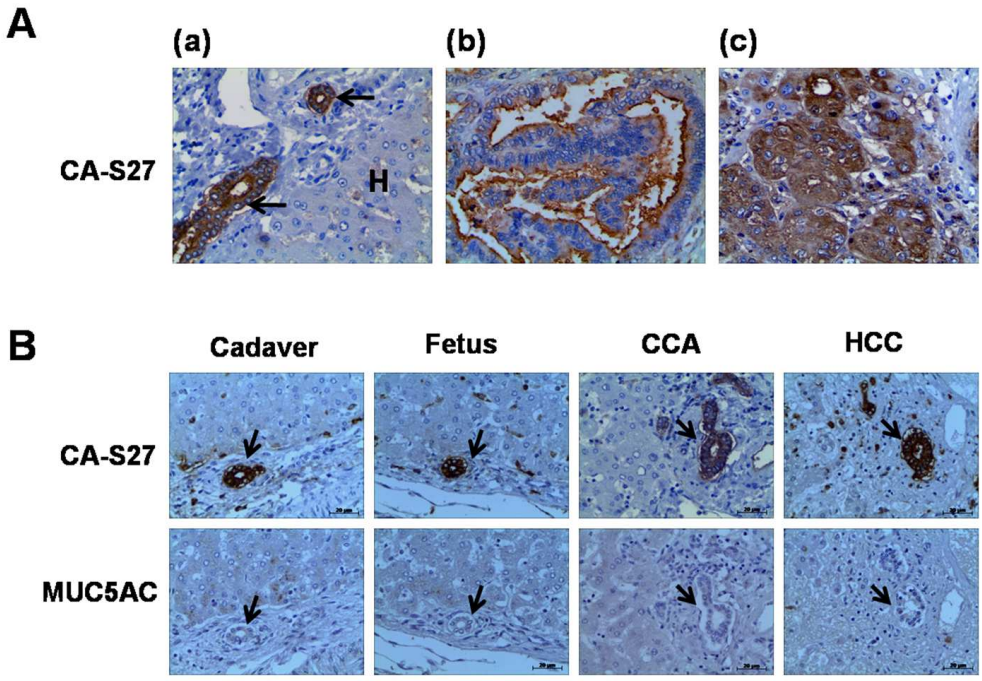

Figure 3 Immunohistochemistry of CA-S27 in CCA tissues indicated bile duct epithelium origin of CA-S27. Immunohistochemistry of CCA patients' tissues using S27-mAb as primary antibody, no CA-S27 reactivity was found in hepatocytes and stromal but the high signal was detected in normal bile duct epithelial cells (A-a), CCA with papillary type (A-b) and non-papillary type (A-c). Serial sections of liver tissues from cadaveric donors, fetus, CCA and hepatoma (HCC) patients were positive with CA-S27 but not MUC5AC (B). Arrows indicate normal bile ducts; H = hepatocyte; HCA = hepatoma
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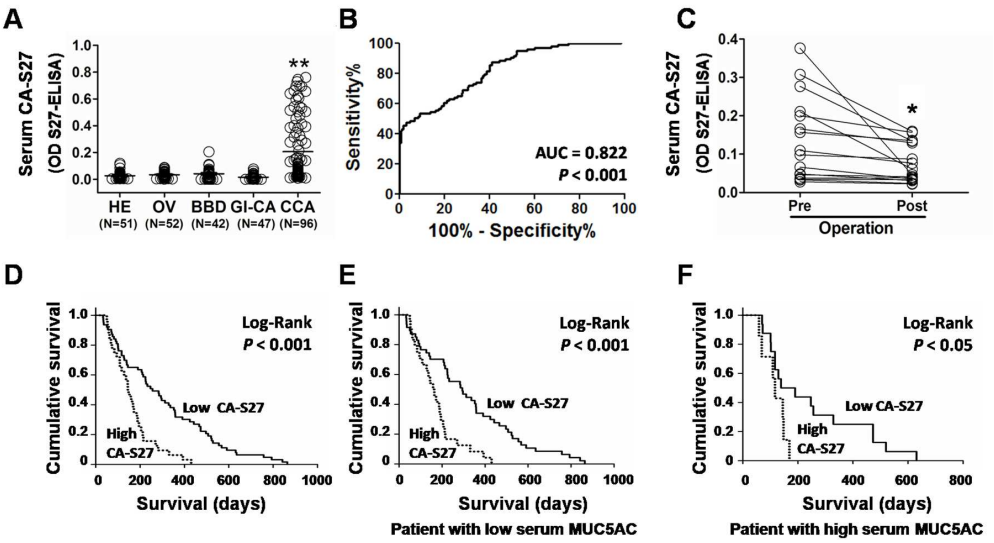

Figure 4 Serum CA-S27 as diagnostic and prognostic indicators for CCA. Level of serum CA-S27 determined by SBA-S27 sandwich ELISA was significantly higher in CCA patients compared with those of healthy person (HE) and patients of OV-infection (OV), benign biliary diseases (BBD) and other gastro-intestinal cancers (GI-CA) (A). ROC analysis of serum CA-S27 revealed the significant distinction between CCA patients and control (P < 0.001) with an area under curve of 0.822 (B). Serum CA-S27 of post-operative serum were significantly decreased compared with those of the corresponded pre-operative sera (N = 17) (C). Survival analysis using Kaplan Meier plot and Log Rank test revealed that CCA patients with high serum CA-S27 had significantly worse survival than those with low serum CA-S27 (D). Regardless to the level of serum MUC5AC, CCA patient with high serum CA-S27 had worse survival compared with the patients with low serum CA-S27 (E-F). *P < 0.05; **P < 0.001 (t-test).
306x168mm (300 x 300 DPI)

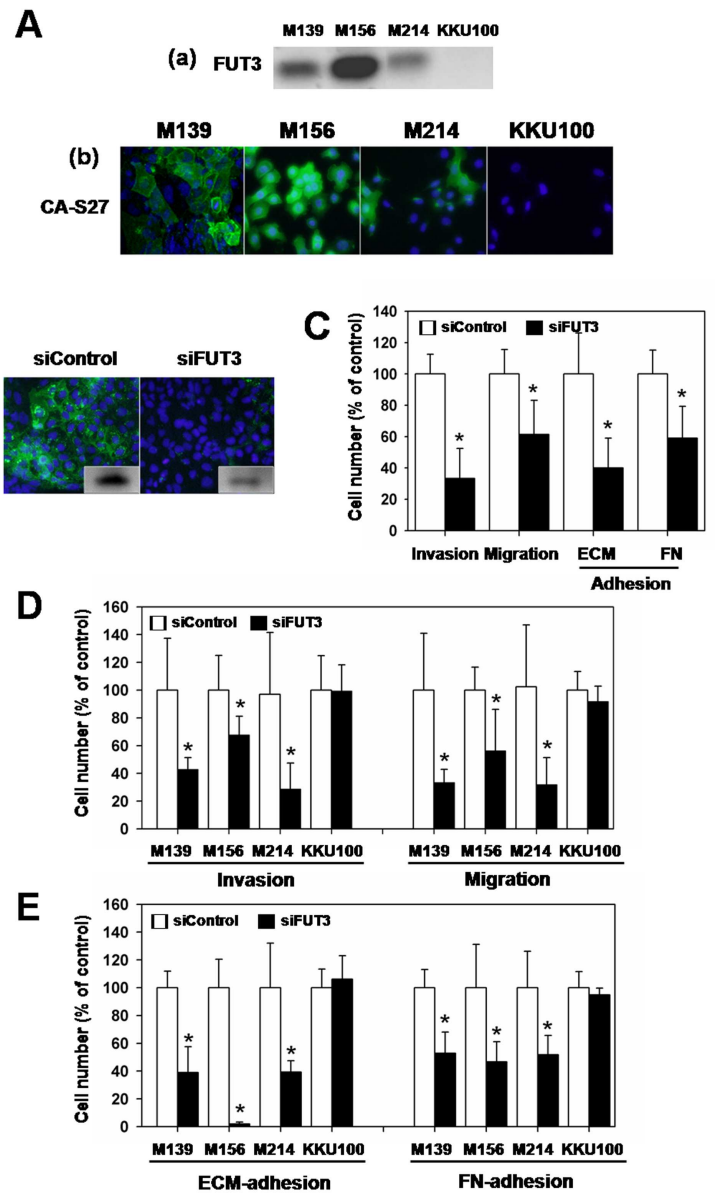

Figure 5 Expression of CA-S27 related to FUT3 activity and metastatic potential of CCA cell lines. CCA cell lines differentially express FUT3 mRNA as determined by semi-qPCR (A-a); the intensity of CA-S27 immunocytofluorecent staining of CCA cell lines (A-b) are corresponded to FUT3 levels. siFUT3 treatment markedly decreased FUT3 mRNA (insert) and CA-S27 immunocytofluorecent staining of KKU-M139 cells (B). The ability of KKU-M139 in invasion, migration, and adhesion to ECM and FN were significantly decreased after FUT3 knocked down (C). Treated CCA cell lines with S27 mAb significantly decreased cell invasion and, migration (D), and cell adhesion to ECM and FN of KKU-M139, KKU-M156, KKU-M214 but not those of KKU-100, compared with those of IgM control-treated (E). *significant different from controls; $P < 0.05$ (t-test). The assays were performed in triplicate and repeated at least twice in separated experiments.
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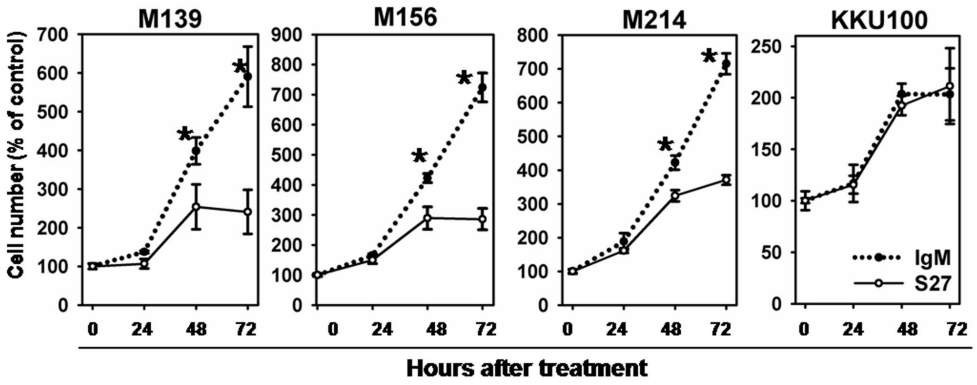

Figure 6 CA-S27 associates with cell proliferation. Treated CCA cell lines with S27 mAb significantly decreased cell proliferation of KKU-M139, KKU-M156, KKU-M214 but not those of KKU-100, compared with those of IgM control-treated. *significant different from controls; $P < 0.05$ (t-test). The assays were performed in triplicate and repeated at least twice in separated experiments.
177x70mm (300 x 300 DPI)

Supplementary Figure 1 Anti-CA19-9 specifically reacted with sialyl Lea was used as the positive control for microarray system.
51x96mm (300 x 300 DPI)