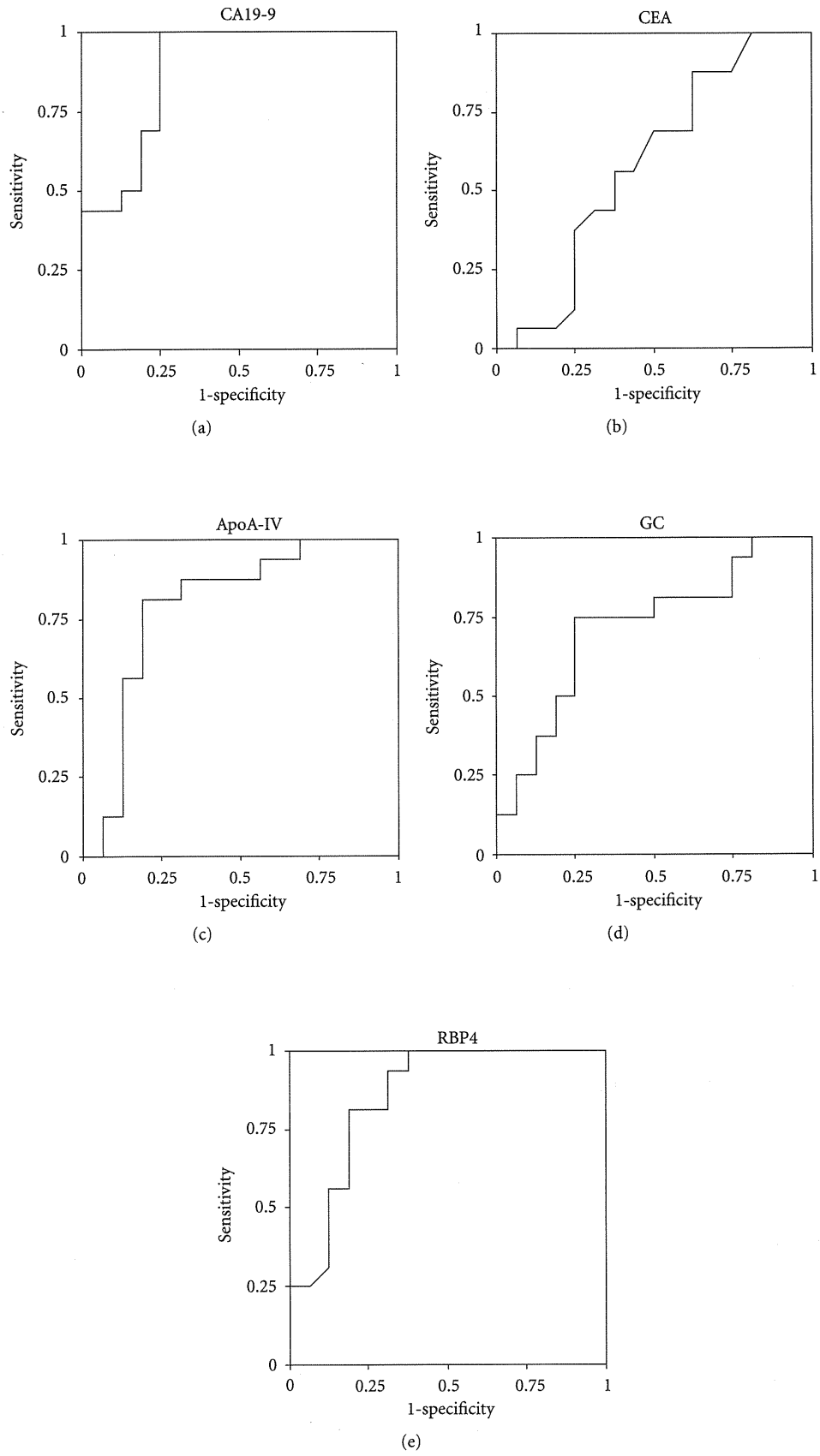

Figure 4: Receiver operating characteristic (ROC) curves for CA19-9, CEA, ApoA-IV, GC, RBP4 Their AUCs are described in the text.

Table 3

(a) Serum tumor marker levels in patients with pancreatic cancer.

Gender	Age (years)	UICC-stage	Tumor size (mm)	CA19-9 (U/mL)	CEA (ng/mL)	ApoA-IV (AU)	GC (AU)	RBP4 (AU)
M	38	IA	10	26.2	2.3	262.1	35.9	54.2
M	50	IB	30	46.5	2.3	8.5	34.3	43.9
M	63	IIA	18	157	1.1	10.2	26.7	37.0
M*	62	IIA	38	9	5	70.7	25.1	41.5
M*	54	IIA	24	11	1.4	87.5	11.3	42.5
M	73	IIA	25	15.5	3.5	203.5	13.6	49.5
F*	76	IIA	26	13.3	1.5	70.7	33.1	43.2
M	65	IIB	32	1579	3.4	352.1	24.3	36.7
M*	74	IIB	27	10.9	3.4	0	28.0	35.3
F	74	IIB	40	43	3.2	54	25.8	38.9
F	68	IIB	27	302	2.1	82.8	40.8	36.4
M	61	IIB	30	10	1.4	134.7	27.3	46.2
M	63	IIB	15	1080	—	144.6	23.9	53.3
M*	73	IIB	50	13.9	2.1	23.2	1.8	43.1
F	62	IIB	25	11.5	1.1	111.7	28.4	43.1
Ave ± SD	63.7 ± 1 0.4		27.8 ± 9.9	221.9 ± 466.2	63.7 ± 10.4	107.8 ± 99.9	25.4 ± 10	50.2 ± 4.2

CA19-9: carbohydrate antigen, CEA: carcinoembryonic antigen, ApoA-IV: apolipoprotein A-IV, GC: vitamin D-binding protein, RBP4: plasma retinol binding protein 4.
The stars indicate the patient who had a normal CA19-9 level and a low ApoA-IV.
Ave: average. SD: standard deviation. AU: arbitrary unit.

(b) Serum tumor marker levels in healthy controls.

Gender	Age (years)	CA19-9 (u/mL)	CEA (ng/mL)	ApoA-IV (AU)	GC (AU)	RBP4 (AU)
M	71	33.9	2.2	157.4	29.5	47.7
M	55	0.1	4.7	243.8	35.0	48.5
M	55	6.4	1	223.0	35.3	54.3
M	55	0.1	3.5	237.1	33.0	48.1
M	60	7.7	0.8	237.1	23.9	44.1
M	60	37.4	7	214.0	39.6	48.8
M	61	7.6	4.8	277.0	16.8	49.7
F	77	7.1	1.1	294.5	24.2	43.7
M	61	7.8	1.3	80.6	33.4	55.3
F	61	6	3	210.1	45.8	56.3
F	65	2.9	2.1	65.7	61.7	53.3
M	66	5.1	0.7	156.7	33.8	44.7
F	62	11.7	1.7	122.9	33.0	50.9
F	64	8.4	1.2	222.9	38.7	50.3
F	71	24.4	2.6	184.7	30.5	56.3
Ave ± SD	62.9 ± 6.3	11.1 ± 11.5	2.5 ± 1.8	195.2 ± 66.9	34.3 ± 10.3	50.2 ± 4.2

CA19-9: carbohydrate antigen, CEA: carcinoembryonic antigen, ApoA-IV: apolipoprotein A-IV, GC: vitamin D-binding protien, RBP4: plasma retinol binding protein
Ave: average. SD: standard deviation. AU: arbitrary unit.

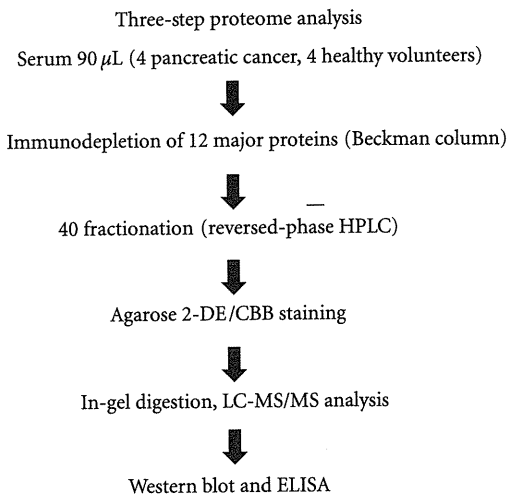

FIGURE 5: Outline of the procedure for the three-step serum proteome analysis.

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Expression and purification of human FROUNT, a common cytosolic regulator of CCR2 and CCR5

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ABSTRACT

Chemokine receptors play pivotal roles for immune cell recruitment to inflammation sites, in response to chemokine gradients (chemotaxis). The mechanisms of chemokine signaling, especially the initiation of the intracellular signaling cascade, are not well understood. We previously identified a cytoplasmic protein FROUNT, which binds to the C-terminal regions of CCR2 and CCR5 to mediate chemokine signaling. Although large amounts of purified protein are required for detailed biochemical studies and drug screening, no method to produce recombinant FROUNT has been reported. In this study, we developed a method for the production of recombinant human FROUNT. Human FROUNT was successfully expressed in *Escherichia coli*, as a soluble protein fused to the folding chaperone Trigger Factor, with a cold shock expression system. The purified FROUNT protein displayed CCR2 binding ability without any additional components, as demonstrated by SPR measurements. A gel filtration analysis suggested that FROUNT exists in a homo-oligomeric state. This high-yield method is cost-effective for human FROUNT production. It should be a powerful tool for further biochemical and structural studies to elucidate GPCR regulation and chemokine signaling, and also will contribute to drug development.

Introduction

G protein-coupled receptors (GPCRs) constitute the largest family of membrane proteins and mediate a multitude of cellular and physiologic responses to specific ligands [1]. Mutations in the genes encoding GPCRs are implicated in numerous diseases, and these receptors presently form the largest class of therapeutic targets [2]. Chemokine receptors play pivotal roles for immune cell recruitment to inflammation sites, in response to chemokine gradients (chemotaxis). This innate immune system is absolutely required for host defense, although when it becomes uncontrolled, it leads to inflammatory disease. Approximately 20 plasma membrane receptors have been characterized as members of the chemokine receptor family, and all of them are GPCRs [3].

Mutational analyses revealed that the cytoplasmic C-terminal domain, especially the membrane-proximal C-terminal region (Pro-C), of chemokine receptors plays an important role in chemotaxis [4–9]. In the cases of CCR2 and CCR5, the truncation of the Pro-C also impairs the chemokine signals, without the loss of cell surface localization [4,5]. We previously identified a 75-kDa cytoplasmic protein, FROUNT, which interacts with the Pro-C regions of CCR2 and CCR5, using a yeast two-hybrid system [10,11]. FROUNT directly binds to activated CCR2 and CCR5 and mediates directional cell migration. Since FROUNT does not bind to the C-terminal regions of CCR1, CCR3 and CXCR4, it was suggested that FROUNT interacts specifically with CCR2 and CCR5 [11]. The mechanisms of chemokine signaling, and especially the initiation of the intracellular signaling cascade, are not well understood. Since FROUNT lacks homology with known GPCR regulators, FROUNT may mediate the chemokine signaling in a novel manner. Clarification of the function of FROUNT will provide new insights into chemokine signaling and general GPCR regulation.

CCR2 and CCR5 are involved in various diseases, including chronic inflammation, cancer progression and viral infection, and thus FROUNT is considered as a promising drug target to treat a wide range of diseases. Various reports have indicated that FROUNT could actually have effective therapeutic applications: (1) We previously reported that macrophage infiltration was inhibited by FROUNT depletion, in a mouse peritonitis model [10]. (2) Belema-Bedada et al. reported that FROUNT is required for the migration and recruitment of CCR2-expressing bone marrow-derived mesenchymal stem cells to injured heart tissue [12]. (3) Satoh et al. showed that the mRNA levels of both FROUNT and

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CCR2 were up-regulated in biopsy tissue samples from patients with heart failure [13]. (4) Golen et al. reported that FROUNT mediates the transendothelial migration of prostate carcinoma cells [14].

Although large amounts of purified protein are required for detailed biochemical studies and drug screening, no method to produce recombinant FROUNT has yet been reported. We report here the first successful expression and purification of human FROUNT. We expressed human FROUNT fused to Trigger factor (TF), by a cold shock expression system in *Escherichia coli* (*E. coli*). The purified FROUNT protein retained the bind ability to CCR2. A gel filtration analysis suggested that FROUNT has oligomeric properties.

Materials and methods

Materials

Restriction enzymes were purchased from Toyobo Co., Ltd. PrimeStar DNA[1] polymerase and pCold TF DNA were purchased from Takara Bio Inc. SYBR® Safe DNA gel stain was purchased from Invitrogen. Molecular weight standards for SDS PAGE were purchased from BioRad. The Gel Filtration Calibration Kit (HMW) was purchased from GE Healthcare. The synthesized peptides, CCR2 Pro-C (EKFRRYLSVFFRKHITKRF) and 3 × FLAG peptide (DYKDDDDKDYKDDDDKDYKDDDDK), were purchased from Hokkaido System Science Co., Ltd. Other reagents were purchased from Nacalai Tesque, Inc. and Wako Chemicals, unless otherwise noted.

Construction of expression vectors

The DNA fragment encoding human FROUNT was amplified by polymerase chain reaction (PCR) and cloned in-frame into the pCold TF DNA vector, between the *Bam*HI and *Sal*I sites. A TEV protease site (ENLYFQG) was inserted just before the human FROUNT gene, by site-directed mutagenesis. The resulting plasmid, named pTF-FNT, generates the FROUNT protein fused with a hexahistidine-tag (His_6-tag) and Trigger factor (TF) at the N-terminus.

Protein expression

The His_6-TF-FROUNT fusion protein was expressed in *E. coli* BL21-CodonPlus™-RP cells (Stratagene) transformed with pTF-FNT. The cells were grown to an OD_{600} of 0.5 at 37 °C, in M9-tryptone medium (12.8 g $Na_2HPO_4{\cdot}7H_2O$, 3.0 g KH_2PO_4, 0.5 g NaCl, 1.0 g NH_4Cl, 10 g tryptone (BD) per liter of H_2O, 1 mM $MgSO_4$, 0.1 M $CaCl_2$, 0.5% glucose, 32 μg/mL chloramphenicol, 100 μg/mL ampicillin) [15]. To induce His_6-TF-FROUNT fusion protein production, 0.1 mM isopropyl-thio-galactopyranoside (IPTG) was added to the culture, which was incubated for an additional 12 h at 15 °C. The cells were collected by centrifugation at 4800 × *g* at 4 °C for 15 min and frozen at −80 °C.

Protein purification

The cell pellet (4 g) was resuspended in 40 mL of A buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 5 mM DTT), containing 1 mL of Protease Inhibitor Cocktail (Nacalai Tesque, Inc.), and lysed by sonication. Brij-35 was added to the cell lysate at a final concentration of 0.03%, and then it was centrifuged at 30,000 × *g* at 4 °C for 15 min. The supernatant was applied to a 10 mL Ni Sepharose 6 Fast Flow (GE Healthcare) column, pre-equilibrated in A buffer containing 0.03% Brij-35. The column was washed with 50 mL of B buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 2 mM DTT, 0.03% Brij-35, 200 mM imidazole), and the fusion protein was eluted with 20 mL of C buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 2 mM DTT, 0.03% Brij-35, 200 mM imidazole). TEV protease (AcTEV™ Protease, Invitrogen) was added to the His_6-TF-FROUNT fusion protein, and the solution was dialyzed against D buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 2 mM DTT, 0.03% Brij-35) at 4 °C for 24 h. The digested sample was loaded on a nickel-affinity column packed with 10 mL of Ni Sepharose 6 Fast Flow resin, to remove the histidine-tagged contaminants, including the uncleaved fusion protein, His_6-TF and TEV protease. The flow-through fraction was further purified by gel-filtration chromatography (HiLoad 26/60, Superdex 200 prep grade, GE Healthcare).

MALDI-TOF MS analysis of the intact protein

The solution containing the purified FROUNT protein was desalted, using a ZipTip C18 pipette tip (Millipore). The MALDI-TOF analysis was performed on a Bruker ultrafleXtreme (Bruker Daltonics) mass spectrometer. The sample (1 μL) was mixed with an equal volume of sinapic acid matrix solution in 50% acetonitrile and 0.1% TFA, and was spotted onto the target plate. Bovine serum albumin was used for calibration. For the average masses obtained in the linear mode, the mass accuracy was set at ~10 ppm.

Limited proteolysis

Recombinant FROUNT was digested with trypsin and chymotrypsin in A buffer at 37 °C for 20 min. The weight ratios of proteases to FROUNT were 1:100 and 1:10. The reactions were quenched by the addition of 0.1% TFA, and the proteolytic products were lyophilized.

N-terminal amino acid sequencing

Intact and digested FROUNT preparations were fractionated on a 15% SDS–PAGE gel and electrotransferred to a PVDF membrane. The bands were excised from the membrane after staining with Coomassie Brilliant Blue R250. After washing with methanol and Milli-Q water, the membrane pieces were analyzed by a protein sequencer (Procise® HT, Applied Biosystems) to determine the N-terminal amino acid sequences of intact and digested FROUNT.

In-gel digestion

Lyophilized samples were fractionated on a 15% SDS–PAGE gel, and the bands were excised from the gel after staining with a Rapid Stain CBB Kit. The gel pieces were diced into about 1 mm^3 pieces, and were digested according to the procedure reported by Ochi et al. [16], with minor modifications. The gel pieces were treated with 50 mM ammonium bicarbonate in 100% (v/v) acetonitrile and vacuum-dried. Sequence Grade Modified Trypsin (Promega) was added at a concentration of 50 μg/mL to the gel pieces, in 6.6% acetonitrile including 50 mM ammonium bicarbonate, and the mixture was incubated at 37 °C O/N. The trypsinized peptides were sequentially extracted from the gels with 0.1% (v/v) TFA in 30% (v/v) acetonitrile, 50% (v/v) acetonitrile, and 80% (v/v) acetonitrile, for 5 min each. The extracted peptides were vacuum-dried, dissolved in 20 μL of 0.1% (v/v) TFA, and desalted with a ZipTip C18 pipette tip.

[1] Abbreviations used: DNA, deoxyribonucleic acid; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; Hepes, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid; OD, optical density; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SPR, surface plasmon resonance; TFA, trifluoroacetic acid; Tris, tris (hydroxymethyl) aminomethane.

MALDI-TOF MS analysis for proteolytic products

MALDI-TOF and MALDI-TOF/TOF analyses were performed on a 4700 MALDI-TOF/TOF Analyzer (Applied Biosystems). The peptide solution (1 μL) was spotted onto the target plate, followed by spotting 1 μL of the matrix solution (3 mg/mL alpha-cyano-4-hydroxycinnamic acid, dissolved in 50% (v/v) acetonitrile and 0.1% (v/v) TFA). Analyses of mass data were performed with the 4000 Series Explorer Software v.3.5 (Applied Biosystems) and the Mascot software v.2.1 (Matrix Science).

SPR analysis

Purified recombinant human FROUNT was analyzed by SPR measurements, using a BIAcore T100 instrument (GE Healthcare). FROUNT was immobilized on the sensor chip CM5 (GE Healthcare) by standard amine-coupling chemistry, resulting in a signal of about 12,000 resonance units. The binding assay was performed in running buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P20, 1 mM DTT) at a flow rate of 30 μL/min at 25 °C. Various concentrations (0.16–5 μM) of CCR2 Pro-C (EKFRRYLSVFFRKHITKRF) or 3 × FLAG (DYKDDDDKDYKDDDDKDYKDDDDK) were injected into the FROUNT-immobilized flow-cell and a non-immobilized, control flow-cell. The data from the non-immobilized flow-cell were used for background subtraction. Equilibrium dissociation constants (K_D) were determined by nonlinear regression analyses, according to a 1:1 binding model. The BIA T100 evaluation software (GE Healthcare) was used for data analyses.

Results and discussion

Expression and purification of recombinant FROUNT

To express and isolate the human FROUNT protein, we constructed several kinds of *E. coli* expression plasmids encoding human FROUNT with various fusion tags, including GST, His_6, and *Strep* (Table 1). However, all of the proteins expressed from these plasmids formed inclusion bodies in *E. coli* under two temperature conditions (16 and 32 °C) (Table 1). Although we tried to purify the His_6-FROUNT-Strep fusion protein from the *E. coli* lysate, which even included a very small amount of the soluble fusion protein, we were not able to obtain a sufficient amount of the purified fusion protein (Table 1). After these attempts, we tried the TF and cold shock expression system [17–19]. TF, a prokaryotic molecular chaperone, assists with the folding of co-expressed proteins [17,18]. The cold shock expression system utilizes the *cspA* promoter to express a target protein at low temperature [19]. The low-temperature induction is effective for proper protein folding and inhibiting cellular proteases. We thus constructed the pTF-FNT vector for the expression of human FROUNT, fused with a His_6-tag and TF at the N-terminus (His_6-TF-FROUNT), using the cold shock vector (Fig. 1). A TEV protease cleavage site was inserted between TF and human FROUNT (Fig. 1), to allow the His_6-tag and the TF to be proteolytically removed by TEV protease, leaving the human FROUNT protein with one additional glycine residue. The *lac* operator was inserted downstream of the *cspA* promoter, to control the expression strictly. The resulting construct is schematically presented in Fig. 1.

E. coli BL21-CodonPlus™-RP cells were transformed with the pTF-FNT vector. The expression of the recombinant human FROUNT fusion protein was accomplished by induction with 0.1 mM IPTG at 15 °C for 24 h. The cells were lysed by sonication, and the soluble total protein was separated from the cell debris and the insoluble protein by centrifugation. Almost all of the His_6-TF-FROUNT (129 kDa) was detected in the soluble fraction by SDS–PAGE (Fig. 2). The His_6-TF-FROUNT was then purified from the soluble total protein by nickel-affinity chromatography (Fig. 3A, lane 2). More than 95% of the fusion protein was cleaved by a TEV protease treatment at 4 °C for 24 h (Fig. 3A, lane 3). The TEV protease-treated solution was passed through a nickel-affinity column to isolate the human FROUNT from the other His_6-tagged contaminants, including the uncleaved fusion protein, His_6-TF and TEV protease (Fig. 3A, lane 4). The recombinant human FROUNT was then purified by gel-filtration chromatography, and was detected as a single band that appeared to run at a MW of ~66 kDa by SDS–PAGE (Fig. 3A, lane 5). The single band was stained with an anti-human FROUNT polyclonal antibody [10] (Fig. 3B). The purification yield was ~3 mg per 1 L flask culture.

Confirmation of the recombinant human FROUNT protein

Mass spectroscopy and N-terminal amino acid sequence analyses were performed, in order to confirm that the purified protein was intact human FROUNT. Whole mass determination of the purified protein by MALDI-TOF mass spectrometry yielded an observed mass of 75077.1 Da, which is close to the mass of 75077.4 Da predicted from the sequence of human FROUNT (Fig. 4A). The N-terminal sequence of the purified protein was found to be "GMEEL", which is the same as intact human FROUNT, with an additional glycine residue from the TEV protease recognition site. Furthermore, the purified protein was treated with trypsin and chymotrypsin, and its fragments were separated by SDS–PAGE (Fig. 4B). Each fragment on the gels was analyzed by N-terminal amino acid sequencing, and after an additional tryptic digestion the peptides extracted from each fragment were

Table 1
Protein over-expression approaches for the production of recombinant human FROUNT.

Construct	Expression			Purification		
	Temperature (°C)	Amount[a](mg/L)	Solubility (%)	Step	Yield (mg/1L culture)	Purity (%)
GST-FROUNT	32	~5	<5	NT[b]		
	16	~5	<5	NT		
His_6-FROUNT	32	~10	<5	NT		
	16	~10	<5	NT		
His_6-FROUNT-Strep[c]	16	~10	<5	Nickel affinity/Strep-Tactin affinity	~0.2[d]	>95
His_6-TF-FROUNT	15	~10	>95	Nickel affinity/tag cleavage/tag removal/gel filtration	~3	>99

[a] Expression amount was estimated by comparing the band intensity with 0.5 μg of BSA on a CBB-stained SDS–PAGE gel.
[b] NT: Not tested.
[c] Strep: Strep-tag, Pro-Ser-His-Pro-Gln-Phe-Glu-Lys.
[d] His_6-FROUNT-Strep has a TEV protease site between His_6 and FROUNT, and a 3C protease site between FROUNT and the Strep-tag. Since it was inefficient to cleave the tags by these proteases, the yield described in this table is that of the fusion protein.

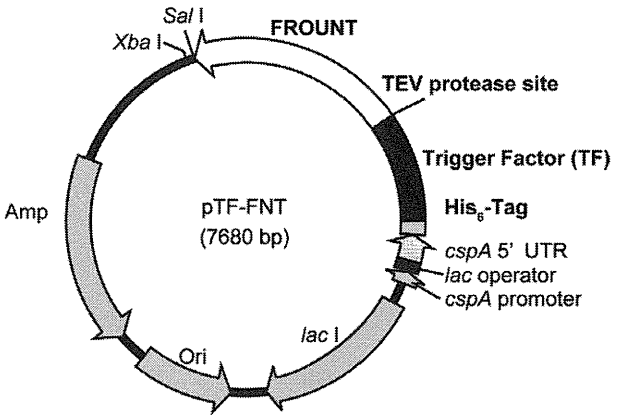

Fig. 1. Plasmid map of pTF-FNT. Structure and restriction map of the plasmid pTF-FNT, for the inducible expression of the His_6-TF-FROUNT gene from the *cspA* promoter. The *Sal*I and *Xho*I sites are indicated. Amp, Ampicillin resistance gene; Ori, *E. coli* origin of replication; *lacI*, *lac* repressor gene.

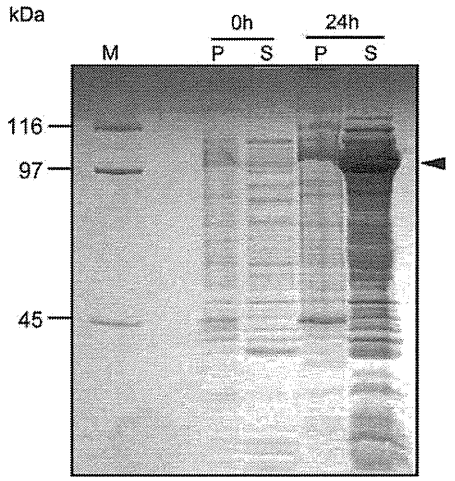

Fig. 2. Expression of FROUNT. Protein expression in recombinant *E. coli* BL21-CodonPlus-RP/pTF-FNT. Lane 1, insoluble cell protein before induction; lane 2, soluble cell protein before induction; lane 3, insoluble cell protein after induction for 24 h; lane 4, soluble cell protein after induction for 24 h. The black arrowhead indicates His_6-TF-FROUNT.

characterized by MALDI-TOF mass spectrometry. The sequences determined by these analyses covered 51% of the total human FROUNT sequence (Fig. 4D). These results indicate that the purified protein contains a full-length of human FROUNT, which was not degraded.

Secondary structure and oligomeric properties of recombinant human FROUNT

To examine the secondary structure of recombinant human FROUNT, we performed far-UV CD spectroscopy. Negative maximal peaks were detected around 208 and 222 nm (Supplementary Fig. 1). These data suggested that the recombinant human FROUNT adopts an α-helical secondary structure.

The oligomeric properties of recombinant human FROUNT were analyzed by size-exclusion chromatography. The purified protein was eluted as a single, symmetric major peak, at a larger molecular mass than the monomeric human FROUNT (75 kDa), between ferritin (440 kDa) and thyroglobulin (669 kDa)(Fig. 5). These data suggested that human FROUNT exists in an oligomeric state. A dynamic light scattering analysis also indicated that FROUNT forms

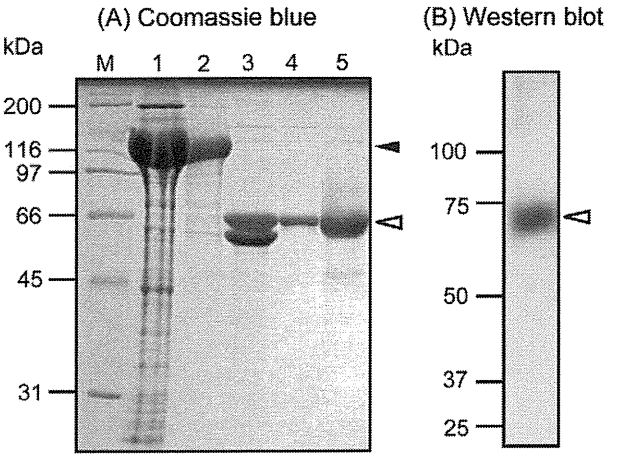

Fig. 3. Purification of FROUNT. (A) Summary of the FROUNT purification process. Lane 1, soluble cell protein after induction; lane 2, eluate from Ni^{2+} affinity resin; lane 3, products after cleavage by TEV protease: His_6-TF (51 kDa) and FROUNT (75 kDa); lane 4, flow-through from the Ni^{2+} affinity resin; lane 5, eluate from the Superdex 200 gel filtration column. Black and white arrowheads indicate His_6-TF-FROUNT and FROUNT, respectively. (B) Western blot analysis of human FROUNT. The purified FROUNT was detected by a polyclonal anti-human FROUNT antibody.

a homo-oligomer, and its molecular mass was estimated to be about 524 kDa (Supplementary Fig. 2). These results suggested that human FROUNT forms a homo-oligomer consisting of seven molecules.

The clustering of CCR2 or CCR5 is thought to be a sensor mechanism for the directed migration of leukocytes, and is regulated by FROUNT [10,11]. Homo- or hetero-dimeric complexes of those receptors are related to chemokine signaling [20–22]. Our FROUNT oligomerization data suggested that FROUNT may be related to receptor oligomerization.

Characterization of recombinant human FROUNT binding with CCR2

We next examined the CCR2-binding properties of recombinant human FROUNT by SPR analyses. Binding studies of CCR2 Pro-C (EKFRRYLSVFFRKHITKRF) were performed, using FROUNT protein that was immobilized on an SPR sensor chip. The 3 × FLAG peptide (DYKDDDDKDYKDDDDKDYKDDDDK) was used as a negative control. In these analyses, the interaction of FROUNT with CCR2 Pro-C was detected dose-dependently, while there were no significant signals with the 3 × FLAG peptide (Fig. 6). A small SPR signal from the non-immobilized, control sensor chip was detected, and was used for baseline correction. The average kinetic rate constants describing the CCR2 Pro-C–FROUNT interaction were calculated, and yielded a dissociation constant of 0.3 μM. These data showed that recombinant human FROUNT is able to interact with CCR2, and its binding needs no additional components.

Conclusions

This is the first report describing the expression and purification of recombinant human FROUNT. Human FROUNT was strongly expressed, as a soluble protein fused to TF, with the cold shock expression system in *E. coli*. Using our method, it was purified well, with a high yield. Using this purified protein, we found that human FROUNT is able to bind to CCR2 without any additional components. We also determined that human FROUNT exists in homo-oligomeric states.

Finally, the availability of milligram amounts of human FROUNT should enable efficient biochemical and structural studies of FROUNT, and may lead to the elucidation of the mechanisms of

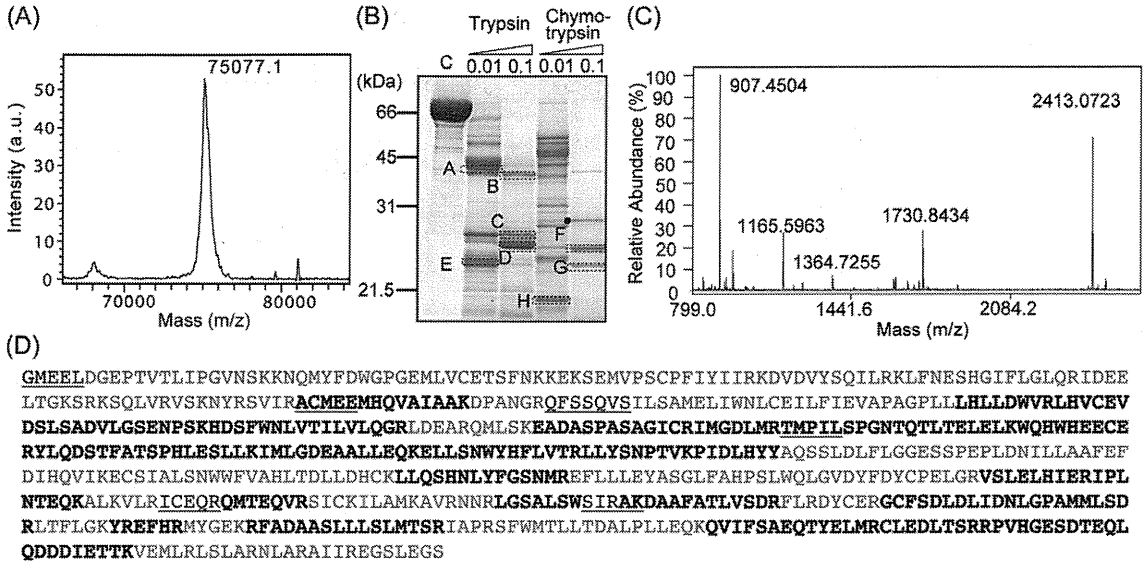

Fig. 4. Mass spectrometry and N-terminal amino acid sequence analyses of FROUNT. (A) Mass spectrum of FROUNT, showing the protein mass of 75 kD. (B) FROUNT was incubated in the absence (C: control) or presence of the indicated proteases for 20 min at 37 °C. The weight ratios of proteases to FROUNT were 1:100 (0.01) and 1:10 (0.1). The fragments that were analyzed by N-terminal sequencing and mass spectrometry are indicated by boxes. (C) A representative MALDI-TOF mass spectrum of an in-gel tryptic digested FROUNT fragment from a gel fraction (the spectral data obtained from band H, Fig. 4B). (D) Amino acids belonging to peptides identified by MALDI-TOF mass spectroscopy (bold) and by the N-terminal amino acid sequence (underlined), mapped on the human FROUNT amino acid sequence.

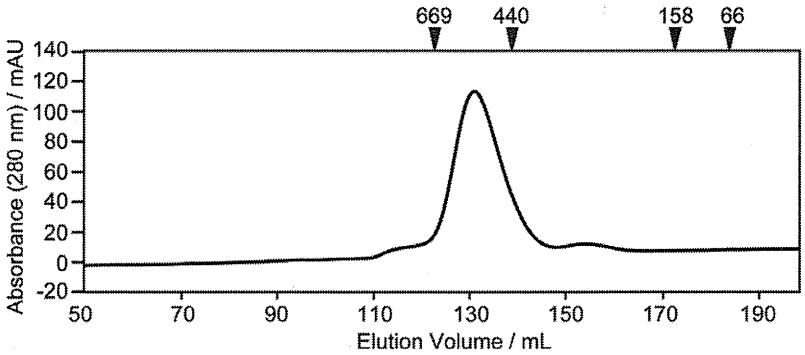

Fig. 5. Gel-filtration analysis. Gel-filtration chromatography profile of purified FROUNT. FROUNT eluted as a nearly single, sharp peak. The elution volumes of gel-filtration standards are indicated in the chromatogram by arrowheads: thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa) and BSA (66 kDa).

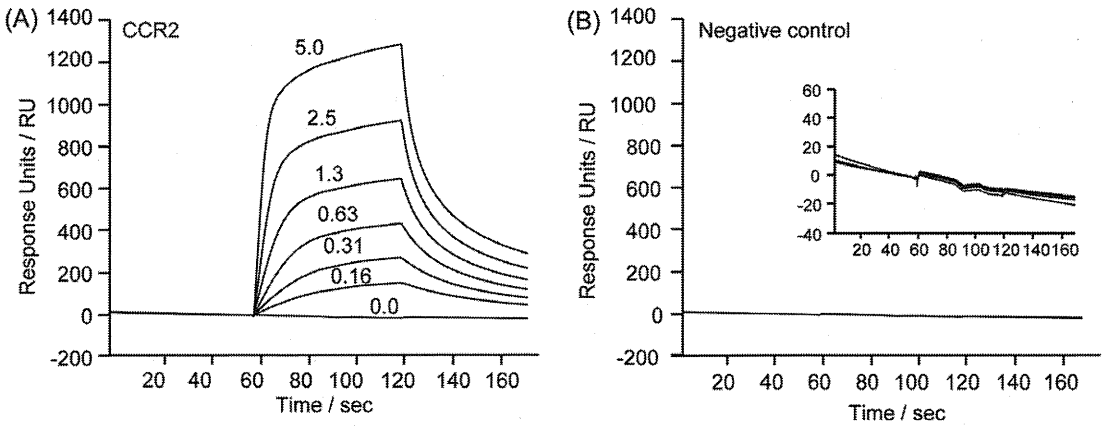

Fig. 6. Binding of FROUNT to CCR2 Pro-C. SPR sensorgrams for the interactions of FROUNT with CCR2 Pro-C (A) and a negative control (3 × FLAG) peptide (B). Peptides (0, 0.16, 0.31, 0.63, 1.4, 2.5 and 5.0 μM) were passed over immobilized FROUNT. The response units were determined by subtracting the blank values on the non-immobilized surface from the values on the FROUNT surface.

chemokine signaling and general GPCR regulation. Furthermore, it will also contribute to drug development to treat a wide range of diseases, including chronic inflammation, cancer progression and viral infection.

Acknowledgments

We are grateful to Dr. Shogo Misumi for the TOF mass spectrum measurements, Dr. Kohichi Kawahara for the N-terminal amino acid sequencing, and Dr. Hiroshi Morioka and Mr. Ryu Takahashi for SPR measurements. This work was supported in part by a Grant-in-Aid from the Targeted Proteins Research Program (TPRP) from the Japan Science and Technology Agency (JST), by a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), by a Sasakawa Scientific Research Grant from the Japan Science Society, and by the Global COE Program-Cell Fate Regulation Research and Education Unit from MEXT.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pep.2010.12.012.

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Original Article

A Novel Serum Carbohydrate Marker on Mucin 5AC

Values for Diagnostic and Prognostic Indicators for Cholangiocarcinoma

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BACKGROUND: The incidence of cholangiocarcinoma (CCA) is increasing globally. Currently, there is no powerful marker for the diagnosis of CCA, which has led to late diagnosis and poor patient outcome. This study was designed to establish a new monoclonal antibody (MoAb) for detecting a serum marker associated with CCA. **METHODS:** Pooled CCA tissue extracts were immunized to germinal center associated nuclear protein (GANP)-transgenic mice. The antibody-producing hybridomas were prepared and initially screened by using an indirect enzyme-linked immunosorbent assay (ELISA). A positive clone that reacted strongly with CCA serum or tumor tissue extract and failed to react with normal human serum and liver extract was selected. **RESULTS:** An S121 immunoglobulin M MoAb that recognized a novel glycan epitope was obtained. Immunohistochemistry of CCA tissues revealed that the MoAb reacted strongly with hyperplastic/dysplastic and neoplastic bile ducts but not with normal bile ducts. In addition, experiments demonstrated that mucin 5AC (MUC5AC) is a core glycoprotein for the S121 epitope. A sandwich ELISA using soybean agglutinin and an S121 MoAb was developed for detecting S121 reactive antigen in patient sera. The level of serum S121 from patients with CCA was reduced significantly after tumor removal, indicating the tumor origin of this antigen. The test was able to distinguish patients with CCA from healthy individuals, active *Opisthorchis viverrini*-infected individuals and patients with various gastrointestinal cancers, hepatoma, and benign hepatobiliary diseases with 87.63% sensitivity, 89.58% specificity, an 80.95% positive predictive value, and a 93.47% negative predictive value. Moreover, high serum S121 levels were related to a poor patient outcome. **CONCLUSIONS:** The sugar antigen recognized by S121 MoAb is a new serum marker for the diagnosis and prognosis of CCA. ***Cancer* 2011;117:3393–403.** © *2011 American Cancer Society.*

KEYWORDS: cholangiocarcinoma, tumor marker, enzyme-linked immunosorbent assay, carbohydrate marker, mucin, mucin 5AC.

Cholangiocarcinoma (CCA) is a rare cancer in Western countries but is considered the major public health problem in the northeast of Thailand, where the incidence of CCA is highest in the world.[1] With unknown factors as the cause, the incidence and mortality rate of CCA are increasing globally.[1-3] CCA is a slow-growing but highly metastatic tumor, which leads to the high mortality rate. Most patients present late and have a median survival of months. The late detection and poor survival after diagnosis has led a need for more powerful markers or techniques for the early diagnosis of CCA. Currently, complete resection is the therapy of choice; however, the difficulty in establishing the diagnosis of CCA preoperatively limits the number of successful treatments. Therefore, the availability of a rapid and formal proof of malignancy by using less invasive procedures, such as a serum marker, remains a constant goal in the diagnosis of CCA.

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There are several serum markers for demonstrating CCA, such as carcinoembryonic antigen (CEA),[4,5] carbohydrate antigen 19-9 (CA 19-9), biliary alkaline phosphatase,[6] and serum mucin 5AC (MUC5AC).[7-9] The questions about their overall accuracy, however, limit the use of these markers for early detection. Moreover, patients without cancer may harbor low levels of these markers in blood. The discovery of a new CCA-associated marker with high sensitivity and specificity remains an important objective.

Protein-based markers potentially are powerful, because they are amenable to simple blood tests and can be tested with routine assays. The monoclonal antibody (MoAb) approach has been used for investigating new markers in several cancers.[10,11] This attractive approach not only aids in the discovery of antigens or markers but also provides a tool for generating several marker-detection methods; moreover, it holds out the possibility of identifying a therapeutic bullet.[12] In the current report, we describe a new MoAb, S121, which specifically detects carbohydrate antigens in tumor tissues and sera from patients with CCA. The epitope is observed as a sugar moiety of mucin MUC5AC. A lectin-captured enzyme-linked immunosorbent assay (ELISA) was developed to determine the level of S121-specific carbohydrate markers in serum. We also explored the potential for using this assay as a diagnostic and prognostic marker for CCA.

MATERIALS AND METHODS

Tissues and Serum

Paraffin-embedded liver tissues and sera from patients with CCA were obtained from the Specimen Bank of the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand. Informed consent was obtained from each patient, and the study protocol was approved by the Ethics Committee for Human Research, Khon Kaen University (HE450525 and HE471214). Preoperative sera were obtained from 97 patients with CCA, 43 patients with benign hepatobiliary diseases and 47 patients with gastrointestinal cancers (12 stomach cancers, 9 pancreatic cancers, 13 colon cancers, 3 carcinomas of the ampulla of Vater, and 10 hepatomas). Preoperative and postoperative serum samples (> 3 months postsurgery) were obtained from 17 patients with CCA. Serum samples from 52 patients who had active opisthorchiasis and from 51 healthy individuals were included as controls. All serum samples were stored at −20°C until analysis.

All cancer specimens were obtained from patients with histologically proven disease. Tumor staging was classified according to the American Joint Committee on Cancer classification and staging system.[13] The diagnosis of benign hepatobiliary disease was based on clinical and histologic records. Opisthorchiasis was defined for asymptomatic individuals who had normal serum liver function tests and had positive detection of *Opisthorchis viverrini* eggs in their feces. Serum samples from healthy individuals were obtained from visitors at the hospital who attended an annual health checkup and were age-matched (based on their average age) with the patients with CCA.

Establishment of S121 MoAb

Two germinal center associated nuclear protein (GANP)-transgenic ($Ganp^{Tg}$) mice[14] were injected intraperitoneally with 50 μg of pooled CCA tumor homogenates (n = 5) in complete Freund adjuvant. Two weeks later, tumor homogenates (50 μg) in incomplete Freund adjuvant were injected subcutaneously. Two weeks after the second boost, the antigen (50 μg) in incomplete Freund adjuvant was prefusion boosted, and cell fusion was performed 4 days later. The antibody-producing hybridomas initially were screened by using a standard, indirect ELISA in pooled sera from patients with CCA or healthy individuals diluted 1:1000 or in crude extracts of CCA tissue or normal liver tissue at a concentration of 50 μg protein/mL as antigen.

A positive clone (S121 MoAb) was selected that reacted strongly with CCA serum or tumor tissue extract but failed to react with normal human serum and normal human liver tissue extract. Large amounts of S121 MoAb were produced in ascetic fluids according to the standard protocol. Briefly, after priming the mice with pristine (Sigma Chemical Company, St. Louis, Mo), the hybridoma was injected intraperitoneally into Balb/c-nude mice (Charles River Japan, Yokohama, Japan) to produce the ascetic fluids. The S121 MoAb was obtained from the ascetic fluids of the mice and further purified using a KAPTIV-M immunoglobulin M (IgM) purification column (Technogen, Piana di Monte Verna, Italy) according to the manufacturer's instructions.

Immunohistochemistry of CCA Tissues Using S121 MoAb

Detection of S121-reactive antigen in CCA tissue sections with the indirect immunoperoxidase method was performed according to the standard protocol. After nonspecific binding was blocked, the sections were incubated

with 5 μg/mL of S121 MoAb at room temperature overnight and with 1:500 horseradish peroxidase (HRP)-conjugated goat antimouse IgM (Southern Biotechnology, Birmingham, Ala) for 1 hour. The immunoreactivity was developed with diaminobenzidine tetrahydrochloride (Sigma Chemical Company) and 0.1% H_2O_2 in 50 mmol/L Tris-HCl, pH 7.8. Sections that were incubated with phosphate-buffered saline (PBS) instead of S121 MoAb were used as negative controls. Anti-MUC1 antibody (Invitrogen, Carlsbad, Calif) was used as a protein-specific antibody control followed by EnVision-system-HRP (Dako, Glostrup, Denmark). The staining frequency of S121-specific antigen was scored semiquantitatively on the basis of the percentage of positive cells as negative (0% positive cells), 1+ (1%-25% positive cells), 2+ (26%-50% positive cells), or 3+ (>50% positive cells).

Characterization of S121-Reactive Epitope

To determine whether the immunoepitope of S121 MoAb was a protein or glycan moiety, protein antigen was digested by trypsin (Invitrogen) or proteinase K (Sigma Chemical Company), whereas the sugar moieties were destroyed by treatment with sodium periodate ($NaIO_4$) (Sigma Chemical Company).[15-17] After deparaffinization and rehydration, the CCA tissue sections were treated either with 10 μg/mL trypsin or 10 μg/mL proteinase K at 37°C, for 1 hour or with 20 mmole/L $NaIO_4$ at 37°C for 2 hours. After washing with PBS, the sections were processed further for immunohistochemistry according to the standard protocol.

For characterization of the antigen epitope in serum, pooled sera (1 mg/mL) from 10 patients with CCA who had different histologic types was serially diluted 2-fold in distilled water, and 1 μL of each diluted sample was dotted onto a nitrocellulose membrane. After drying at room temperature, the membrane was treated with 10 μg/mL trypsin or proteinase K at 37°C for 1 hour. For deglycosylation, the membrane was incubated with 20 mmole/L $NaIO_4$ in 50 mM sodium acetate buffer, pH 4.5, at 37°C for 2 hours. Then, the membrane was washed 3 times for 10 minutes each with 0.3% Tween-20 in PBS (TPBS) and incubated with 5% skim milk in PBS for nonspecific blocking at 37°C for 1 hour. After 3 washings for 10 minutes each in TPBS, the membrane was incubated for 1 hour with 0.5 μg/mL S121 MoAb in TPBS and with 1:10,000 HRP-conjugated goat antimouse IgM at room temperature for 1 hour. Then, the membrane was developed with the Enhanced Chemiluminescence (ECL) Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, United Kingdom). The images of ECL signals were taken with an ImageQuant 400 image analyzer and were analyzed using ImageQuant TL analysis software (GE Healthcare).

Gel-Filtration Chromatography

To determine the apparent molecular weight of S121 antigen, pooled sera from patients with CCA (100 μL) were fractionated on a 0.5 × 10 cm sepharose-6B gel-filtration chromatography column (Pharmacia Biotech, Uppsala, Sweden) using 25 mM sodium phosphate buffer, pH 7.4, in 150 mM NaCl with a constant flow rate of 0.3 mL per minute. The 0.5-mL eluted fractions were collected, and the absorbance at 280 nm was determined. The level of S121-specific antigen in each fraction was determined with a soybean agglutinin (SBA)-captured ELISA using S121 MoAb.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Immunoblot

Pooled sera (30 μg) from patients with CCA were placed on 4% sodium dodecyl sulfate (SDS)-polyacrylamide gels and electrophoresed at 20 mA for 2 hours in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) running buffer according to the method published by Laemmli.[18] Then, the proteins were transferred onto a polyvinylidene fluoride membrane and probed by S121 MoAb as described above (see Characterization of S121-Reactive Epitope).

Glycoconjugate Microarray

The S121-specific sugar was analyzed by using a glycoconjugate microarray that consisted of 98 known sugar compounds as described previously.[19,20] Indocarbocyanine-labeled antimouse IgM (Jackson ImmunoResearch Laboratories, West Grove, Pa) was preincubated with 1 μg/mL S121 MoAb in a probing buffer (25 mM Tris–HCl, pH 7.4; 0.8% NaCl; 1% Triton-X; 1 mM $MnCl_2$; and 1 mM $CaCl_2$) to yield a final dilution of 1:2000. The mixture (100 μL) was applied to the glycoconjugate microarray and incubated at 20°C for 3 hours. The microarray was analyzed by using an evanescent-field fluorescence-assisted scanner (SC-profiler; GP Biosciences, Yokohama, Japan).

Identification of S121 Antigen

Pooled serum samples from patients with CCA were used as a source of S121 antigen. First, albumin and immunoglobulin were depleted from the pooled sera using the

Proteo Extract Albumin/IgG Removal Kit (Calbiochem, Darmstadt, Germany) according to the manufacturer's instructions; then, they were passed through a 0.2-mL S121-immunobilized agarose bead column. The unbound proteins were washed with a ×10 column volume of PBS. The bound protein was eluted with 1 × SDS-PAGE sample buffer and further separated by 4% SDS-PAGE.[18] The pooled serum samples from healthy individuals were processed in the same manner and were used as controls (the S121-negative sample). Gel from the S121-reactive band in patient sera and the corresponding gel from healthy individuals were excised for mass spectroscopy.

Mass Spectrometry

Samples were in-gel digested with trypsin. The digested peptides were desalted by using Zip Tips C18 (Millipore; Bedford, Mass) and were analyzed with nanoelectrospray ionization liquid chromatography (LC)/tandem mass spectrometry (MS/MS) using the LC Packings Ultimate instrument on a QSTAR Pulsar i mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, Calif). The identified peptide was searched by using the Mascot search engine (Matrix Science, Tokyo, Japan). The proteins that were identified in CCA samples were subtracted from the proteins that were identified in controls.

Lectin-Captured ELISA for S121-Specific Antigens in Sera From Patients With CCA

Fifty microliters of 40 μg/mL SBA (Sigma-Aldrich, Inc., St. Louis, Mo) in 50 mM carbonate buffer, pH 9.6, were coated into an individual well of a 96-well microtiter plate (Corning Incorporated, Corning, NY). After overnight incubation at 4°C in a moisture chamber, the plate was washed 5 times with 0.05% Tween-20 in normal saline. Unbinding sites were blocked with 200 μL of 2% bovine serum albumin (BSA) in 0.05% Tween-20 in PBS (PBST) at 37°C for 1 hour. After washing, serum samples (1:10 dilution; 50 μL) in 1% BSA-PBST were added, incubated at 37°C for 1 hour, then incubated with 50 μL of 1 μg/mL S121 MoAb for 1 additional hour. After washing, 50 μL of 1:4000 HRP-conjugated goat anti-mouse-IgM were added and incubated at 37°C for 1 hour. After washing, freshly prepared 3,3′,5,5′-tetramethyl benzidine (Sigma–Aldrich Inc.) substrate solution (100 μL) was added, and the plate was incubated in the dark for 15 minutes at room temperature; then, 50 μL of 2N sulfuric acid were added to stop the reaction. The optical density was read at 450 nm. All samples were processed in duplicate.

MU5AC-S121 Sandwich ELISA

To determine whether S121 antigen has a glycan moiety on MUC5AC in serum, a sandwich ELISA using anti-MUC5AC MoAb (clone 22C5)[7] and S121 MoAb was performed. Anti-MUC5AC MoAb (10 μg/mL) was coated onto a 96-well microtiter plate overnight. The subsequent processes were similar to those described for the lectin-captured ELISA.

Statistical Analysis

Statistical analysis was performed using the SPSS software package (version 16.0; SPSS, Chicago, Ill) and SigmaStat software (version 3.1; Systat Software, San Jose, Calif). The S121-specific antigens in sera from patients with CCA were compared with those from the control groups using the Mann-Whitney *U* test. The chi-square test was used to compare the differences in clinicopathologic findings from patients with CCA. A receiver operating characteristic (ROC) curve was constructed to compare the ability of serum S121 antigen to distinguish between the patients with CCA and the control groups.[21] The Youden index was used to select a cutoff value for the optical density (OD) that would indicate the diagnostic values of the test. Patient survival was calculated from the time of surgical resection to death. Survival analyses were performed using the Kaplan-Meier method, and survival was compared using the log-rank test. All P values $< .05$ were considered statistically significant.

RESULTS

More than 400 antibody-producing clones were screened. Of these, a MoAb designated S121 was identified that had high reactivity to pooled sera from patients with CCA but not to sera from healthy individuals. The subclass of this MoAb was named IgM/k.

S121-Specific Antigen Is Highly Expressed in Neoplastic Bile Ducts of CCA Tissue

Immunohistochemistry for S121-specific antigens was performed in 45 histologically proven CCA tumor tissues. Hepatocytes and almost all normal bile duct epithelial cells had negative immunoreactivity for the S121-specific antigen (Fig. 1A), whereas premalignant (Fig. 1B) and malignant (Fig. 1C,D) bile ducts exhibited strong positive staining. Forty-two of 45 CCA tissues (93%) had high reactivity that was both intense and frequent. Almost all

S121-reactive staining was distributed diffusely in the cytoplasm and densely at the apical surface. Some Kupffer cells and inflammatory cells exhibited positive staining. There was no statistical correlation between S121-positive tissues and tumor staging or histologic type among patients with CCA (data not shown).

S121 MoAb Recognizes High-Molecular-Weight Antigen in Sera

To identify the antigen of S121 MoAb, pooled serum samples from patients with CCA were fractionated based on their molecular weight using Sepharose 6B column chromatography. The S121-reactive fractions were observed mainly in the void volume (excluded fraction), suggesting a high molecular weight of S121-reactive antigen, as indicated in Figure 2A. An immunoblot analysis of serum proteins after 4% SDS-PAGE using S121 MoAb revealed an intense band at the top of the gel with an apparent molecular weight > 500 kDa (Fig. 2B).

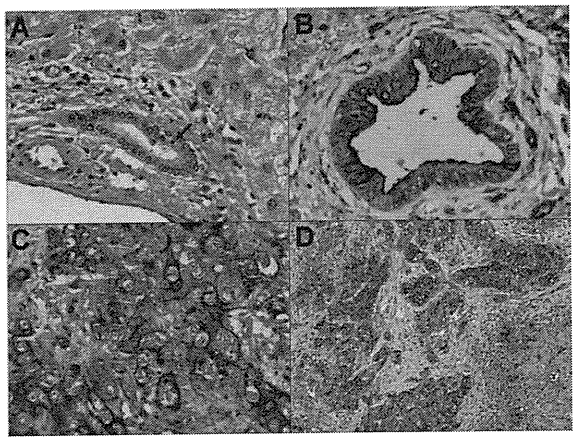


Figure 1. These photomicrographs illustrate the immunoperoxidase staining of S121 monoclonal antibody (mAb) in tissue samples of cholangiocarcinoma (CCA). (A) Normal bile duct epithelium did not stain with the S121 mAb (arrow), whereas S121-positive staining was observed in (B) hyperplasia/dysplasia and (C,D) CCA bile duct epithelium (original magnification, × 400 in A-C, × 100 in D).

S121 MoAb Recognizes Carbohydrate Moieties of the Antigen

Immunoblotting of serum samples from patients with CCA using S121 MoAb revealed positive reactivity in specimens that were treated with trypsin or proteinase K. In contrast, immunoreactivity was diminished in samples that were treated with sodium periodate (Fig. 3A). Similar observations were obtained with the immunohistochemistry of S121 when sections of tissue from CCA tumors were treated with trypsin, proteinase K, or sodium periodate (Fig. 3B). Positive immunohistochemical staining of S121 was retained after the protein antigens were digested with trypsin or proteinase K, whereas the signal was reduced when the sugar moieties were destroyed with sodium periodate. These results indicate the significance of the carbohydrate moieties as immunoepitopes of S121 MoAb. To demonstrate the specificity of trypsin, proteinase K, and sodium periodate treatments, the anti-MUC1 antibody, which recognizes protein fractions, was used instead of S121 MoAb. Immunoreactivity for anti-MUC1 was the reverse of what we observed for S121 MoAb. Positive immunostaining for anti-MUC1 antibody was observed in samples that were treated with sodium

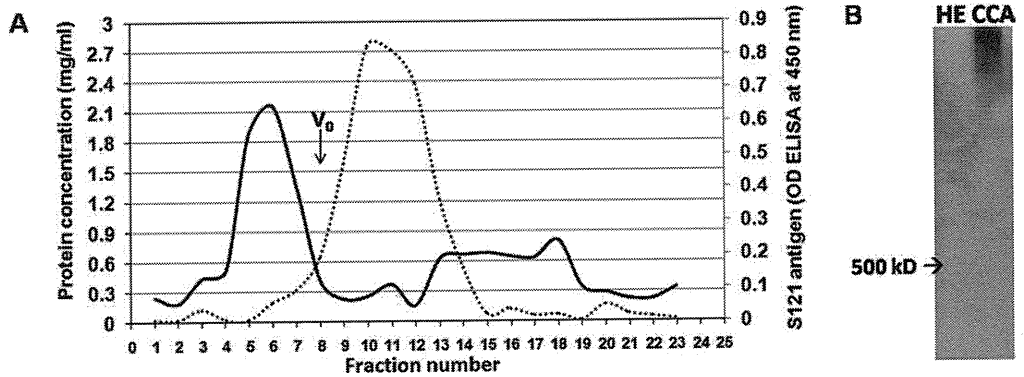


Figure 2. The S121 epitope was identified in high-molecular-weight protein fractions. (A) Pooled serum samples from patients with cholangiocarcinoma (CCA) were fractionated on a Sepharose 6B column, and the absorbance of protein at 280 nm (dotted line) and S121-specific antigen levels (solid line) were determined. S121 antigen was observed mainly in the void fractions. (B) This immunoblot of pooled sera from healthy individuals (HE) was compared with serum samples from patients with CCA. The S121 antigen was identified only in sera from patients with CCA at an apparent molecular weight >500 kDa. OD indicates outer diameter; ELISA, enzyme-linked immunosorbent assay.

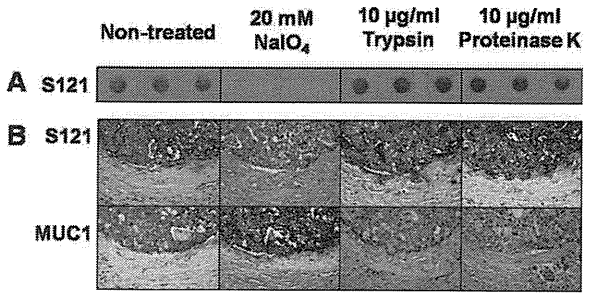


Figure 3. These images characterize S121-reactive antigen in (A) pooled serum samples from patients with cholangiocarcinoma and (B) tumor tissues that were treated either with protease (trypsin and proteinase K) or with periodate oxidization as described in the text (see Materials and Methods). After treatment, S121 antigen levels in each sample were determined using (A) immunoblotting and (B) immunohistochemistry. A monoclonal antibody against the protein part of mucin 1 (MUC1) was used as a control for the specific reactions of enzyme and sodium periodate treatment.

periodate, but immunostaining was reduced markedly in samples that were treated with trypsin or proteinase K (Fig. 3B). Taken together, these findings strongly suggest that the S121 MoAb recognizes an epitope of carbohydrate itself or an epitope that is associated with carbohydrate.

The potential structure of the glycan unit recognized by S121 MoAb was investigated further using a glycoconjugate microarray.[19,20] Of 100 glycan structures that were immobilized in the array, no known glycan moiety or common tumor markers reported as CCA marker, such as sialyl-Lewis A (sLe^a), Le^y, Le^x, sLe^x, sialyl-Tn antigen, etc, reacted with S121 MoAb. Therefore, it is probable that S121 MoAb recognizes a new carbohydrate-associated antigen that has not yet been identified.

MUC5AC Mucin Was Identified as the Core Glycoprotein of Carbohydrate Moieties Recognized by S121 MoAb

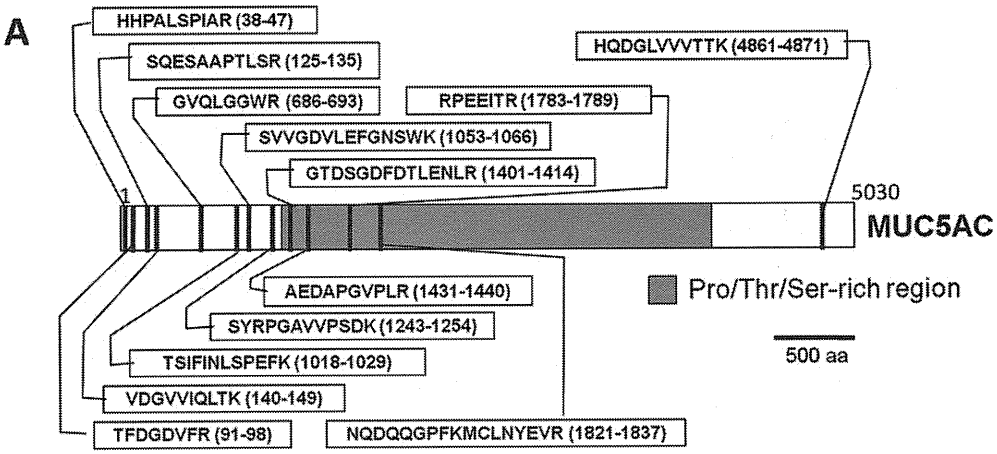
To identify the core protein of the S121 sugar epitope, S121 antigen in sera from patients with CCA was purified by S121 MoAb-affinity chromatography and separated further by SDS-PAGE. The LC/MS/MS analysis revealed that 14 peptide were sequences generated from tryptic digested CCA serum coinciding with those of MUC5AC (Fig. 4A). To confirm that the S121-recognizing glycan epitope was a component of MUC5AC mucin, a sandwich ELISA using anti-MUC5AC (22C5-MoAb) and S121 MoAb was performed. The reactivity of S121 MoAb obtained from the sandwich ELISA system using MUC5AC antibody (22C5 MoAb) and S121 MoAb was similar to that of obtained with the lectin-captured ELISA with S121 MoAb, as illustrated in Figure 4B. In addition, $NaIO_4$-treated serum samples exhibited lower reactivity for S121 MoAb obtained from the anti-MUC5AC captured sandwich ELISA system. This result indicated that the sugar moieties of MUC5AC mucin are the epitopes recognized by S121 MoAb.

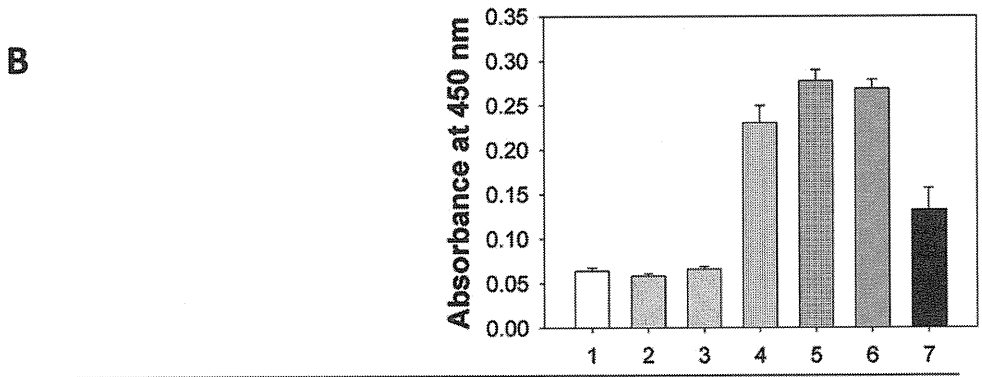
Value of Serum S121 Antigen as a Diagnostic Indicator of CCA

A lectin-capture ELISA was developed to determine the levels S121-specific antigen in serum. Checkerboard studies were performed to determine the optimal concentrations of S121 MoAb and to test sera with a fixed dilution (1:4000) of HRP-conjugated goat antimouse-IgM. Plates were coated with various concentrations of SBA (10-50 µg/mL) and reacted with different dilutions of test sera. The ELISA system using 50 µL of 40 µg/mL SBA at 1:10 dilution of sera and using 50 µL of 1 µg/mL S121 MoAb yielded the highest absorbance for CCA sera and the lowest absorbance for normal, healthy sera. Therefore, this system was used for subsequent studies.

The levels of S121 antigen determined by SBA-captured ELISA in serum samples from patients with CCA and from the control groups are illustrated in Figure 5A. The median serum S121 value was elevated significantly in samples from patients with CCA compared with the median value in samples from the control groups (patients with gastrointestinal cancer, patients with benign hepatobiliary diseases, patients with opisthorchiasis, and healthy individuals; $P < .001$). An analysis of the ROC curve was performed to determine the best cutoff S121 antigen value that distinguished patients with CCA from individuals in the control groups. On the basis of ROC curve analysis, an area under the curve of 0.956 (95% confidence interval [CI], 0.934-0.977) is illustrated in Figure 5B. Using a cutoff OD of 450 nm at an area under the curve of 0.11 produced sensitivity of 87.63% (85 of 97 patients) and specificity of 89.58% (172 of 192 patients) with a positive predictive value of 80.95% (85 of 105 patients) and a negative predictive value of 93.47% (172 of 192 patients). Serum S121 levels were not associated with age, sex, histopathology, or tumor staging among the patients as determined by univariate analysis (data not shown).

To demonstrate the tumor origin of the S121 antigen identified in serum, the association of S121 antigen detected in serum and tumor tissues was investigated further. Serum levels of S121 antigen from patients with CCA were determined before and after they underwent tumor resection. Seventeen patients with CCA who did





Detectors	HRP conjugated anti-IgM	-	+	+	-	+	+	+
	HRP conjugated SBA	+	-	-	+	-	-	-
Primary Ab	S121 MoAb	-	-	-	-	+	+	+
Antigens	CCA pooled serum	-	-	-	+	+	+	-
	$NaIO_4$ treated CCA pooled serum	-	-	-	-	-	-	+
Captures	SBA	-	+	-	-	+	-	-
	Anti-MUC5AC (22C5)	+	-	+	+	-	+	+

Figure 4. (A) The identified peptides from S121-purified antigen mapped on mucin 5AC (MUC5AC) are shown. Most peptides were hit at the N-terminus of MUC5AC, which is not a highly glycosylated part. Pro indicates proline; Thr, threonine; Ser, serine. (B) The presence of MUC5AC was determined in sera from patients with cholangiocarcinoma (CCA) using a sandwich enzyme-linked immunosorbent assay (ELISA) (lane 4, anti-MUC5AC and horseradish peroxidase [HRP]-conjugated soybean agglutinin [SBA]) and yielded results similar to those obtained with a lectin-captured ELISA of S121 antigen (lane 5, SBA and S121 MoAb) and from MUC5AC captured with an S121 MoAb ELISA (lane 6, anti-MUC5AC and S121 MoAb). Lanes 1, 2, and 3 were negative controls for each ELISA system. Treatment of serum with $NaIO_4$ at 37°C for 2 hours (lane 7) reduced the reactivity of S121 MoAb obtained from the anti-MUC5AC-captured sandwich ELISA system. IgM indicates immunoglobulin M; Ab, antibody.

not receive any treatment after tumor resection were included in this study. The elevated S121 level observed as OD in preoperative serum samples from patients with CCA was 0.324 ± 0.134 and decreased significantly to 0.239 ± 0.065 after tumor removal ($P < .001$) (Fig. 5C).

High Serum S121 Antigen Indicates a Worse Prognosis for Patients With CCA

In total, 97 patients with CCA who had different demographic characteristics were included in this study (Table 1). Patients with CCA were categorized according to their

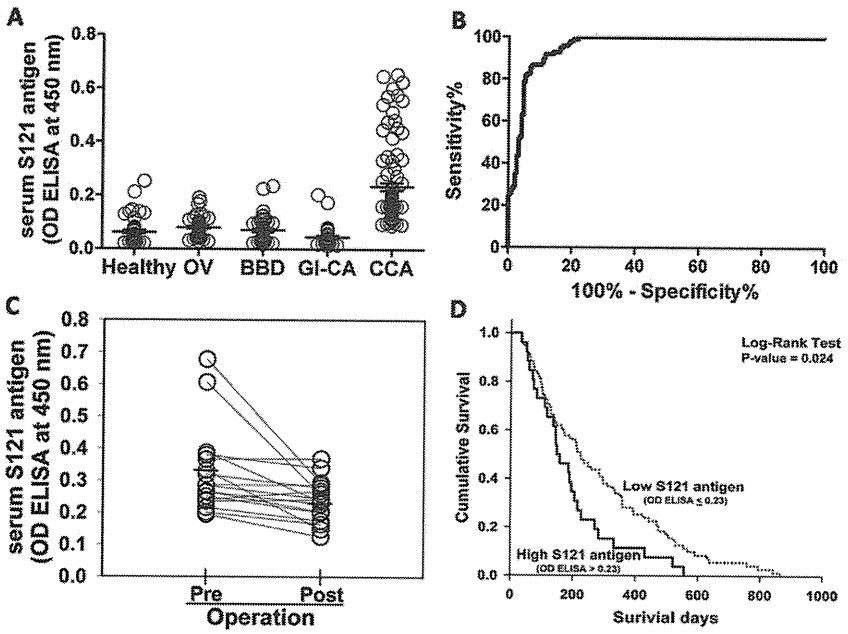


Figure 5. The determination of S121 antigen levels in serum is illustrated. (A) S121 antigen levels in sera from patients with cholangiocarcinoma (CCA) (n = 97) were determined by using a soybean agglutinin-captured enzyme-linked immunosorbent assay (ELISA) and were compared with the levels in healthy individuals (HE) (n = 51), in liver fluke-infected patients (OV) (n = 52), in patients with benign biliary diseases (BBD) (n = 43), and in patients with other gastrointestinal cancers (GI-CA) (n = 47). OD indicates outer diameter. (B) Receiver operating characteristic (ROC) analysis yielded an area under the ROC curve of 0.956 (95% confidence interval, 0.934-0.977) and a *P* value < .001. (C) The level of S121 antigen decreased significantly after tumor removal (n = 17; *P* < .001; Wilcoxon signed-rank test). (D) Kaplan-Meier analysis indicated that patients with CCA who had low serum S121 antigen levels (OD of ELISA, ≤0.23) had better survival than patients who had high serum S121 levels (OD of ELISA, >0.23; *P* = .024; log-rank test).

Table 1. Demographic Characteristics of Patients With Cholangiocarcinoma

Characteristic	No. of Patients (%)
Age, y	
≤56	45 (46.4)
>56	52 (53.6)
Sex	
Men	69 (71.1)
Women	28 (28.9)
Histopathology	
Papillary	22 (22.7)
Nonpapillary	75 (77.3)
Tumor stage	
I-III	14 (14.4)
IVA-IVB	83 (85.6)

mean serum S121 levels of the CCA into a group with low serum S121 (OD, ≤0.23 nm) and a group with high serum S121 (OD, >0.23 nm). If patients survived for <30 days, then their deaths were considered perioperative, and those patients were excluded from the analysis. A log-rank analysis indicated that patients with CCA who had low serum S121 antigen levels had significantly better survival than the patients who had high serum S121 antigen levels (*P* = .024) (Fig. 5D). The respective median survival was 224 ± 21 days (95% CI, 183-265 days) and 148 ± 52 days (95% CI, 95-200 days) for CCA patients in the low and high serum S121 groups, respectively.

DISCUSSION

In this report, we detail the discovery of a new MoAb that specifically reacts with a novel carbohydrate moiety of a high-molecular-weight glycoprotein(s) that was identified in tumor tissues and sera from patients with CCA. By using crude extracts from pooled CCA tissues as immunogens for generating MoAbs and using serum samples from patients with CCA as antigens for MoAb screening, the S121 MoAb, which recognizes a novel carbohydrate epitope on a tumor-originated antigen, was established. We demonstrated that the recognized S121 antigen is the carbohydrate moiety of a high-molecular-weight glycoprotein(s). Antigenicity of the S121antigen was abolished when the antigen was

treated with $NaIO_4$, a treatment known to oxidize and disrupt sugar conformation; whereas treatment with a he proteolytic enzyme (trypsin or proteinase K) had no effect on antigenicity of the S121 antigen. This result strongly suggests that the S121 epitope has a carbohydrate structure. According to the glycoconjugated microarray results, the S121 MoAb did not recognize any of the known sugar moieties on the array, including sLe^a or CA 19-9, which are common tumor markers of many cancers, including CCA. Hence, it is possible that the S121 antigen is a novel carbohydrate moiety that is highly expressed in CCA.

In general, identifying the sugar structure on mucins, such as MUC family proteins, is very difficult, and the technology for such analysis has not been established, because those carbohydrates are composed of densely branched sugar chains that are synthesized with high diversity on the mucin core protein. The sugar array technology developed by Tateno et al[19] may be the most powerful tool for identifying sugar epitopes; however, although nearly 100 distinguished sugar chain structures were displayed on our array chip, no reactivity against S121 MoAb was revealed; therefore, the epitope structure for S121 may be novel. Further study using other approaches will be needed to identify the sugar chain structure of the S121 epitope.

The S121-reactive antigen was excluded in the void volume of a Sepharose 6B column in gel-filtration chromatography and appeared at the top of a 4% SDS-PAGE gel; therefore, it may be a glycan epitope of the high-molecular-weight glycoprotein that we identified as MUC5AC mucin using S121 MoAb affinity purification and LC/MS/MS analysis. In the current study, almost all CCA tissues (93%) expressed S121 antigen in immunohistochemistry analysis. It was demonstrated previously that MUC5AC is expressed aberrantly in CCA tissues and is associated with the type, histologic grade, and advanced stage of intrahepatic CCA.[8] To our knowledge, there have been no reports on the upstream signal of MUC5AC expression in CCA. However, recently, the Kruppel-like zinc-finger GLI1 was identified as the regulator of the MUC5AC mucin in pancreatic ductal adenocarcinoma cells.[22] GLI1 up-regulated MUC5AC, attenuated E-cadherin-mediated cell-cell adhesion, and promoted cell migration and invasion. Our current results also suggest that CCA cells may have specific O-glycoenzyme up-regulation that causes the novel sugar chain modification on MUC5AC. This may be 1 of the most important mechanisms to be clarified in future studies. Currently, our analysis of the specific glycoenzyme genes in CCA is underway.

The value of S121-reactive antigen is emphasized by our finding that it could be detected in patients' sera with high sensitivity and specificity. In addition, this antigen originated from CCA tissue, because it was not observed in the normal bile duct or hepatocytes but was detected strongly in premalignant bile duct epithelium and CCA tissues. Moreover, serum levels of S121 were reduced after tumor removal. Serum S121 levels were high in samples from patients with CCA compared with the levels in samples from the control groups, which included healthy individuals, liver fluke-infected patients, patients with benign biliary diseases, and patients with gastrointestinal tract cancers. Our ROC analysis indicated that the serum S121 antigen could be used to diagnose CCA with 87.63% sensitivity and 89.58% specificity. These data suggest that serum S121 antigen can be used as a tumor marker for CCA. Moreover, the serum S121 level can be used as a prognostic marker, because who high serum S121 levels (OD, >0.23 nm) were associated with poor survival in patients with CCA.

In the past decade, there have been several attempts to identify a better tumor marker for CCA. In addition to CEA and CA 19-9, which are the commonly used tumor markers for CCA,[4,23-26] biliary alkaline phosphatase,[6] and MUC5AC[7-9] reportedly have been used as candidate markers for CCA with varied sensitivity and specificity.[6,9,27,28] Sensitivity from 50% to 80% and specificity from 80% to 90% have been reported for CA 19-9,[4,5,25] whereas serum MUC5AC reportedly had 60% to 70% sensitivity and 90% to 97% specificity for diagnosing CCA.[7-9] In the current study, the S121epitope was at least as sensitive as a carbohydrate moiety as the core protein of MUC5AC mucin. Detection of the sugar moiety provided better sensitivity, because the detection of serum S121 yielded 87.63% sensitivity and 89.58% specificity with 80.95% positive predictive value and 93.47% negative predictive value for the diagnosis of CCA. The advantage of serum S121 compared with CEA and CA 19-9 is that high levels of the S121 antigen were observed only in serum from patients with CCA but not in serum from patients who had gastric cancer, pancreatic cancer, colon cancer, carcinoma of the ampulla of Vater, and hepatoma; whereas high levels of CEA and CA 19-9 have been reported not only in patients with CCA but also in patients with many different cancers and chronic inflammatory conditions (eg, pancreatitis).[24,29,30]

Although the molecular function of the sugar-associated epitope recognized by S121 MoAb has not been determined, the S121 antigenic moiety may play a significant role in the pathogenesis of CCA. The association between the S121 antigen and the pathogenesis of CCA is supported by many aspects of the current study. First, the antigen was detected only in pathogenic bile duct epithelium. Immunohistochemistry using the S121 MoAb revealed that the S121 antigen was not present in normal bile ducts or and hepatocytes but was expressed progressively in hyperplastic/dysplastic bile duct epithelium and CCA. Second, the S121 antigen was detected at significantly higher levels in serum from patients with CCA compared with the levels detected in serum from individuals in the non-CCA control groups. Moreover, tumor resection significantly reduced the level of S121 in serum. Third, higher serum levels of S121 antigen were associated with a worse prognosis in patients with CCA.

In summary, this study established a MoAb that we designated as S121, which recognizes a not-yet-identified, carbohydrate-associated epitope that appeared specifically in CCA tumor cells. We have established that the MoAb is applicable for studies using immunohistochemistry of paraffin-embedded sections, immunoblotting, and ELISA. The MoAb is useful as a tool for detecting S121 antigen, which is elevated in sera from patients with CCA. The ability of S121 MoAb to differentiate between neoplastic-bile duct and normal bile duct suggests the potential application of S121 MoAb in a therapeutic approach.

CONFLICT OF INTEREST DISCLOSURES

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