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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REFERENCES

1. Shaib Y, El-Serag HB. The epidemiology of cholangiocarcinoma. *Semin Liver Dis.* 2004;24:115-125.
2. Shaib YH, Davila JA, McGlynn K, El-Serag HB. Rising incidence of intrahepatic cholangiocarcinoma in the United States: a true increase? *J Hepatol.* 2004;40:472-477.
3. Patel T. Increasing incidence and mortality of primary intrahepatic cholangiocarcinoma in the United States. *Hepatology.* 2001;33:1353-1357.
4. Qin XL, Wang ZR, Shi JS, Lu M, Wang L, He QR. Utility of serum CA19-9 in diagnosis of cholangiocarcinoma: in comparison with CEA. *World J Gastroenterol.* 2004;10:427-432.
5. Ramage JK, Donaghy A, Farrant JM, Iorns R, Williams R. Serum tumor markers for the diagnosis of cholangiocarcinoma in primary sclerosing cholangitis. *Gastroenterology.* 1995;108:865-869.
6. Bhudhisawasdi V, Muisuk K, Areejitranusorn P, et al. Clinical value of biliary alkaline phosphatase in nonjaundiced cholangiocarcinoma. *J Cancer Res Clin Oncol.* 2004;130:87-92.
7. Bamrungphon W, Prempracha N, Bunchu N, et al. A new mucin antibody/enzyme-linked lectin-sandwich assay of serum MUC5AC mucin for the diagnosis of cholangiocarcinoma. *Cancer Lett.* 2007;247:301-308.
8. Boonla C, Wongkham S, Sheehan JK, et al. Prognostic value of serum MUC5AC mucin in patients with cholangiocarcinoma. *Cancer.* 1. 2003;98:1438-1443.
9. Wongkham S, Sheehan JK, Boonla C, et al. Serum MUC5AC mucin as a potential marker for cholangiocarcinoma. *Cancer Lett.* 2003;195:93-99.
10. Krueger P, Nitz C, Foster R, et al. A new small cell lung cancer (SCLC)-specific marker discovered through antigenic subtraction of neuroblastoma cells. *Cancer Immunol Immunother.* 2003;52:367-377.
11. Krueger P, Nitz C, Moore J, Foster R, Gelber O, Gelber C. Monoclonal antibody identifies a distinctive epitope expressed by human multiple myeloma cells. *J Immunother.* 2001;24:334-344.
12. Van Aarsen LA, Leone DR, Ho S, et al. Antibody-mediated blockade of integrin alpha v beta 6 inhibits tumor progression in vivo by a transforming growth factor-beta-regulated mechanism. *Cancer Res.* 2008;68:561-570.
13. Greene FL, Page DL, Flaming ID, et al. eds. AJCC Cancer Staging Manual. 6th ed. New York: Springer-Verlag; 2002.
14. Sakaguchi N, Kimura T, Matsushita S, et al. Generation of high-affinity antibody against T cell-dependent antigen in the Ganp gene-transgenic mouse. *J Immunol.* 2005;174:4485-4494.
15. Bara J, Decaens C, Loridon-Rosa B, Oriol R. Immunohistological characterization of mucin epitopes by pretreatment of gastro-intestinal sections with periodic acid. *J Immunol Methods.* 1992;149:105-113.
16. Cao Y, Blohm D, Ghadimi BM, Stosiek P, Xing PX, Karsten U. Mucins (MUC1 and MUC3) of gastrointestinal and breast epithelia reveal different and heterogeneous tumor-associated aberrations in glycosylation. *J Histochem Cytochem.* 1997;45:1547-1557.
17. Gil J, Alvarez R, Vinuela JE, et al. Inhibition of in vivo tumor growth by a monoclonal IgM antibody recognizing tumor cell surface carbohydrates. *Cancer Res.* 1990;50:7301-7306.
18. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970;227:680-685.
19. Tateno H, Mori A, Uchiyama N, et al. Glycoconjugate microarray based on an evanescent-field fluorescence-assisted detection principle for investigation of glycan-binding proteins. *Glycobiology.* 2008;18:789-798.
20. Tateno H, Ohnishi K, Yabe R, et al. Dual specificity of Langerin to sulfated and mannosylated glycans via a single

C-type carbohydrate recognition domain. *J Biol Chem.* 2010;285:6390-6400.
21. Zweig MH, Campbell G. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clin Chem.* 1993;39:561-577.
22. Inaguma S, Kasai K, Ikeda H. GLI1 facilitates the migration and invasion of pancreatic cancer cells through MUC5AC-mediated attenuation of E-cadherin [published online ahead of print October 25, 2010]. Oncogene. 2010.
23. Nakeeb A, Lipsett PA, Lillemoe KD, et al. Biliary carcinoembryonic antigen levels are a marker for cholangiocarcinoma. *Am J Surg* 171:147-152, 1996; discussion 152-143.
24. Ni XG, Bai XF, Mao YL, et al. The clinical value of serum CEA, CA19-9, and CA242 in the diagnosis and prognosis of pancreatic cancer. *Eur J Surg Oncol.* 2005;31:164-169.
25. Patel AH, Harnois DM, Klee GG, LaRusso NF, Gores GJ. The utility of CA 19-9 in the diagnoses of cholangiocarcinoma in patients without primary sclerosing cholangitis. *Am J Gastroenterol.* 2000;95:204-207.
26. Saito K, Fujii Y, Kawakami S, et al. Increased expression of sialyl-Lewis A correlates with poor survival in upper urinary tract urothelial cancer patients. *Anticancer Res.* 2003;23: 3441-3446.
27. Uenishi T, Yamazaki O, Tanaka H, et al. Serum cytokeratin 19 fragment (CYFRA21-1) as a prognostic factor in intrahepatic cholangiocarcinoma. *Ann Surg Oncol.* 2008;15:583-589.
28. Watanabe H, Enjoji M, Nakashima M, et al. Clinical significance of serum RCAS1 levels detected by monoclonal antibody 22-1-1 in patients with cholangiocellular carcinoma. *J Hepatol.* 2003;39:559-563.
29. Del Favero G, Fabris C, Panucci A, et al. Carbohydrate antigen 19-9 (CA 19-9) and carcinoembryonic antigen (CEA) in pancreatic cancer. Role of age and liver dysfunction. *Bull Cancer.* 1986;73:251-255.
30. Del Favero G, Fabris C, Plebani M, et al. CA 19-9 and carcinoembryonic antigen in pancreatic cancer diagnosis. *Cancer.* 1986;57:1576-1579.

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

Kaori Esaki [a], Yuya Terashima [b,c], Etsuko Toda [c], Sosuke Yoshinaga [a], Norie Araki [a], Kouji Matsushima [c], Hiroaki Terasawa [a,*]

[a] Faculty of Life Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan
[b] Central Lab, ECI Inc., Meguro-ku, Tokyo, Japan
[c] Department of Molecular Preventive Medicine, Graduate School of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo, Japan

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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

* Corresponding author. Fax: +81 96 371 4310.
E-mail address: terasawa@gpo.kumamoto-u.ac.jp (H. Terasawa).

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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.

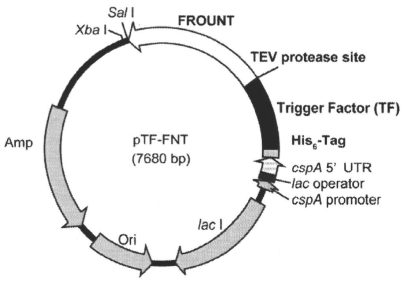

Fig. 1. Plasmid map of pTF-FNT. Structure and restriction map of the plasmid pTF-FNT, for the inducible expression of the His_6-TF-FROUNT gene from the *cspA* promoter. The *Sal*I and *Xho*I sites are indicated. Amp, Ampicillin resistance gene; Ori, *E. coli* origin of replication; *lacI*, *lac* repressor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

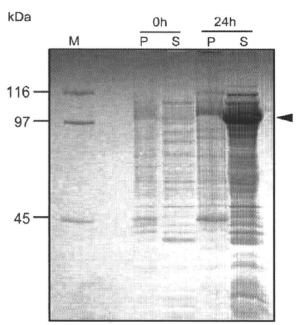

Fig. 2. Expression of FROUNT. Protein expression in recombinant *E. coli* BL21-CodonPlus-RP/pTF-FNT. Lane 1, insoluble cell protein before induction; lane 2, soluble cell protein before induction; lane 3, insoluble cell protein after induction for 24 h; lane 4, soluble cell protein after induction for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

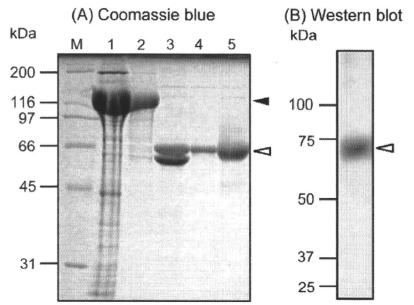

Fig. 3. Purification of FROUNT. (A) Summary of the FROUNT purification process. Lane 1, soluble cell protein after induction; lane 2, eluate from Ni^{2+} affinity resin; lane 3, products after cleavage by TEV protease: His_6-TF (51 kDa) and FROUNT (75 kDa); lane 4, flow-through from the Ni^{2+} affinity 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

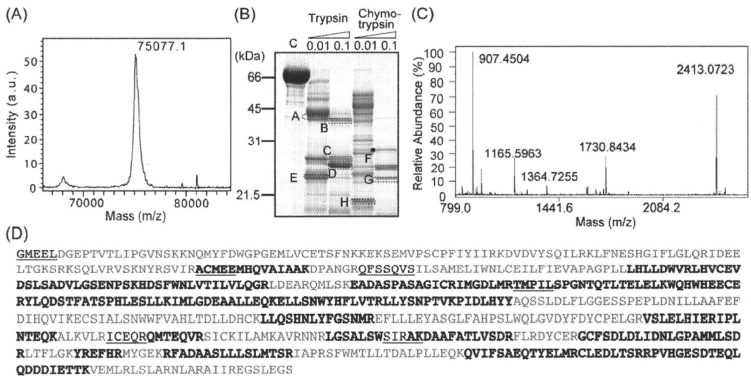
(A)
75077.1
Intensity (a. u.)
Mass (m/z)
(B)
Trypsin
Chymo-trypsin
C 0.01 0.1 0.01 0.1
(kDa)
66
45
31
21.5
A
B
C
D
E
F
G
H
(C)
907.4504
2413.0723
1730.8434
1165.5963
1364.7255
Relative Abundance (%)
Mass (m/z)
(D)
GMEELDGEPTVTLIPGVNSKKNQMYFDWGPGEMLVCETSFNKKEKSEMVPSCPFIYIIRKDVDVYSQILRKLFNESHGIFLGLQRIDEE
LTGKSRKSQLVRVSKNYRSVIRACMEEMHQVAIAAKDPANGRQFSSQVSILSAMELIWNLCEILFIEVAPAGPLLLHLLDWVRLHVCEV
DSLSADVLGSENPSKHDSFWNLVTILVLQGRLDEARQMLSKEADASPASAGICRIMGDLMRTMPILSPGNTQTLTELELKWQHWEECE
RYLQDSTFATSPHLESLLKIMLGDEAALLEQKELLSNWYHFLVTRLLYSNPTVKPIDLHYYAQSSLDLFLGGESSPEPLDNILLAAFEF
DIHQVIKECSIALSNWWFVAHLTDLLDHCKLLQSHNLYFGSNMREFLLLEYASGLFAHPSLWQLGVDYFDYCPELGRVSLELHIERIPL
NTEQKALKVLRICEQRQMTEQVRSICKILAMKAVRNNRLGSALSWSIRAKDAAFATLVSDRFLRDYCERGCFSDLDLIDNLGPAMMLSD
RLTFLGKYREFHRMYGEKRFADAASLLLSLMTSRIAPRSFWMTLLTDALPLLEQKQVIFSAEQTYELMRCLEDLTSRRPVHGESDTEQL
QDDDIETTKVEMLRLSLARNLARAIIREGSLEGS

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.

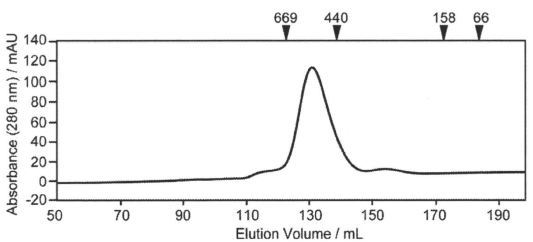

Fig. 5. Gel-filtration analysis. Gel-filtration chromatography profile of purified FROUNT. FROUNT eluted as a nearly single, sharp peak. The elution volumes of gel-filtration standards are indicated in the chromatogram by arrowheads: thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa) and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

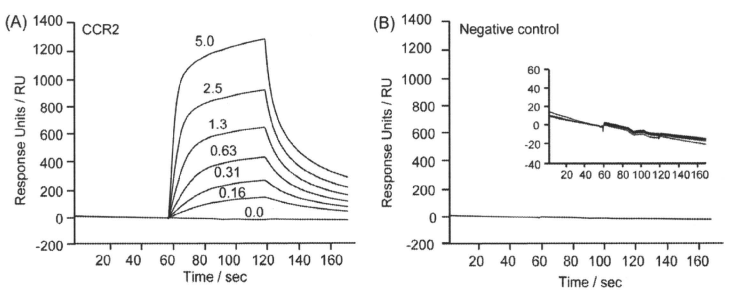

Fig. 6. Binding of FROUNT to CCR2 Pro-C. SPR sensorgrams for the interactions of FROUNT with CCR2 Pro-C (A) and a negative control (3 × FLAG) peptide (B). Peptides (0, 0.16, 0.31, 0.63, 1.4, 2.5 and 5.0 μM) were passed over immobilized FROUNT. The response units were determined by subtracting the blank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

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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.

References

[1] D.M. Rosenbaum, S.G. Rasmussen, B.K. Kobilka, The structure and function of G-protein-coupled receptors, Nature 459 (2009) 356–363.
[2] R.M. Eglen, R. Bosse, T. Reisine, Emerging concepts of guanine nucleotide-binding protein-coupled receptor (GPCR) function and implications for high throughput screening, Assay Drug Dev. Technol. 5 (2007) 425–451.
[3] S.J. Allen, S.E. Crown, T.M. Handel, Chemokine: receptor structure, interactions, and antagonism, Annu. Rev. Immunol. 25 (2007) 787–820.
[4] H. Arai, F.S. Monteclaro, C.L. Tsou, C. Franci, I.F. Charo, Dissociation of chemotaxis from agonist-induced receptor internalization in a lymphocyte cell line transfected with CCR2B. Evidence that directed migration does not require rapid modulation of signaling at the receptor level, J. Biol. Chem. 272 (1997) 25037–25042.
[5] K. Kraft, H. Olbrich, I. Majoul, M. Mack, A. Proudfoot, M. Oppermann, Characterization of sequence determinants within the carboxyl-terminal domain of chemokine receptor CCR5 that regulate signaling and receptor internalization, J. Biol. Chem. 276 (2001) 34408–34418.
[6] C. Le Gouill, J.L. Parent, C.A. Caron, R. Gaudreau, L. Volkov, M. Rola-Pleszczynski, J. Stankova, Selective modulation of wild type receptor functions by mutants of G-protein-coupled receptors, J. Biol. Chem. 274 (1999) 12548–12554.
[7] J. Sai, G. Walker, J. Wikswo, A. Richmond, The IL sequence in the LLKIL motif in CXCR2 is required for full ligand-induced activation of Erk, Akt, and chemotaxis in HL60 cells, J. Biol. Chem. 281 (2006) 35931–35941.
[8] S.G. Mueller, J.R. White, W.P. Schraw, V. Lam, A. Richmond, Ligand-induced desensitization of the human CXC chemokine receptor-2 is modulated by multiple serine residues in the carboxyl-terminal domain of the receptor, J. Biol. Chem. 272 (1997) 8207–8214.
[9] C. Otero, P.S. Eisele, K. Schaeuble, M. Groettrup, D.F. Legler, Distinct motifs in the chemokine receptor CCR7 regulate signal transduction. Receptor trafficking and chemotaxis, J. Cell Sci. 121 (2008) 2759–2767.
[10] Y. Terashima, N. Onai, M. Murai, M. Enomoto, V. Poonpiriya, T. Hamada, K. Motomura, M. Suwa, T. Ezaki, T. Haga, S. Kanegasaki, K. Matsushima, Pivotal function for cytoplasmic protein FROUNT in CCR2-mediated monocyte chemotaxis, Nat. Immunol. 6 (2005) 827–835.
[11] E. Toda, Y. Terashima, T. Sato, K. Hirose, S. Kanegasaki, K. Matsushima, FROUNT is a common regulator of CCR2 and CCR5 signaling to control directional migration, J. Immunol. 183 (2009) 6387–6394.
[12] F. Belema-Bedada, S. Uchida, A. Martire, S. Kostin, T. Braun, Efficient homing of multipotent adult mesenchymal stem cells depends on FROUNT-mediated clustering of CCR2, Cell Stem Cell 2 (2008) 566–575.
[13] M. Satoh, T. Akatsu, Y. Ishkawa, Y. Minami, M. Nakamura, A novel activator of C–C chemokine, FROUNT, is expressed with C–C chemokine receptor 2 and its ligand in failing human heart, J. Card. Fail. 13 (2007) 114–119.
[14] K.L. van Golen, C. Ying, L. Sequeira, C.W. Dubyk, T. Reisenberger, A.M. Chinnaiyan, K.J. Pienta, R.D. Loberg, CCL2 induces prostate cancer transendothelial cell migration via activation of the small GTPase Rac, J. Cell Biochem. 104 (2008) 1587–1597.
[15] A. Koide, C.W. Bailey, X. Huang, S. Koide, The fibronectin type III domain as a scaffold for novel binding proteins, J. Mol. Biol. 284 (1998) 1141–1151.
[16] H. Ochi, I. Horiuchi, N. Araki, T. Toda, T. Araki, K. Sato, H. Murai, M. Osoegawa, T. Yamada, K. Okamura, T. Ogino, K. Mizumoto, H. Yamashita, H. Saya, J. Kira, Proteomic analysis of human brain identifies α-enolase as a novel autoantigen in Hashimoto's encephalopathy, FEBS Lett. 528 (2002) 197–202.
[17] G. Stoller, K.P. Rucknagel, K.H. Nierhaus, F.X. Schmid, G. Fischer, J.U. Rahfeld, A ribosome-associated peptidyl-prolyl cis/trans isomerase identified as the trigger factor, EMBO J. 14 (1995) 4939–4948.
[18] K. Nishihara, M. Kanemori, H. Yanagi, T. Yura, Overexpression of trigger factor prevents aggregation of recombinant proteins in *Escherichia coli*, Appl. Environ. Microbiol. 66 (2000) 884–889.
[19] G. Qing, L.C. Ma, A. Khorchid, G.V. Swapna, T.K. Mal, M.M. Takayama, B. Xia, S. Phadtare, H. Ke, T. Acton, G.T. Montelione, M. Ikura, M. Inouye, Cold-shock induced high-yield protein production in *Escherichia coli*, Nat Biotechnol 22 (2004) 877–882.
[20] M. Mellado, J.M. Rodriguez-Frade, A.J. Vila-Coro, S. Fernandez, A. Martin de Ana, D.R. Jones, J.L. Toran, A.C. Martinez, Chemokine receptor homo- or heterodimerization activates distinct signaling pathways, EMBO J. 20 (2001) 2497–2507.
[21] L. El-Asmar, J.Y. Springael, S. Ballet, E.U. Andrieu, G. Vassart, M. Parmentier, Evidence for negative binding cooperativity within CCR5-CCR2b heterodimers, Mol. Pharmacol. 67 (2005) 460–469.
[22] M. Rafei, Y.A. Berchiche, E. Birman, M.N. Boivin, Y.K. Young, J.H. Wu, N. Heveker, J. Galipeau, An engineered GM-CSF-CCL2 fusokine is a potent inhibitor of CCR2-driven inflammation as demonstrated in a murine model of inflammatory arthritis, J. Immunol. 183 (2009) 1759–1766.

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Proteomic Differential Display Analysis Shows Up-regulation of 14-3-3 Protein Sigma in Human Scirrhous-type Gastric Carcinoma Cells

YASUHIRO KURAMITSU[1], BYRON BARON[1], SHIGEFUMI YOSHINO[2], XIULIAN ZHANG[1], TOSHIYUKI TANAKA[1], MASAKAZU YASHIRO[3], KOSEI HIRAKAWA[3], MASAAKI OKA[2] and KAZUYUKI NAKAMURA[1]

Departments of [1]Biochemistry and Functional Proteomics and [2]Surgery and Clinical Science, Yamaguchi University Graduate School of Medicine, Ube, Japan; [3]Department of Surgical Oncology, Osaka City University Graduate School of Medicine, Osaka, Japan

Abstract. *This study performed proteomic differential display analysis of human scirrhous-type gastric carcinoma (SGC) cell lines and normal gastric mucosa (NGM) tissues by using two-dimensional gel electrophoresis (2-DE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). The human SGC cell lines were OCUM-1, OCUM-2M, OCUM-2MLN, OCUM-2D, OCUM-D3, OCUM-9 and OCUM-12. Among the SGC cell lines and the NGM tissues, 28 protein spots were found whose expression levels were different from the results of 2-DE; 19 protein spots appeared higher, and 9 other protein spots appeared lower in SGCs than in NGM tissues. These spots were analysed by LC-MS/MS analysis, and identified by a peptide sequence tag. Identified increased spots included elongation factor 1-beta, 14-3-3 sigma, tropomyosin alpha-4 chain, protein DJ-1, nucleoside diphosphate kinase A, elongation factor Tu and peroxiredoxin-1. Western blot analysis showed increased protein level of 14-3-3 sigma in SGCs. And although OCUM-1 and AGS (gastric cancer) showed up-regulation of 14-3-3 sigma, MiaPaca-2 (pancreatic cancer), Huh-7 (HCC) and NCI-H2052 (malignant pleural mesothelioma) showed very weak expression of 14-3-3 sigma. The up-regulation of 14-3-3 sigma may play an important role in SGS carcinogenesis and progression and may be used as a diagnostic biomarker of SGS.*

Scirrhous-type gastric carcinoma (SGC) diffusely infiltrates into a broad region of the stomach accompanied by extensive stromal fibrosis and is frequently associated with metastasis to lymph nodes and peritoneal dissemination (1), showing a markedly poor prognosis with 5-year survival rates in the range of 10-15% (2). Although a novel oral fluorouracil derivative S-1 has recently been proposed as first-line chemotherapy for SGC patients, still many advanced SGC patients survive less than a year (3). Therefore, it is important to understand the biology of SGC and to find a molecular target for its therapy.

Proteome refers to the total protein complement of a genome (4). Anderson *et al.* defined proteomics as the use of quantitative protein-level measurements of gene expression to characterise biological processes (disease processes and drug effects) and decipher the mechanisms of gene expression control (5). The combination of two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) is a sophisticated method of high-throughput proteomic analysis. Many proteomic studies using 2-DE and MS have identified various proteins that may be involved in the pathogenic mechanism of cancer in cancer cell lines, cancer tissues and sera from cancer patients (6, 7).

In the present study, proteomic differential display analysis using 2-DE and LC-MS/MS was performed to examine the difference in proteome between SGC cells and normal gastric mucosa tissues, with the aim of identifying the protein(s) specific to SGC which may be useful for developing an appropriate therapy.

Materials and Methods

Scirrhous-type gastric carcinoma cell lines and culture conditions. The study used seven human SGC cell lines (OCUM-1, OCUM-2M, OCUM-2MLN, OCUM-2D, OCUM-D3, OCUM-9 and OCUM-12), AGS (a human gastric adenocarcinoma cell line), MiaPaCa-2 (a human pancreatic adenocarcinoma cell line) and Huh-7 (a human hepatocellular carcinoma cell line). They were maintained in continuous *in vitro* culture in Dulbecco's modified Eagle medium

Correspondence to: Yasuhiro Kuramitsu, MD, Ph.D., Department of Biochemistry and Functional proteomics, Yamaguchi University Graduate School of Medicine, 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan. Tel: +81 836222213, Fax: +81 836222212, e-mail: climates@yamaguchi-u.ac.jp

Key Words: LC-MS/MS, 2-DE, peptide sequence tag.

(D-MEM) (high glucose; Wako, Osaka, Japan) with 2 mM L-glutamine and 10% FBS at 37°C in a 5% CO_2 atmosphere. NCI-H2052 (a human malignant pleural mesothelioma cell line) was maintained in continuous *in vitro* culture in RPMI 1640 medium (Nissui, Tokyo, Japan) with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES and 90% 1.0 mM sodium pyruvate and 10% FBS at 37°C in a 5% CO_2 atmosphere.

Normal gastric mucosa tissues. Five non-cancerous gastric mucosa tissues were obtained from patients who were diagnosed with non-scirrhous-type gastric carcinoma and underwent surgical gastric resection at the Department of Surgery II, Yamaguchi University Hospital between January and December 2008. Written informed consent was obtained from all patients before surgery. None of the patients received any preoperative therapy such as chemotherapy or radiation. The study protocol was approved by the Institutional Review Board for Human Use of the Yamaguchi University School of Medicine.

Sample preparation. Cells and tissues were homogenised in lysis buffer (50 mM Tris-HCl, pH 7.5, 165 mM sodium chloride, 10 mM sodium fluoride, 1 mM sodium vanadate, 1 mM PMSF, 10 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% NP-40) on ice. Suspensions were incubated for 1 h at 4°C, centrifuged at 21,500 × *g* for 30 min at 4°C and the supernatants were stored at –80°C until use (8).

Two-dimensional gel electrophoresis (2-DE). The 2-DE was carried out according to the method described by Tanaka *et al*. (9). Briefly, for the first dimension, isoelectric focusing (IEF) was performed in an IPGphor 3 IEF unit (GE Healthcare, Buckinghamshire, UK) on 11 cm, immobilised, pH 3-10 linear gradient strips (Bio-Rad, Hercules, CA, USA) at 50 μA/strip. In the second dimension, SDS-PAGE was performed on a precast polyacrylamide gel with a linear concentration gradient of 5-20% (Bio-Rad), run at 200 V.

Fluorescent gel staining. After 2-DE, the gels were subjected to fixing solution with 40% ethanol and 10% acetic acid for 2 h. The gels were stained with a fluorescent gel staining, Flamingo™ Fluorescent Gel Stain (Bio-Rad) overnight. Stained gels were washed with Milli-Q (Millipore Bedford, MA, USA) water three times.

Image analysis and spot picking. The positions of the protein spots on the gels were recorded by the ProEXPRESS 2D Proteomic Imaging System (PerkinElmer Inc., Waltham, MA, USA). Expression levels of the proteins were quantified by analysing the intensity of each spot with Progenesis PG240 software (PerkinElmer Inc.) (9). The differences in expression between SGC cell lines and NGM tissues were analysed statistically by the Student's *t*-test. The 2-DE analysis was performed five times. After statistical analysis, the gels were re-stained with See Pico™ (Benebiosis Co., Ltd, Seoul, Korea), and the selected spots, whose expression was significantly different between SGC cell lines and NGM tissues, were cut and removed for the MS analysis.

In-gel digestion. In-gel digestion was carried out according to the method described by Tanaka *et al*. (9). Briefly, the sample in the gel piece was reduced twice in 50% ACN, 50 mM ammonium bicarbonate, and 5 mM DTT for 10 min. The gel piece was dehydrated in 100% ACN twice for 30 min, and then rehydrated with an in-gel digestion reagent containing 10 μg/ml sequencing-grade-modified trypsin (Promega, Madison, WI, USA) in 30% ACN, 50 mM ammonium bicarbonate, and 5 mM DTT.

LC-MS/MS. An Agilent 1100 LC/MSD Trap XCT (Agilent Technologies, Palo Alto, CA, USA) was used for HPLC and MS/MS. Twenty-five μl of each sample was applied and separated on a column (Zorbax 300SB-C18, 75 μm, 150 mm, Agilent Technologies). Protein identification was performed in the Agilent Spectrum MILL MS proteomics workbench against the Swiss-Prot protein database search engine (http://kr.expasy.org/sprot/) and the MASCOT MS/MS ions search engine (http://www.matrixscience.com/search_form_select.html) (10, 11).

Western blot analysis. After electrophoresis, gels were transferred electrophoretically onto PVDF membranes (Immobilon-P; Millipore, Bedford, MA, USA) and blocked for 1 h at room temperature with TBS containing 5% skimmed milk. Primary antibodies were an anti-14-3-3 sigma, which is an affinity purified goat polyclonal antibody raised against a peptide mapping near the amino terminus of 14-3-3 sigma of human origin (dilution range 1:200) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and an anti-actin goat polyclonal antibody as a loading control (dilution range 1:200) (Santa Cruz Biotechnology). Membranes were incubated with the primary antibodies overnight at 4°C, washed three times with TBS containing 0.05% Tween-20 and once with TBS, and then incubated with a horseradish peroxidase-conjugated secondary antibody (dilution range 1:5,000; Jackson ImmunoResearch Lab., West Grove, PA, USA) for 1.5 h at room temperature, and developed with a chemifluorescence reagent (ECL Plus Western Blotting Detection Reagents; GE Healthcare). The immunoreactive protein bands were then obtained by using the ProEXPRESS 2D Proteomic Imaging System (PerkinElmer Inc.) (12).

Results

Detection and identification of the spots with altered expression between SGC cell lines and NGM tissues using 2-DE gels. Protein expressions in the human SGC cell lines (OCUM-1, OCUM-2M, OCUM-2MLN, OCUM-2D, OCUM-D3, OCUM-9 and OCUM-12) and five NGM tissues was assessed. Protein spots on the 2-DE gels were visualised and differences in spot intensities between SGC cell lines and NGM tissues were analysed with Progenesis PG240 software for each gel. The expression of 29 protein spots differed between SGC cell lines and NGM tissues. Of those, 19 protein spots appeared to have higher levels (Figure 1A) and 9 protein spots appeared to have lower levels (Figure 1B) in SGC cell lines than in NGM tissues. The spots whose expression levels were higher or lower by more than 1.5 fold ($p<0.05$) are indicated by circles. The expression levels of these spots are shown in Table I. The bar charts were developed by normalisation of the volume, which was calculated relative to total spot volume and the error bars show standard deviations. The identification of these 28 protein spots with different expression levels was accomplished by measuring tryptic

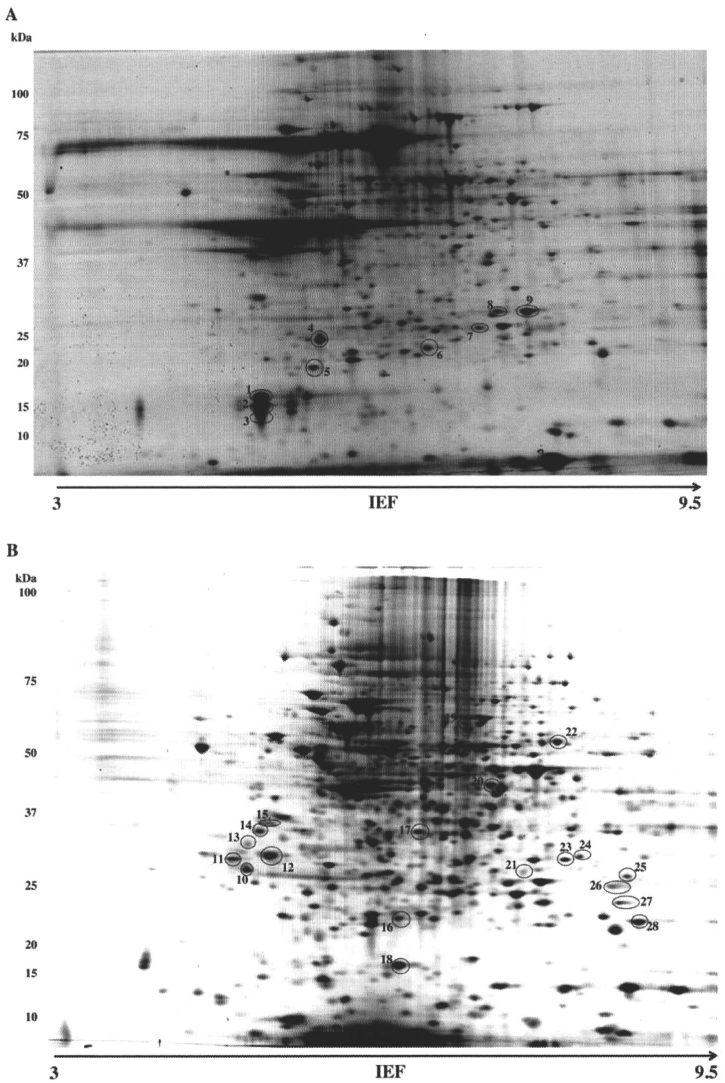

Figure 1. *2-DE patterns of NGM tissue and human SGC cell line stained with Flamingo Gel Stain. (A) Protein spots whose expression levels were down-regulated in human SGC cell lines are shown (spots 1-9). (B) Protein spots whose expression levels were up-regulated in human SGC cell lines are shown (spots 10-28).*

Table I. *Identification of proteins which are differentially expressed between scirrhous-type gastric cancer (SGC) cell lines and normal gastric mucosal tissues.*

Spot no.	Protein name	Accession no.	Theoretical pI	Theoretical Mr	Distinct peptides	Sequence coverage (%)	MS/MS search score	Change in SGCs (folds)
1	Gastrokine-1	Q9NS71	5.90	21,999	13	32	188.25	Decrease (0.13)
2	Gastrokine-1	Q9NS71	5.90	21,999	15	34	214.21	Decrease (0.03)
3	Gastrokine-1	Q9NS71	5.90	21,999	12	32	175.00	Decrease (0.00)
4	Apolipoprotein A-I	P02647	5.56	30,778	14	41	168.98	Decrease (0.13)
5	ATP synthase subunit d, mitochondrial	O75947	5.21	18,491	4	22	55.90	Decrease (0.08)
6	ATP synthase subunit alpha, mitochondrial	P25705	9.16	59,751	3	7	31.73	Decrease (0.70)
7	Ig lambda chain C regions	P01842	6.91	11,237	2	23	25.59	Decrease (0.18)
8	Carbonic anhydrase 1	P00915	6.59	28,870	4	18	58.24	Decrease (0.09)
9	Carbonic anhydrase 2	P00918	6.87	29,246	6	16	72.52	Decrease (0.23)
10	14-3-3 protein sigma	P31947	4.68	27,774	16	59	230.99	Increase (4.49)
11	Elongation factor 1-beta	P24534	4.50	24,764	7	25	95.06	Increase (26.84)
12	Tropomyosin alpha-4 chain	P67936	4.67	28,522	4	21	56.01	Increase (54.30)
13	Proliferating cell nuclear antigen	P12004	4.57	28,769	7	33	87.36	Increase (36.06)
14	Glucosidase 2 subunit beta	P14314	4.33	59,426	7	14	88.94	Increase (83.12)
15	Nucleophosmin	P06748	4.64	32,575	7	25	97.91	Increase (1.50)
16	Protein DJ-1	Q99497	6.33	19,891	6	34	78.77	Increase (5.65)
17	Carbonyl reductase 3	O75828	5.82	30,850	4	17	65.53	Increase (3.91)
18	Nucleoside diphosphate kinase A	P15531	5.83	17,149	5	41	72.89	Increase (3.65)
19	Bifunctional purine biosynthesis protein PURH	P31939	6.27	64,616	6	14	79.19	Increase (2.28)
20	Elongation factor Tu, mitochondrial	P49411	7.26	49,542	18	37	266.81	Increase (2.13)
21	S-methyl-5'-thioadenosine phosphorylase	Q13126	6.75	31,236	6	19	84.66	Increase (3.32)
22	UDP-glucose 6-dehydrogenase	O60701	6.73	55,024	13	26	175.30	Increase (2.56)
23	Electron transfer flavoprotein subunit alpha, mitochondrial	P13804	8.62	35,080	11	45	165.67	Increase (5.89)
24	Pyrroline-5-carboxylate reductase 1, mitochondrial	P32322	7.18	33,361	5	16	65.06	Increase (3.86)
25	Proteasome subunit alpha type-4	P25789	7.58	29,484	1	4	13.83	Increase (2.42)
26	Eukaryotic translation initiation factor 4H	Q15056	6.67	27,385	2	12	31.33	Increase (2.61)
27	3-hydroxyacyl-CoA dehydrogenase type-2	Q99714	7.65	26,923	4	20	62.91	Increase (2.39)
28	Peroxiredoxin-1	Q06830	8.27	22,111	5	27	71.87	Increase (1.71)

peptide masses using the Agilent 1100 LC-MS/MS Trap XCT system in the positive ion mode and carrying out a database search in the Agilent Spectrum MILL MS proteomics workbench against the Swiss-Prot protein database search engine and the MASCOT MS/MS ions search engine, as summarised in Table I. Each sample provided good spectra of amino acid sequences. Figure 2 shows MS and MS/MS spectra of trypsin-digested spot 10 in Figure 1B. The MS/MS spectrum was identified as the partial tryptic peptide SAYQEAMDISK from 14-3-3 sigma processed with a spectrum MILL workbench. The spectrum of the precursor showed a double charged peptide ion.

Up-regulation of proteins in SGC cell lines compared with NGM tissues. Western blot analysis of 14-3-3 sigma was performed in all SGC cell lines and NGM tissues. Their expression tended to be higher (Figure 3A), although the expression of actin tended to be same or lower in SGC cell lines than in NGM tissues (Figure 3B). The up-regulation of 14-3-3 sigma was not detected in the human pancreatic cancer cell line MiaPaca-2, the human hepatocellular carcinoma cell line Huh-7 or the human malignant pleural mesothelioma cell line NCI-H2052 (Figure 4A), although the expression of actin tended to be same in all cell lines(Figure 4B). The non-scirrhous-type gastric carcinoma cell line AGS cells also showed up-regulation of 14-3-3 sigma, but this up-regulation was smaller than in SGC cells (Figure 4A).

Discussion

This study described a proteomic differential display analysis of seven human SGC cell lines compared to human NGM tissues. Nine protein spots were found to be down-regulated in SGC cell lines and these protein spots were identified as gastrokine-1, apolipoprotein A-I, mitochondrial ATP synthase subunit d, mitochondrial ATP synthase subunit alpha, Ig lambda chain C regions, carbonic anhydrase 1 and 2. Nineteen protein spots were found to be up-regulated in SGC cell lines (Table I). By Western blotting, up-regulation of 14-3-3 sigma was confirmed in the seven human SGC cell lines. Albeit OCUM-1 and AGS (gastric cancer) showed up-regulation of 14-3-3 sigma, MiaPaca-2 (pancreatic cancer), Huh-7 (HCC)

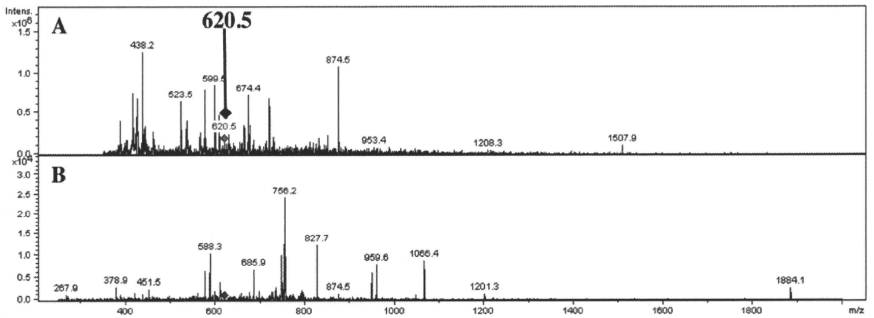

Figure 2. *MS and MS/MS spectra of trypsin-digested spot 10. (A) LC-MS spectra of trypsin-digested spot 10; 14-3-3 sigma; precursor ion m/z is 620.5. (B) LC-MS/MS spectrum of a precursor ion with m/z 620.5 marked by a diamond in (A). The MS/MS spectrum was identified as the partial tryptic peptide SAYQEAMDISK from 14-3-3 sigma processed with a spectrum MILL workbench.*

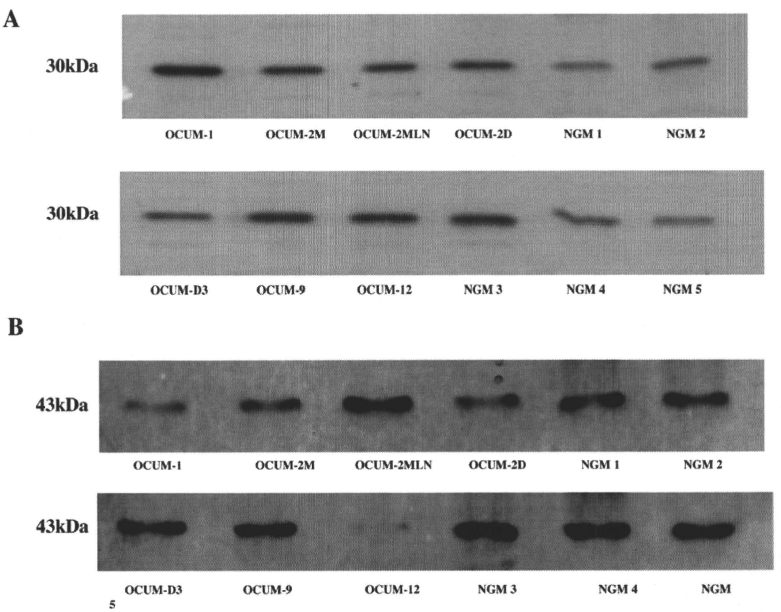

Figure 3. *Western blot analysis for 14-3-3 protein sigma in human SGC cell lines and NGM tissues. (A) 14-3-3 protein sigma expression level was significantly up-regulated in human SGC cell lines compared to NGM tissues. (B) Actin expression levels were unchanged. Ten μg of protein was used.*

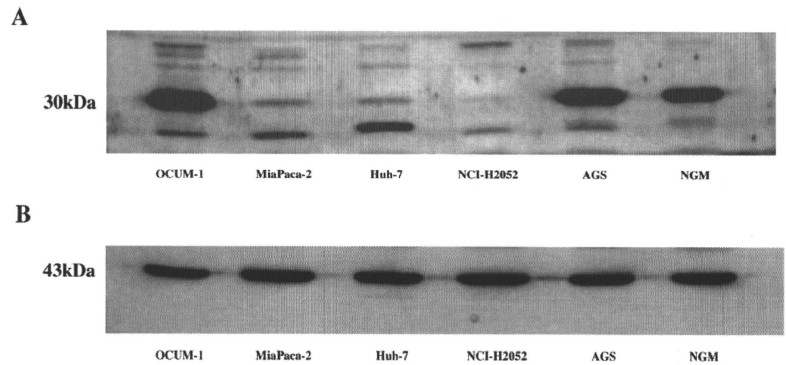

Figure 4. *Western blot analysis for 14-3-3 protein sigma in pancreatic cancer, hepatocellular carcinoma, malignant pleural mesothelioma and gastric cancer cell lines. (A) 14-3-3 protein sigma expression level was not up-regulated in pancreatic cancer, hepatocellular carcinoma and malignant pleural mesothelioma cell lines. (B) Actin expression levels were unchanged. Ten μg of protein was used.*

and NCI-H2052 (malignant pleural mesothelioma) showed markedly weak expression of 14-3-3 sigma.

The 14-3-3 protein sigma that was only expressed in epithelial cells is a member of 14-3-3 family. It performs a checkpoint control by promoting G2 arrest following DNA damage. Down-regulation of 14-3-3 sigma has been detected in human lung, prostate, ovary, urinary bladder, breast, liver, and skin cancers (13-19). In contrast, up-regulation of 14-3-3 sigma has been detected in lung cancer, head and neck squamous cell carcinoma and oral squamous cell carcinoma (20-22). This controversy about the regulation of 14-3-3 sigma in cancer cells needs to be clarified in future studies.

References

1 Nakazawa K, Yashiro M and Hirakawa K: Keratinocyte growth factor produced by gastric fibroblasts specifically stimulates proliferation of cancer cells from scirrhous gastric carcinoma. Cancer Res *63*: 8848-8852, 2003.

2 Otsuji E, Kuriu Y, Okamoto K, Ochiai T, Ichikawa D, Hagiwara A and Yamagishi H: Outcome of surgical treatment for patients with scirrhous carcinoma of the stomach. Am J Surg *188*: 327-332, 2004.

3 Nakata B, Mitachi Y, Tsuji A, Yamamitsu S, Hirata K, Shirasaka T and Hirakawa K: Combination phase I trial of a novel oral fluorouracil derivative S-1 with low-dose cisplatin for unresectable and recurrent gastric cancer (JFMC27-9902). Clin Cancer Res *10*: 1664-1669, 2004.

4 Wasinger VC, Cordwell SJ, Cerpa-Poljak A, Yan JX, Gooley AA, Wilkins MR, Duncan MW, Harris R, Williams KL and Humphery-Smith I: Progress with gene-product mapping of the Mollicutes: Mycoplasma genitalium. Electrophoresis *16*: 1090-1094, 1995.

5 Anderson NL and Anderson NG: Proteome and proteomics: new technologies, new concepts, and new words. Electrophoresis *19*: 1853-1861, 1998.

6 Kuramitsu Y and Nakamura K: Proteomic analysis of cancer tissues: shedding light on carcinogenesis and possible biomarkers. Proteomics *6*: 5650-5661, 2006.

7 Kuramitsu Y and Nakamura K: Current progress in proteomic study of hepatitis C virus-related human hepatocellular carcinoma. Expert Rev Proteomics *2*: 589-601, 2005.

8 Mori-Iwamoto S, Kuramitsu Y, Ryozawa S, Mikuriya K, Fujimoto M, Maehara S, Maehara Y, Okita K, Nakamura K and Sakaida I: Proteomics finding heat shock protein 27 as a biomarker for resistance of pancreatic cancer cells to gemcitabine. Int J Oncol *31*: 1345-1350, 2007.

9 Tanaka T, Kuramitsu Y, Fujimoto M, Naito S, Oka M, and Nakamura K: Down-regulation of two isoforms of ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1) correlates with high metastatic potentials of human SN12C renal cell carcinoma cell clones. Electrophoresis *29*: 2651-2659, 2008.

10 Takashima M, Kuramitsu Y, Yokoyama Y, Iizuka N, Fujimoto M, Sakaida I, Okita K, Oka M and Nakamura K: Proteomic analysis of autoantibodies in patients with hepatocellular carcinoma. Proteomics *6*: 3894-3900, 2006.

11 Takashima M, Kuramitsu Y, Yokoyama Y, Iizuka N, Fujimoto M, Nishisaka T, Sakaida I, Okita K, Oka M, and Nakamura K: Overexpression of alpha enolase in hepatitis C virus-related hepatocellular carcinoma: association with tumor progression as determined by proteomic analysis. Proteomics *5*: 1686-1692, 2005.

12 Kuramitsu Y, Harada T, Takashima M, Yokoyama Y, Hidaka I, Iizuka N, Toda T, Fujimoto M, Zhang X, Sakaida I, Okita K, Oka M and Nakamura K: Increased expression, and phosphorylation of liver glutamine synthetase in well-differentiated hepatocellular carcinoma tissues of patients infected with hepatitis C virus. Electrophoresis *27*: 1651-1658, 2006.

13 Moreira JM, Ohlsson G, Rank FE and Celis JE: Down-regulation of the tumor suppressor protein 14-3-3 sigma is a sporadic event in cancer of the breast. Mol Cell Proteomics *4*: 555-569, 2005.
14 Moreira JM, Gromov P and Celis JE: Expression of the tumor suppressor protein 14-3-3 sigma is down-regulated in invasive transitional cell carcinomas of the urinary bladder undergoing epithelial-to-mesenchymal transition. Mol Cell Proteomics *3*: 410-419, 2004.
15 Liu Y, Chen Q and Zhang JT: Tumor suppressor gene 14-3-3sigma is down-regulated whereas the proto-oncogene translation elongation factor 1delta is up-regulated in non-small cell lung cancers as identified by proteomic profiling. J Proteome Res *3*: 728-735, 2004.
16 Iwata N, Yamamoto H, Sasaki S, Itoh F, Suzuki H, Kikuchi T, Kaneto H, Iku S, Ozeki I, Karino Y, Satoh T, Toyota J, Satoh M, Endo T and Imai K: Frequent hypermethylation of CpG islands and loss of expression of the 14-3-3 sigma gene in human hepatocellular carcinoma. Oncogene *19*: 5298-5302, 2000.
17 Osada H, Tatematsu Y, Yatabe Y, Nakagawa T, Konishi H, Harano T, Tezel E, Takada M and Takahashi T: Frequent and histological type-specific inactivation of 14-3-3 sigma in human lung cancers. Oncogene *21*: 2418-2424, 2002.
18 Mhawach P, Greloz V, Assaly M and Herrmann F: Immunohistochemical expression of 14-3-3 sigma protein in human urological and gynecological tumors using a multi-tumor microarray analysis. Pathol Int *55*: 77-82, 2005.
19 Cheng L, Pan CX, Zhang JT, Zhang S, Kinch MS, Li L, Baldridge LA, Wade C, Hu Z, Koch MO, Ulbright TM and Eble JN: Loss of 14-3-3sigma in prostate cancer and its precursors. Clin Cancer Res *10*: 3064-3068, 2004.
20 Qi W, Liu X, Qiao D, and Martinez JD: Isoform-specific expression of 14-3-3 proteins in human lung cancer tissues. Int J Cancer *113*: 359-363, 2005.
21 Villaret DB, Wang T, Dillon D, Xu J, Sivam D, Cheever MA and Reed SG: Identification of genes overexpressed in head and neck squamous cell carcinoma using a combination of complementary DNA subtraction and microarray analysis. Laryngoscope *110*: 374-381, 2000.
22 Hayashi E, Kuramitsu Y, Fujimoto M, Zhang X, Tanaka T, Uchida K, Fukuda T, Furumoto H, Ueyama Y and Nakamura K: Proteomic profiling of differential display analysis for human oral squamous cell carcinoma: 14-3-3 σ protein is upregulated in human OSCC and dependent on the differentiation level. Proteomics Clinical Applications *3*: 1338-1347, 2009.

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Staining with Highly Sensitive Coomassie Brilliant Blue SeePico™ Stain after Flamingo™ Fluorescent Gel Stain is Useful for Cancer Proteomic Analysis by Means of Two-dimensional Gel Electrophoresis

YASUHIRO KURAMITSU[1], EIKO HAYASHI[1,2], FUTOSHI OKADA[3], XIULIAN ZHANG[1], TOSHIYUKI TANAKA[1], YOSHIYA UEYAMA[2] and KAZUYUKI NAKAMURA[1]

Departments of [1]Biochemistry and Functional Proteomics and [2]Oral and Maxillofacial Surgery, Yamaguchi University Graduate School of Medicine, Ube, Japan; [3]Department of Biochemistry and Molecular Biology, Yamagata University Graduate School of Medical Science, Yamagata, Japan

Abstract. *Highly sensitive Coomassie brilliant blue SeePico™ Stain was applied for proteomic analysis using two-dimensional gel electrophoresis (2-DE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). After staining with Flamingo™ Fluorescent Gel Stain, the images of the protein spots were analyzed, and 424 protein spots were detected. After washing with Milli-Q water three times, the gels were re-stained with SeePico™ Stain and the images of the protein spots were analyzed; 272 spots were detected. To assess whether SeePico™ Stain alters MS analysis, a spot was picked up and was analyzed by LC-MS/MS. The MS analysis showed good protein identification. These results show a possible role for SeePico™ Stain in cancer proteomics using 2-DE and MS.*

Proteomics is an established molecular profiling technology that may significantly accelerate human cancer research. Development of high-throughput proteomic analysis provides a new tool to study biomarkers and the pathogenesis of cancer. Proteomic differential display analysis has been used to characterize the molecular events occurring in cancer (1). Two-dimensional electrophoresis (2-DE) and mass spectrometry (MS) are important techniques in proteomics to identify comparatively protein expression profiles which may be associated with cancer. 2-DE simultaneously separates thousands of proteins from complex biological samples according to the isoelectric point in the first dimension and molecular weight in the second dimension (2). For the discovery of biomarker and target proteins, it is most important to be able to display extremely small amount of proteins. For this purpose, nowadays, applications of very sensitive fluorescent dyes for 2-DE have been developed (3). After gel image analysis, particular protein spots should be picked up for MS analysis. This gel picking procedure is a very important step, because if the wrong spots are picked up, incorrect MS analysis results are obtained. Commercial gel spot picker machines are very popular for gel spot picking, and automatically select and cut the spots (4). However, since these machines are very expensive, many investigators still use Coomassie brilliant blue (CBB) staining or silver staining after fluorescent staining. CBB staining is not sensitive and the gel image pattern obtained by silver staining is not completely the same as that obtained using fluorescent staining. In the present study we re-stained spots with highly sensitive CBB SeePico™ Stain for the 2-DE gels which had been firstly stained with Flamingo Gel Stain, and selected the protein spots with the aim of the identification of the protein by MS analysis.

Correspondence to: Yasuhiro Kuramitsu, MD, Ph.D., Department of Biochemistry and Functional Proteomics, Yamaguchi University Graduate School of Medicine, 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan. Tel: +81 836222213, Fax: +81 836222212, e-mail: climates@yamaguchi-u.ac.jp

Key Words: Two-dimensional gel electrophoresis, LC-MS/MS, Flamingo Gel Stain, SeePico, high sensitive Coomassie brilliant blue.

Materials and Methods

Sample preparation. QR-32 was kindly provided by the Laboratory of Pathology, Cancer Institute, Hokkaido University School of Medicine. QR-32 is a transplantable murine fibrosarcoma cell line which has been described previously (5). The nuclear proteins from QR-32 were extracted by means of NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (PIERCE Biotechnology, Rockford, IL, USA) according to the manufacturer's instruction.

2-DE. Eighty μg of protein were used for each 2-DE. For the first dimension, isoelectric focusing (IEF) was performed in an IPGphor

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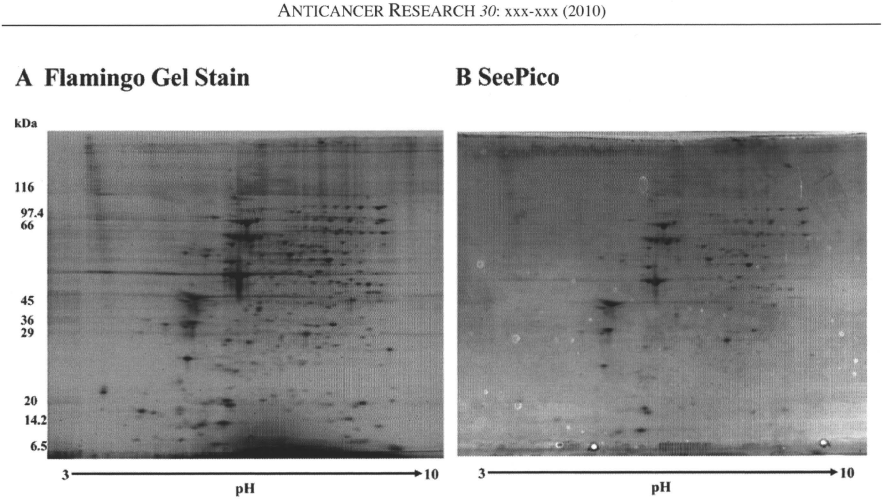

Figure 1. *2-DE patterns of QR32 cell nuclear fraction stained with Flamingo Gel Stain™ and SeePico Stain™. Panel A shows the 2-DE patterns of the nuclear fraction of QR-32 stained with Flamingo Gel Stain™. Panel B shows the 2-DE patterns of the same nuclear fraction of QR-32 stained with SeePico Gel Stain™. Proteins (80 μg) were separated by 2-DE using a pH 3-10 gradient in the first dimension, followed by second-dimensional separation on a precast polyacrylamide gel with a linear concentration gradient of 5-20%.*

3 IEF unit (GE Healthcare, Buckinghamshire, UK) on 11 cm, immobilized, pH 3-10 linear gradient strips (BIO RAD, Hercules, CA, USA) at 50 μA/strip. Samples of QR-32 nuclear proteins were mixed with 200 μl of rehydration buffer (8 M urea, 2% CHAPS, 0.01% bromophenol blue, 1.2% Destreak reagent (GE Healthcare) and 0.5% IPG buffer (GE Healthcare) and loaded into the IPGphor strip holder (GE Healthcare). IEF was performed using the following voltage program: rehydration for 10 h (no voltage), a stepwise increase from 0 to 500 V for 4 h, 500 to 1,000 V for 1 h, 1,000 to 8,000 V for 4 h, a linear increase from 8,000 V for 20 min, and a final phase of 500 V from 20,000 to 30,000 Vh. In the second dimension, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a precast polyacrylamide gel with a linear concentration gradient of 5-20% (BIO RAD), run at 200 V (6).

Fluorescent gel staining. After 2-DE, the gels were subjected to fixing with 40% ethanol and 10% acetic acid for 2 h. The gels were stained with Flamingo™ Fluorescent Gel Stain (BIO RAD) overnight. Stained gels were washed with Milli-Q water three times. Agitation was carried out at all stages (7).

Highly sensitive CBB gel staining. After recording images, the gels were washed with Milli-Q water three times, then stained with a highly sensitive CBB gel stain, SeePico™ (Benebiosis Co., Ltd, Seoul, Korea) overnight. Stained gels were washed with Milli-Q water three times. Agitation was carried out at all stages (8).

Image analysis and spot picking. The positions of the protein spots on the gels were recorded by using a ProEXPRESS 2D Proteomic Imaging System (PerkinElmer Inc., Waltham, MA, USA). Expression levels of the proteins were quantified by analyzing the intensity of each spot with Progenesis SameSpot software (Nonlinear Dynamics Ltd. Newcastle Upon Tyne, UK) (8). The differences between expression of spots on the gel stained with Flamingo Gel Stain and on the gel stained with SeePico™ were analyzed. The selected spot whose stained intensity with SeePico™ was moderate, was cut and removed from the gels stained with SeePico™ for the liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

In-gel digestion. The SeePico™ dye was removed from the gel piece by rinsing three times in 60% methanol, 50 mM ammonium bicarbonate, and 5 mM dithiothreitol (DTT) for 15 min. The sample in the gel piece was reduced twice in 50% acetonitrile (ACN), 50 mM ammonium bicarbonate, and 5 mM DTT for 10 min. The gel piece was dehydrated in 100% ACN twice for 30 min, and then rehydrated with an in-gel digestion reagent containing 10 μg/ml sequencing-grade-modified trypsin (Promega, Madison, WI, USA) in 30% ACN, 50 mM ammonium bicarbonate, and 5 mM DTT. This procedure for in-gel digestion was performed overnight at 30°C. The samples were lyophilized overnight with the use of Labconco Lyph-lock 1L Model 77400 (Labconco, Kansas, MO, USA). Lyophilized samples were dissolved in 0.1% formic acid (9).

LC-MS/MS. Protein samples dissolved in 0.1% formic acid were centrifuged at 21,500 ×g for 5 min and the supernatant was stored at –80°C until use. An Agilent 1100 LC/MSD Trap XCT (Agilent Technologies, Palo Alto, CA, USA) was used for HPLC and MS/MS. Twenty-five microliters of each sample were applied and separated on a column (Zorbax 300SB-C18, 75 μm, 150 mm; Agilent Technologies). The Agilent 1100 capillary pump was operated under the following conditions: solvent A: 0.1% formic