Identification of Cystatin SN as a novel tumor marker for colorectal cancer

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Abstract. The goal of this study was to investigate Cystatin SN, a cysteine protease inhibitor, as a novel tumor marker for colorectal cancer (CRC). Gene expression profiles of mRNA from normal tissues and cancer cell lines were performed. Twenty-eight monoclonal antibodies for Cystatin SN were generated and serum Cystatin SN was quantified using ELISA in sera from 159 patients with CRC and 40 healthy controls. Cystatin SN was highly expressed in colon cancer cells. Employing a receiver-operating characteristic curve, we obtained an area under the curve of 0.708 for Cystatin SN, 0.819 for carcinoembryonic antigen (CEA) and 0.703 for carbohydrate antigen 19-9 (CA19-9). The combination assay of Cystatin SN, CEA and CA19-9 showed 62.9% sensitivity and 90.0% specificity. Especially, the sensitivity of the combination assay in stages I and II detection, in which stages curative operation would be possible, was improved over that of the assay testing only for CEA and CA19-9 (from 37.5 to 42.5% in stage I, from 49.0 to 60.8% in stage II). Furthermore, Western blot analysis revealed that Cystatin SN was increased in the urine from patients with CRC. Our results suggest the possibility of utilizing this novel tumor marker that can be tested in urine samples. These observations suggest that Cystatin SN in combination with CEA and CA19-9 is a useful tumor marker for detecting early stage CRC and that it is a unique urinary excretory protein, suggesting that Cystatin SN might be a novel candidate for use in mass screening for CRC.

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Abbreviation: CRC, colorectal cancer; ROC curve, receiver-operating characteristic curve; FOBT, fecal occult blood test; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; CHO, Chinese Hamster Ovary; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; CV, coefficient of variation

Key words: colorectal cancer, Cystatin SN, tumor marker, early detection, carcinoembryonic antigen

Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide and continues to be a serious public health concern. It is thought that if early detection of CRC would be possible, curative surgery could be performed (1). Hence, reduction of CRC mortality would be achieved. Various screening methods for detection of CRC are available (2). The fecal occult blood test (FOBT) is a simple test for CRC mass screening, however, it requires testing of two or more samples to ensure a more accurate result because cancers and precancerous growths only bleed intermittently (3). Fecal DNA testing showed higher sensitivity than FOBT (4), but was expensive (5). Colonoscopy is the most sensitive approach to early detection. However, colonoscopy is invasive and involves significant costs, risks, and inconvenience (6,7). Serologic biomarkers can be analyzed relatively non-invasively and economically and have the potential to greatly enhance mass screening programs. Carcinoembryonic antigen (CEA) is available for CRC

detection (8) and is the only marker widely accepted to be of diagnostic value in CRC, although it cannot be used alone for diagnosing or ruling out CRC (9,10). As the sensitivity of CEA for early CRC is low, its usefulness as a diagnostic tool is limited. It has been proposed that it would be an advantage to combine CEA with other markers in order to raise the diagnosis accuracy (11). Therefore, new cancer biomarkers are needed that will further enhance early detection of the disease, non-invasively and cost-effectively.

Cystatin SN is a member of the Cystatin superfamily of cysteine proteinase inhibitors. Cystatins are reversible, competitive inhibitors of cysteine proteinases. Their inhibitory profiles, as well as their affinities for target enzymes, vary for different cysteine proteinases (12).

In this study, we identified up-regulation of the Cystatin SN gene in colorectal cancer cells by microarray analysis and investigated the utility of Cystatin SN as a novel tumor marker.

Materials and methods

Tissue, serum and urinary samples. We evaluated patients with CRC and endoscopically negative controls who underwent colonoscopy at Yokohama City University Hospital, Yokohama Kosei Hospital, Yokohama Municipal Citizen's Hospital and Saiseikai Yokohama City Nanbu Hospital from April 2006 through November 2007 after informed consent. Venous blood samples and urinary samples were obtained. Tissue samples were obtained at surgery or during colonoscopy. Exclusion criteria were renal dysfunction, hepatic dysfunction, previous history of malignancy and being a smoker. A total of 159 CRC cases (107 males and 52 females; age range, 38-90 years) and 40 healthy controls (21 males and 19 female; age range, 29-85 years) were studied. CRC was diagnosed by histological analysis. The patients with CRC were divided into four groups, stage I to stage IV, using the TNM staging system approved by the International Union Against Cancer and the American Joint Committee on Cancer. The 159 cancer cases were grouped to the following stages: Stage I, 40; stage II, 51; stage III, 40; stage IV, 26; unknown, 2. The study was conducted with the approval of the Ethics Committee of each hospital.

Reagents. Recombinant Cystatin C was purchased from BioVendor (Modrice, Czech Republic), Cystatin SA and Cystatin S were from R&D systems (Minneapolis, MN, USA).

Microarray analysis and real-time RT-PCR. Total RNA from tissues or cells was extracted with Isogen reagent (Nippon gene, Tokyo, Japan). Purchased RNA from 30 different adult normal tissues, five different fetal tissues, and total RNA from 38 cancer cell lines were analyzed using the HG-U133A array (Affymetrix, Santa Clara, CA, USA) containing probes for 22,000 human genes. Further information on the source can be provided on request or is available at http://www.lsbm.org/db/index.html. Microarray analysis was done essentially as described previously (13,14). Aliquots of 5 mg of total RNA were reverse transcribed into cDNA, using Superscript II (Invitrogen, Carlsbad, CA, USA) with oligo-(dT) primers. Quantitative real-time PCR was done using ABI Prism 7700 (Applied Biosystems, Forester City, CA, USA). The expression level of Cystatin SN was first calculated as the relative ratio to ß-actin in each sample.

Generation of anti-Cystatin SN antibodies and standard. Three kinds of antigen were prepared for developing antibodies, and stable transfected Chinese Hamster Ovary (CHO) cells were established for the Cystatin SN standard in the enzyme-linked immunosorbent assay (ELISA). The Cystatin SN cDNA sequence was based on GenBank accession NM001898. Synthetic peptides were prepared in Peptide Institute (Osaka, Japan). Sequences corresponding to non-conserved regions among Cystatin SN, Cystatin D and Cystatin C were synthesized, and then conjugated to keyhole limpet hemocyanin (KLH) as antigens. Second, recombinant *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) was prepared for immunization as described previously (15-18). Recombinant virus particles expressing Cystatin SN as a fusion protein on the surface glycoprotein gp64 were directly immunized in mice. Third, phCMV vector (Stratagene, La Jolla, CA, USA) was used for expression in CHO cells. Transfection was performed with TransIT (Takara Bio, Kyoto, Japan). Secreted recombinant protein in the medium was purified by a HiTrap chelating Nickel column (GE Healthcare, Piscataway, NJ, USA), then used as antigen and standard. BALB/c mice were each immunized with three kinds of antigen by intraperitoneal injection, and hybridomas were screened as previously described (19). The resultant selection yielded 28 clones as described in Table I. Five of eight antibodies produced in response to peptide antigen were able to react with the soluble form of Cystatin SN (the best of which was termed PPMX0203). Immunization with full length antigen resulted in 10 clones (the best of which was termed PPMX0204), of which 4 reacted weakly to a closely homologous protein of Cystatin C in the Cystatin family. Ten clones were obtained in response to recombinant virus.

Immunoblotting and immunohistochemistry. Proteins in the biopsy specimens obtained during colonoscopy were extracted using the T-PER tissue protein extraction reagent (Pierce Biotechnology, Rockford, IL, USA) with 1 mM Na_3VO_4, 25 mM NaF and one tablet of proteinase inhibitor cocktail, 'complete mini' (Roche Diagnostics, Indianapolis, IN, USA). Protein concentrations were determined using the Bio-Rad Protein Assay Reagent (Bio-Rad, Richmond, CA, USA). The extracted proteins or urinary sample were separated by SDS-PAGE, transferred onto a PVDF membrane (GE Healthcare) and probed with primary antibody PPMX0206. The ECL detection kit (GE Healthcare) was used for the detection. The membrane was re-probed with anti-G3PDH (Trevigen, Gaithersburg, MD, USA) to normalize the sample preparations.

Formalin-fixed and paraffin-embedded samples were subjected to immunohistochemistry by an Envision kit (Dako, Carpinteria, CA, USA) with monoclonal antibody (mAb) PPMX0205. Sections were counter-stained with hematoxylin.

Preparation and procedure of ELISA. The mAb PPMX0203 was digested by pepsin into $F(ab')_2$ as previously described (20). The $F(ab')_2$ moiety of PPMX0203 (100 μl of a 5 μg/ml

Table I. Anti-Cystatin SN monoclonal antibodies generated by us.

Animals	Antigen	Antibodies	Clone
Balb/C mice	Synthetic peptide	Monoclonal	8[a]
Balb/C-gp64 transgenic mice	gp64 fusion BV	Monoclonal	10[b]
Balb/C mice	Full length	Monoclonal	10[c]

[a]PPMX0203 is included, [b]PPMX0205 and PPMX0206 are included, [c]PPMX0204 is included. Budded baculovirus, BV.

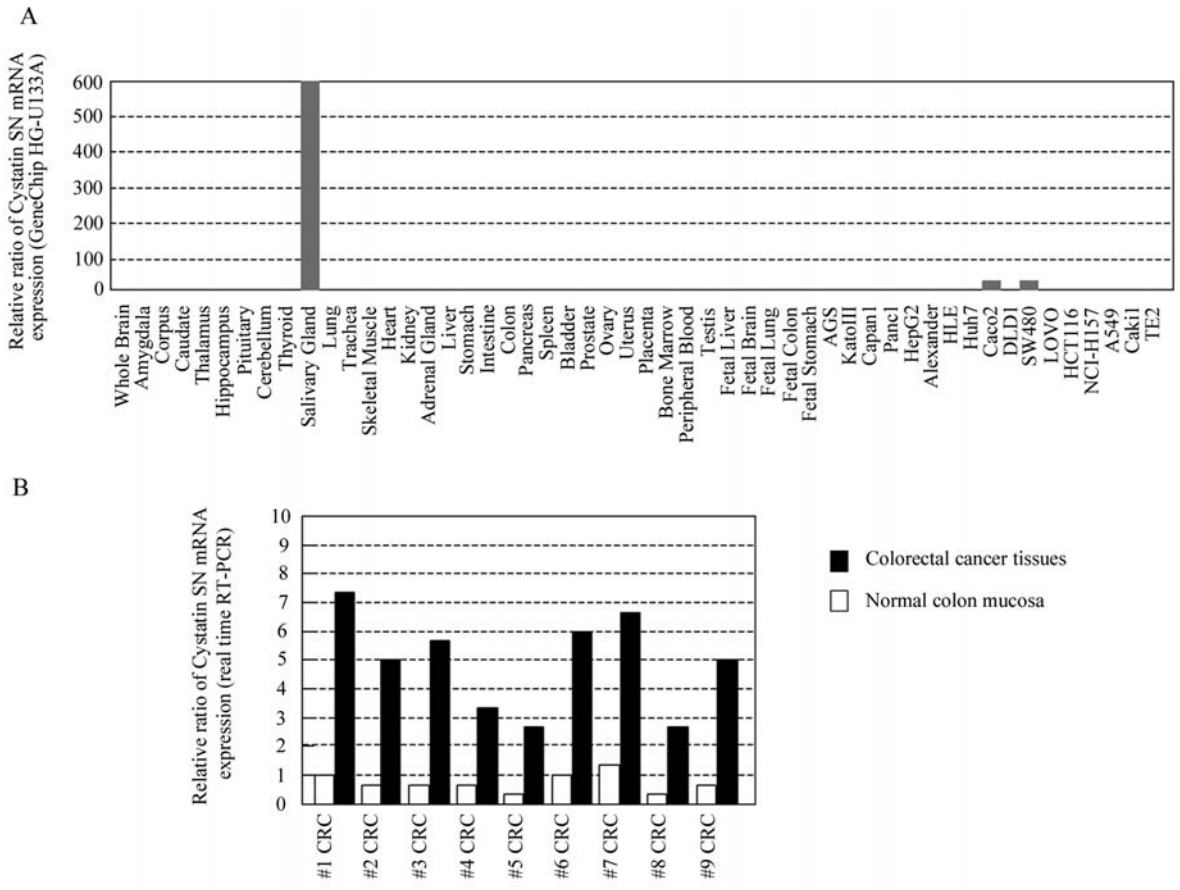

Figure 1. Expression profile of Cystatin SN mRNA in normal tissues and cancer cells. (A) Abstract of *Cystatin SN* mRNA levels analyzed using GeneChip HG-U133A with pooled total RNA from 35 normal tissues and 38 cancer cell lines. (B) Real-time RT-PCR on nine pairs of CRC tissues and adjacent normal colon mucosa. The expression level of *Cystatin SN* was calculated as the relative ratio of *Cystatin SN* to ß-actin in each sample.

solution in 0.9% NaCl solution) was coated onto a microtiter plate, MaxiSope (Nalge Nunc, Roskilde, Denmark). The plates were washed with a washing buffer, 0.05% Tween-20 in PBS, followed by blocking with 1% casein in PBS, pH 7.4. Plates were coated with 150 μl/well of Immunoassay Stabilizer (Advanced Biotechnologies, Columbia, MD, USA) and then dried.

For preparation of detecting antibody-enzyme conjugates, PPMX0204 was digested by pepsin into a $F(ab')_2$ as described above. Monomeric Fab was conjugated with horseradish peroxidase (HRP) by maleimide on thiol groups at the hinge region of the Fab fragment (21).

Sample dilution buffer (150 μl), PBS, 0.5% bovine serum albumin (Pierce Biotechnology), 3 mM EDTA, 0.05% Tween-20, 0.1% ProClin (Supleco, Bellefonte, PA, USA) was added to the antibody-coated microtiter plates. Each standard or serum (20 μl) were added to the wells and incubated. After the wells were washed with washing buffer (400 μl), Fab'-enzyme conjugate (150 μl) was added and incubated. The wells were washed again and then 150 μl of TMB substrate solution (ScyTeK Laboratories, Logan, UT, USA) was added. After incubation, 150 μl of stop solution (ScyTeK Laboratories) was added and the absorbance at 450 nm was measured by a microplate reader system (Towa Labo, Tokyo, Japan). Experiments were performed in duplicate except where noted otherwise.

Carcinoembryonic antigen assay. CEA and carbohydrate antigen 19-9 (CA19-9) were measured by a commercially available assay (SRL laboratory, Tokyo, Japan).

Table II. The standard curve values.

	Absorbance at 450 nm						
	Well 1	Well 2	Well 3	Mean	SD	CV	NET
Standards							
0.0	0.02	0.02	0.02	0.02	0.00	3.5%	0.00
0.2	0.08	0.07	0.07	0.07	0.00	2.8%	0.06
0.5	0.13	0.13	0.12	0.12	0.01	2.8%	0.11
1.0	0.25	0.24	0.24	0.24	0.01	3.8%	0.23
2.5	0.50	0.52	0.51	0.51	0.01	2.3%	0.49
5.0	0.96	0.97	1.01	0.98	0.02	2.5%	0.96
10	1.99	1.99	2.01	1.99	0.01	0.5%	1.98

The absorbance at 450 nm of each standard concentration was measured in triplicate (Wells 1, 2 and 3). The NET values used for calibration are the mean minus blank values.

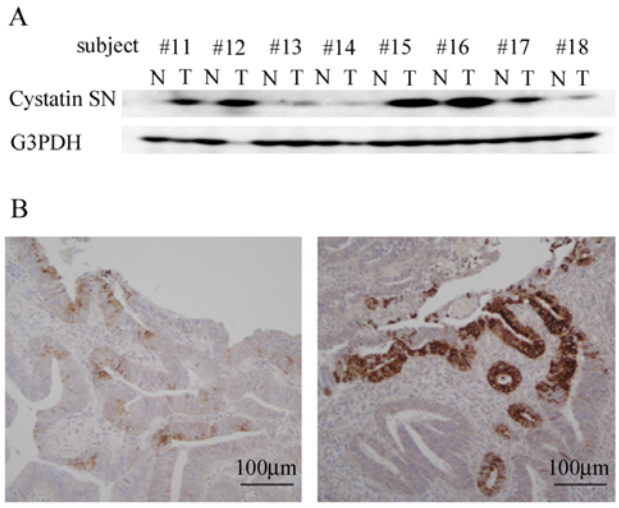

Figure 2. Protein expression analysis of Cystatin SN on CRC tissues. (A) Western blotting of colorectal cancer (T) and adjacent normal (N) tissue were performed. Biopsy specimens were prepared as described in Materials and methods; 10 mg of protein was resolved on 5-20% PAGE gels. The proteins were blotted onto PVDF membrane and detected with mAb PPMX0206. (B) Immunohistochemical staining of CRC tissues (right) and adenomatous polyp (left) obtained from CRC patients. Immunohistochemical staining was performed with the mAb PPMX0205. Magnification, x 200.

Statistical analysis. The association between the clinicopathological factors and expression levels of Cystatin SN were assessed with the Mann-Whitney U test. P-values of <0.05 were considered significant.

Results

Cystatin SN mRNA is specifically up-regulated in colorectal cancer cells. To identify targets for a novel tumor marker in CRC, gene expression profiles of RNA from 35 normal tissues and 38 cancer cell lines were prepared. The goal was to identify genes that are up-regulated in colon cancer cells and encode a secreted protein that can be used to detect them in serum samples. These genes should also exhibit little or no expression in vital organs to minimize the background interference. Genes with the desired expression profile were screened by extensive bioinformatics analysis to determine their structural and functional classifications along with their potential for producing a secreted protein. The *Cystatin SN* gene expression pattern fulfilled the desired characteristics. This gene was overexpressed in the colon cancer cell lines, Caco2 and SW480, as well as strongly expressed in normal salivary glands (Fig. 1A). In addition, Cystatin SN is a member of the Cystatin superfamily, which is one of the cysteine protease inhibitors. To verify overexpression of *Cystatin SN* in CRC, we analyzed *Cystatin SN* expression in nine pairs of CRC tissues and adjacent normal colon mucosa by quantitative real-time RT-PCR. More than a 3-fold up-regulation of *Cystatin SN* expression was observed in all pairs (Fig. 1B).

Expression of Cystatin SN protein in human colorectal cancer tissues. We generated 28 kinds of mAb to Cystatin SN in order to identify the protein expression. Immunoblotting using mAb PPMX0206 showed elevated protein expression in CRC tissues compared with adjacent normal mucosa (Fig. 2A). These results demonstrated that Cystatin SN protein is up-regulated in CRC tissues compared with normal mucosa.

An immunohistochemical analysis using the mAb PPMX0205 demonstrated that the overexpression of Cystatin SN is specific for cancerous areas in CRC tissue and that weak expression of Cystatin SN is found on adenomatous areas in adenoma tissue (Fig. 2B). A strong expression was observed at the membranous lesion of CRC cells, suggesting that this was a membranous secretory protein. These results suggest that Cystatin SN might be a novel tumor marker for CRC. There was no correlation between staining pattern and histological grade (data not shown).

Development of a Cystatin SN assay system. A Cystatin SN ELISA-based assay system was developed using PPMX0203 as the capture antibody and PPMX0204 as the detection antibody. The standard curve values are shown in Table II. A representative calibration curve based on Cystatin SN concentrations of 0.25-10 ng/ml is shown in Fig. 3A. The

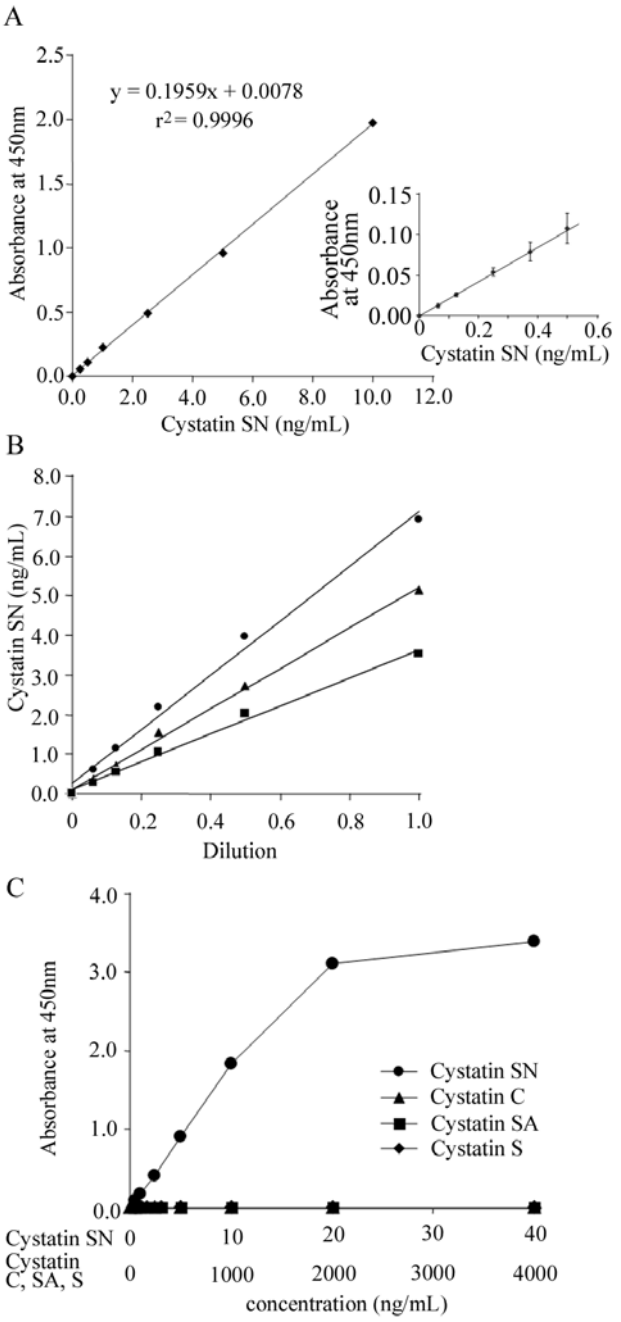

Figure 3. (A) Calibration curve of the ELISA system for Cystatin SN. Calibration values using purified Cystatin SN (in ng/ml): 0.0, 0.25, 0.5, 1.0, 2.5, 5.0 and 10. The equation for the line is $y = 0.1959x + 0.0078$; $r^2 = 0.999$. Inset shows a Cystatin detection limit of 0.0625 ng/ml in the ELISA system. (B) Dilution curves of sera showed good linearity. Three different sera were serially diluted four times with sample dilution buffer (final, one-sixteenth dilution). Each point was measured in duplicate. Serially diluted sera showed good linearity with $r^2 > 0.99$. (C) Cross-reactivity with other closely related Cystatin C, SA or S were examined by the Cystatin SN ELISA system. Cystatin C, SA or S (each 0 to 4000 ng/ml) were added at a hundred times the concentration in the Cystatin SN standard calibration reaction. There was no cross-reactivity observed at any concentration and the absorbance at 450 nm was equal to that of buffer alone, whereas Cystatin SN at 40 ng/ml had an absorbance of >3.3.

absorbance at 450 nm plotted against the amounts of Cystatin SN exhibited a linear relationship. Intra-assay precision was investigated through the analysis (in sets of 5) of three different serum samples, resulting in a coefficient of variation (CV) ≤3.8% at all Cystatin SN concentrations tested. The mean ± SD concentrations measured (and CV) were as follows: Sample 1, 0.98±0.04 ng/ml (3.8%); sample 2, 1.73±0.04 ng/ml (2.6%); sample 3, 3.10±0.10 ng/ml (3.3%) (Table III). Inter-assay precision was investigated through the analysis of three independently performed experiments resulting in a CV ≤8.8% at all Cystatin concentrations tested. The mean ± SD concentrations measured (and CV) were as follows: Sample 1, 0.95±0.07 ng/ml (6.9%); sample 2, 1.55±0.14 ng/ml (8.8%); sample 3, 2.93±0.24 ng/ml (8.0%) (Table IV).

Analytical recovery, assay linearity, assay specificity and cross reactivity. Recovery of exogenously added Cystatin SN from sera ranged from 86.1 to 107.2%. Dilution curves of sera showed good linearity (Fig. 3B).

Bilirubin C or bilirubin F (up to 20 mg/l), or chyle (up to 1,670 units as formazine) had no effect on the present ELISA. Cross-reactivity with Cystatin C, Cystatin SA or Cystatin S was not seen with concentrations up to 4,000 ng/ml, which correspond to 100 times the concentration of Cystatin SN (Fig. 3C).

Presence of Cystatin SN proteins elevated in serum and urine samples. We established a highly sensitive sandwich immunoassay to assess serum levels of the Cystatin SN in patients with CRC (n=159) and controls (n=40) who were proved CRC-free by colonoscopy. In addition, we determined the levels of the established tumor markers CEA and CA19-9 using a commercial immunoassay. Fig. 4 shows serum levels of Cystatin SN, CEA and CA19-9. The median Cystatin SN level in disease-free controls was 1.12 ng/ml. For the patients with CRC, a median level of 1.70 ng/ml was found. We arbitrarily fixed the cutoff level at 2.5 ng/ml for Cystatin SN. The difference between disease-free controls and patients with CRC was highly significant (Mann-Whitney U test, $P<0.0001$) and the serum concentrations of CEA and CA19-9 in patients with CRC was significantly high (Mann-Whitney U test, $P<0.0001$).

In regard to demonstrating the presence of CRC, the sensitivity of the Cystatin SN test was 27.7%, whereas those for CEA and CA19-9 were 50.3 and 23.9%, respectively. The specificity for Cystatin SN was 95.0%, whereas those for CEA and CA19-9 were 97.5 and 95.0%, respectively. The combination of Cystatin SN, CEA and CA19-9 assay showed 62.9% sensitivity and 90.0% specificity (Table VA). The patients with CRC were divided into stage I to stage IV, using the TNM staging system approved by the International Union Against Cancer and the American Joint Committee on Cancer. The combination assay for CEA and CA19-9 revealed increased sensitivity with advanced stage. Similarly, the combination assay for Cystatin SN, CEA and CA19-9 revealed increased sensitivity with advanced stage (Table VB). In particular, the sensitivity of the combination assay for Cystatin SN, CEA and CA19-9 was higher than that for CEA and CA19-9 in stages I and II. Thus, the sensitivity of stage I detection increased from 37.5 to 42.5% and the sensitivity of stage II detection increased from 49.0 to 60.8%. Surgical removal of the primary tumor and resectable metastases is the only option for obtaining a cure (22). If the sensitivity of

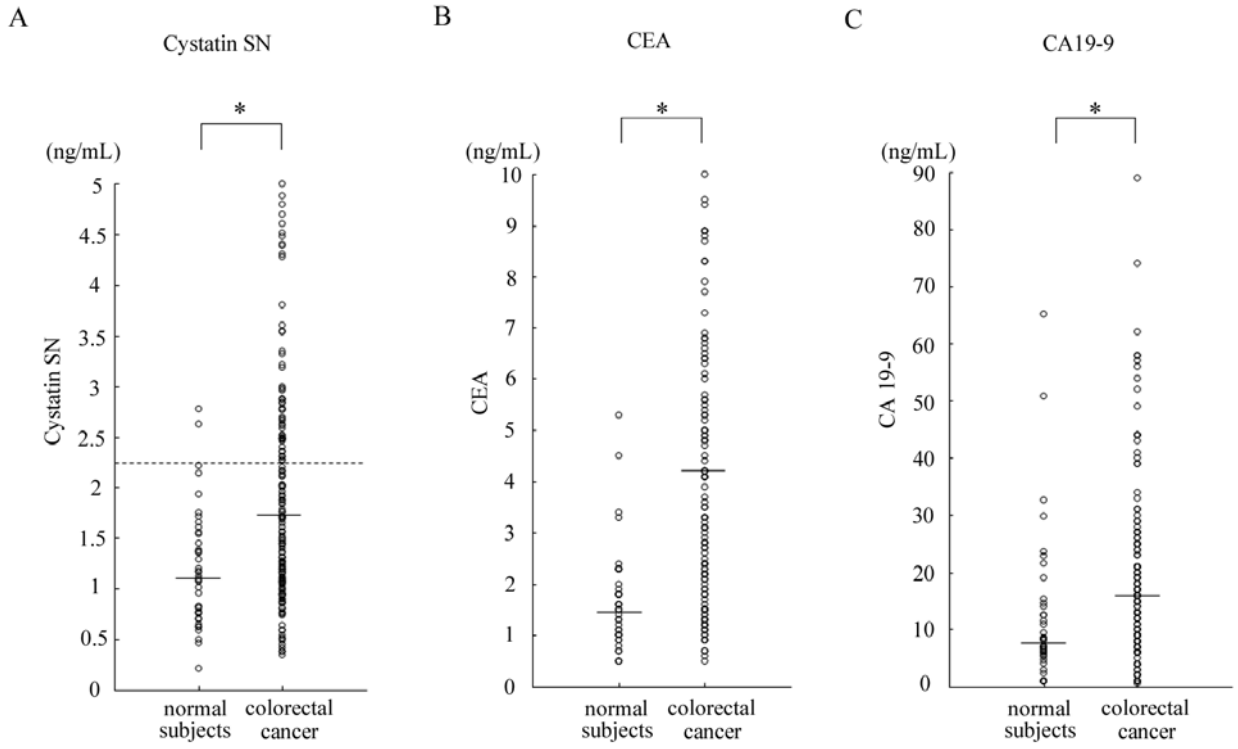

Figure 4. Serum concentration of Cystatin SN (A), CEA (B) and CA19-9 (C) in patients with CRC and controls. Serum concentration of Cystatin SN was measured by an ELISA method. We fixed the cut-off level of Cystatin SN, indicated by a broken line in the panel, at 2.5 ng/ml. In addition, serum concentrations of CEA and CA19-9 were measured by commercial immunoassays. The difference between disease-free controls and patients with CRC was highly significant (*P<0.0001). Horizontal line, median value for each group.

Table III. Intra-assay for our Cystatin SN system.

Sample No.	Well 1	Well 2	Well 3	Well 4	Well 5	SD	CV	Mean
1	0.94	0.94	0.99	0.98	1.03	0.98	0.04	3.8%
2	1.81	1.74	1.69	1.71	1.71	1.73	0.04	2.6%
3	2.92	3.16	3.12	3.12	3.18	3.10	0.10	3.3%

The assay of each serum was done in sets of 5.

Table IV. Inter-assay for our Cystatin SN system.

	Experiments					
Sample No.	1st	2nd	3rd	Mean	SD	CV
1	0.94	0.94	0.98	0.98	0.04	3.8%
2	1.81	1.74	1.71	1.73	0.04	2.6%
3	2.92	3.16	3.12	3.10	0.10	3.3%

Three individual sera (n=3) were measured on the three different days. CV was between 6.9 to 8.8%.

stages I and II detection could be improved we believe that early detection of CRC would be possible and curative operations could be performed. Hence, reduction of CRC mortality would be feasible. Fig. 5 illustrates the relationship between the specificity and sensitivity of the Cystatin SN assay for the detection of CRC, represented by a receiver-operating characteristic (ROC) curve (23). The diagnostic accuracy of the test is expressed by the area under the ROC curve. Values may range between 1.0 (perfect separation of the two groups, i.e., apparently healthy donors and patients with CRC) and 0.5 (no difference between the two groups). The corresponding areas under the curve were 0.708 for Cystatin SN, 0.819 for CEA and 0.703 for CA19-9.

Cystatin SN is a low molecular weight protein of 14 kDa (12) and it has therefore been hypothesized that it may be excreted in the urine. As mentioned, the serum Cystatin SN concentration in CRC is high. We assumed that the urinary Cystatin SN concentration also would be high in CRC and investigated the utility of urinary Cystatin SN as a biomarker for CRC. Using urine samples from patients with CRC and disease-free controls, immunoblotting analysis revealed an increased concentration of Cystatin SN in the urine of patients with CRC compared with urine from controls (Mann-Whitney

Table V. Sensitivity and specificity of CEA, CA19-9 and Cystatin SN.

A, Sensitivity and specificity

	Sensitivity (%)	Specificity (%)
CEA	50.3% (80/159)	97.5% (39/40)
CA19-9	23.9% (38/159)	95.0% (38/40)
Cystatin SN	27.7% (44/159)	95.0% (38/40)
CEA and/or CA19-9	54.1% (86/159)	92.5% (37/40)
Cystatin SN and/or CEA and/or CA19-9	62.9% (100/159)	90.0% (36/40)

B, Sensitivity for each stage

	Stage I (n=40)	Stage II (n=51)	Stage III (n=40)	Stage IV (n=26)
CEA	27.5% (11/40)	49.0% (25/51)	52.5% (21/40)	88.5% (23/26)
Cystatin SN	15.0% (6/40)	35.3% (18/51)	27.5% (11/40)	30.8% (8/26)
CEA and /or CA19-9	37.5% (15/40)	49.0% (25/51)	55.0% (22/40)	92.3% (24/26)
Cystatin SN and/or CEA and/or CA19-9	42.5% (17/40)	60.8% (31/51)	67.5% (27/40)	92.3% (24/26)

The patients with CRC were divided into stage I to stage IV, using the TNM staging system approved by the International Union Against Cancer and the American Joint Committee on Cancer.

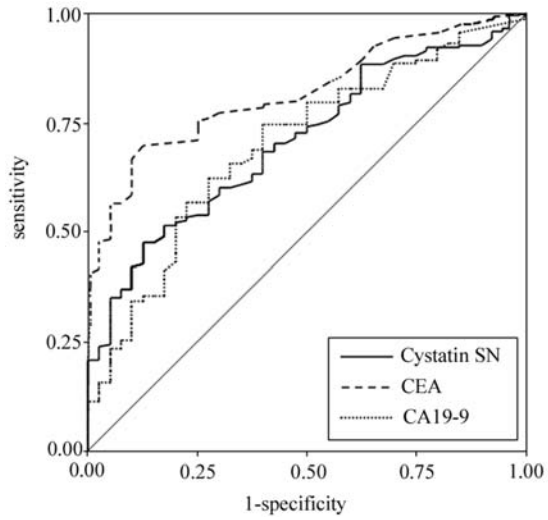

Figure 5. ROC curves of CEA, CA19-9 and Cystatin SN of 159 CRC samples and 40 healthy control samples determined by ELISA as described in Materials and methods. ROC curves were derived by plotting the relationship between the specificity and the sensitivity at various cutoff levels. The area under the curve was 0.708 for Cystatin SN, 0.819 for CEA and 0.703 for CA19-9.

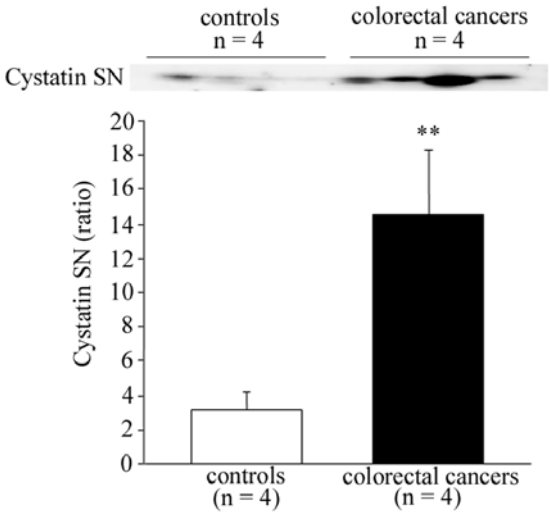

Figure 6. Cystatin SN is up-regulated in urinary samples from the patient with CRC compared with control samples. Identical amounts (10 μl) of urine samples were loaded and immunoblotting with mAb PPMX0206 was performed. Urinary Cystatin SN of CRC is significantly higher than that of normal controls (**P<0.05). Values are expressed as average ± standard error (SE).

U test, P<0.05) (Fig. 6). These results indicate that Cystatin SN might be excreted via the urine, suggesting the possibility of using urine samples to test for this marker.

Discussion

In this study, we showed that Cystatin SN mRNA is highly expressed in colorectal cancer cells. We generated mAbs for Cystatin SN and expression of Cystatin SN was observed on cell membranes in CRC tissues compared with normal mucosa. In bioinformatics analysis, a secreted protein is the most logical biomarker candidate; however, it is often not practically possible to utilize potential markers in serum due to unforeseeable high background interference. In gene expression profiling, Cystatin SN was highly expressed in normal salivary glands along with colon cancer cells, suggesting that salivary gland-derived Cystatin SN might increase the serum levels of Cystatin SN in control subjects.

However, using mAb PPMX0203 and PPMX0204 we detected very low levels of Cystatin SN in disease-free controls. We speculate that the Cystatin SN in normal salivary glands does not secrete into plasma in great amounts. The diagnostic accuracy of the serum Cystatin SN assay for CRC seems to be useful, especially in a combination assay for Cystatin SN, CEA and CA19-9. Thus, the sensitivity using a combination assay for Cystatin SN, CEA and CA19-9 was higher than that of an assay for only CEA and CA19-9 in stages I and II. The five-year survival rates of CRC was 68-100% for stage I, 58-90% for stage II, 33-76% for stage III and <5-9% for stage IV (22). With the prognosis for advanced disease so poor and survival for early disease so favorable, early detection remains the primary option for CRC control (1). If the sensitivity of stages I and II detection can be improved, it is thought that early detection of CRC would be possible and curative operation could be performed. Hence, CRC mortality would be reduced. However, further studies are needed to fully assess the promising role of the Cystatin SN.

In this study, we also demonstrated that Cystatin SN is excreted via urine and that patients with CRC excrete increased levels compared with controls. These results open the possibility of using urine samples to test for this novel tumor marker. No other tumor markers for CRC are tested in urine samples. However, the development of the ELISA system for quantification of Cystatin SN in urine samples is difficult at the present time. Therefore, further studies are necessary to validate and improve this marker for clinical application and for development of the ELISA system for urine samples.

In conclusion, we identified up-regulation of the Cystatin SN gene in colorectal cancer cells by microarray analysis. Twenty-eight kinds of mAb for Cystatin SN were generated and an ELISA system was developed. Elevated levels of Cystatin SN were found in serum from patients with CRC (sensitivity 27.7%, specificity 95.0%). The combination assay for Cystatin SN, CEA and CA19-9 showed 62.9% sensitivity and 90.0% specificity. Especially, the combination assay improved the sensitivity of stages I and II detection, in which stages curative surgery would be possible, over the assay detecting only CEA and CA19-9 (from 37.5 to 42.5% in stage I, from 49.0 to 60.8% in stage II). Furthermore, Western blot analysis revealed that Cystatin SN was increased in the urine of patients with CRC.

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References

1. Ouyang DL, Chen JJ, Getzenberg RH and Schoen RE: Noninvasive testing for colorectal cancer: a review. Am J Gastroenterol 100: 1393-1403, 2005.
2. Inger DB: Colorectal cancer screening. Prim Care 26: 179-187, 1999.
3. Collins JF, Lieberman DA, Durbin TE and Weiss DG: Accuracy of screening for fecal occult blood on a single stool sample obtained by digital rectal examination: a comparison with recommended sampling practice. Ann Intern Med 142: 81-85, 2005.
4. Imperiale TF, Ransohoff DF, Itzkowitz SH, Turnbull BA and Ross ME: Fecal DNA versus fecal occult blood for colorectal-cancer screening in an average-risk population. N Engl J Med 351: 2704-2714, 2004.
5. Parekh M, Fendrick AM and Ladabaum U: As tests evolve and costs of cancer care rise: reappraising stool-based screening for colorectal neoplasia. Aliment Pharmacol Ther 27: 697-712, 2008.
6. Woolf SH: The best screening test for colorectal cancer-a personal choice. N Engl J Med 343: 1641-1643, 2000.
7. Winawer S, Fletcher R, Rex D, *et al*: Colorectal cancer screening and surveillance: clinical guidelines and rationale - Update based on new evidence. Gastroenterology 124: 544-560, 2003.
8. Bast RC Jr, Ravdin P, Hayes DF, *et al*: 2000 update of recommendations for the use of tumor markers in breast and colorectal cancer: clinical practice guidelines of the American Society of Clinical Oncology. J Clin Oncol 19: 1865-1878, 2001.
9. European Group on Tumour Markers: Tumour markers in gastrointestinal cancers - EGTM recommendations. Anticancer Res 28: 2811-2815, 1999.
10. American Society of Clinical Oncology: 1997 update of recommendations for the use of tumor markers in breast and colorectal cancer. J Clin Oncol 16: 793-795, 1998.
11. Carpelan-Holmström M, Louhimo J, Stenman UH, Alfthan H, Järvinen H and Haglund C: Estimating the probability of cancer with several tumor markers in patients with colorectal disease. Oncology 66: 296-302, 2004.
12. Tseng CC, Tseng CP, Levine MJ and Bobek LA: Differential effect toward inhibition of papain and cathepsin C by recombinant human salivary cystatin SN and its variants produced by a baculovirus system. Arch Biochem Biophys 380: 133-140, 2000.
13. Midorikawa Y, Tsutsumi S, Taniguchi H, *et al*: Identification of genes associated with dedifferentiation of hepatocellular carcinoma with expression profiling analysis. Jpn J Cancer Res 93: 636-643, 2002.
14. Hippo Y, Taniguchi H, Tsutsumi S, *et al*: Global gene expression analysis of gastric cancer by oligonucleotide microarrays. Cancer Res 62: 233-240, 2002.
15. Tanaka T, Takeno T, Watanabe Y, *et al*: The generation of monoclonal antibodies against human peroxisome proliferator-activated receptors (PPARs). J Atheroscler Thromb 9: 233-242, 2002.
16. Watanabe Y, Tanaka T, Uchiyama Y, *et al*: Establishment of a monoclonal antibody for human LXRalpha: Detection of LXRalpha protein expression in human macrophages. Nucl Recept 1: 1, 2003.
17. Watanabe Y, Jiang S, Takabe W, *et al*: Expression of the LXRalpha protein in human atherosclerotic lesions. Arterioscler Thromb Vasc Biol 25: 622-627, 2005.
18. Saitoh R, Ohtomo T, Yamada Y, *et al*: Viral envelope protein gp64 transgenic mouse facilitates the generation of monoclonal antibodies against exogenous membrane proteins displayed on baculovirus. J Immunol Methods 322: 104-117, 2007.
19. Köhler G and Milstein C: Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256: 495-497, 1975.
20. Parham P: On the fragmentation of monoclonal IgG1, IgG2a, and IgG2b from BALB/c mice. J Immunol 131: 2895-2902, 1983.
21. Ishikawa E, Yoshitake S, Imagawa M and Sumiyoshi A: Preparation of monomeric Fab'-horseradish peroxidase conjugate using thiol groups in the hinge and its evaluation in enzyme immunoassay and immunohistochemical staining. Ann NY Acad Sci 420: 74-89, 1983.
22. Link KH, Sagban TA, Mörschel M, *et al*: Colon cancer: survival after curative surgery. Langenbecks Arch Surg 390: 83-93, 2005.
23. Zweig MH and Campbell G: Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. Clin Chem 39: 561-577, 1993.