Table 3. Alterations of plasma Apo AI and C9 in various diseases

	n	Apo AI			C9		
		Average†	SD	*P* value‡	Average†	SD	*P* value‡
Healthy control	109	4679.5	3265.6		527.2	674.7	
Colorectal cancer	100	2318.4	2015.0	1.71×10^{-9}	1792.7	1628.1	3.82×10^{-11}
Gastric cancer	105	2812.0	2357.5	3.00×10^{-6}	1629.6	1533.9	3.31×10^{-10}
Hepatocellular carcinoma	14	2621.6	2260.8	0.007	477.0	332.4	0.651
Esophageal cancer	10	3074.6	1572.6	0.014	1639.1	1036.7	0.008
Pancreatic cancer	14	2934.3	2214.8	0.016	1436.8	1125.6	0.010
Cholangiocarcinoma	18	1674.6	1377.0	1.26×10^{-8}	2519.6	2086.8	0.001
Pancreatitis	8	1925.9	1970.3	0.005	1564.4	1502.5	0.093

†Determined by RPPM (in arbitrary units). ‡Compared with healthy controls (student's *t*-test).

proteins in colorectal cancer patients were reproducible, even in this independent cohort (Table 3). The reduction of plasma Apo AI protein seems not to be specific to colorectal cancer, and was observed in patients with various cancers as well as chronic pancreatitis. The increment of plasma C9 protein was also not specific to colorectal cancer patients; patients with gastric cancer, esophageal cancer, pancreatic cancer and cholangiocarcinoma also showed a statistically significant increase of plasma C9 protein. The AUC value of C9 for colorectal cancer patients over healthy individuals (0.796) was higher than that of CEA (0.762; Fig. 4). The combination with C9 improved the AUC value of CEA from 0.762 to 0.852.

Discussion

The context of circulating blood proteins may represent underlying physiological and pathogenic conditions. Therefore, the blood proteome is considered an ample source of biomarker discovery. In order to identify a new biomarker that can be used for a non-invasive and inexpensive blood test of colorectal cancer, we first compared plasma proteome data using 2DICAL. We found that 10 proteins showed statistically significant differences between colorectal cancer patients and controls (Table 2). The differences of Apo AI and C9 were then verified by immunoblotting with relevant antibodies (Fig. 1c). Apo AI is the major protein component of plasma high density lipoprotein.[19] Apo AI has been repeatedly reported to be downregulated in the plasma samples of patients with various cancers including ovarian and pancreatic cancers.[20,21]

Any biomarker candidates identified by genomic or proteomic approaches must be validated in a statistically sufficient number of cases and controls using a different quantitative method before being considered for clinical application.[22,23] Accordingly, we determined the relative plasma levels of Apo AI and C9 in 345 individuals using RPPM (Fig. 3) and confirmed the results in an independent cohort consisting of 378 plasma samples collected from healthy controls and patients with various diseases (Table 3). The collection and storage of all the plasma samples were performed under the same protocol to exclude any sampling biases. Conventionally, ELISA has been used for such validation, but the standard sandwich ELISA assay requires two antibodies that do not interfere with each other. As a result, the development of ELISA usually takes several months for every biomarker candidate protein. And more importantly, ELISA requires a relative large volume (~100 μL) of samples. Because the supply of clinical materials is often limited, it may be unfavorable to use hundreds of microlitters of precise samples for preliminary experiments. Our high-density RPPM requires a minimal sample volume of the nanoliter order and one antibody. RPPM is an alternative validation method that can determine the clinical utility of candidate biomarker protein in a single experiment.[12]

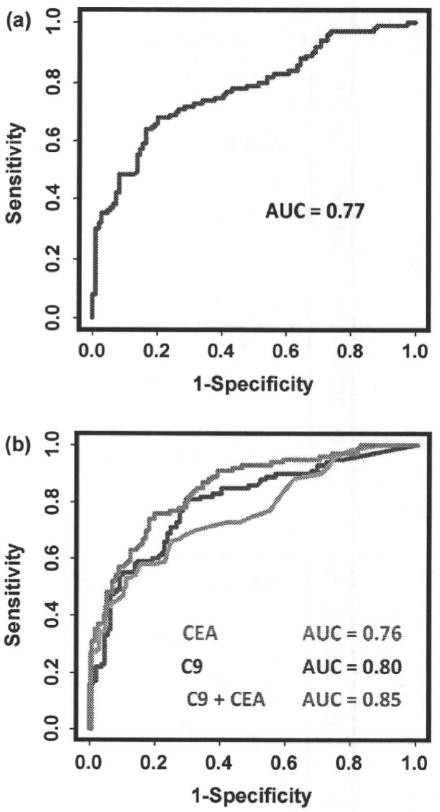

Fig. 4. Confirmation in an independent cohort. (a) Receiver operator characteristic (ROC) analysis of apolipoprotein AI (Apo AI; colorectal cancer patients [*n* = 100] over healthy controls [*n* = 109]). AUC, area under the curve. (b) ROC analysis of carcinoembryonic antigen (CEA), plasma complement component-9 (C9) and their combination (colorectal cancer patients [*n* = 100] over healthy controls [*n* = 109]).

Although protein microarray is a newly established technique and still requires improvement regarding validity and standardization,[24] it has been successfully used for analyzing clinical specimens of prostate cancer,[25] breast cancer,[26] rhabdomyosarcoma[27] and acute myeloid leukemia.[28,29] More recently, Grote *et al.*[30] used protein microarrays for the measurement of serum and plasma CA19-9. They printed a total of 149 sera and plasma samples obtained from pancreatic cancer patients, patients with chronic pancreatitis and healthy controls onto

nitrocellulose-coated slide glasses and obtained results comparable to conventional ELISA. They used 200-μm pins, and signals were detected using diaminobenzidine as a chromogen. We were able to spot as many as 6144 protein samples into a glass slide using a 100-μm innovative screw-shaped pin and hydrophobic surface technologies. The hydrophobic surface of microarrays mediates tight interaction with proteins and prevents protein spot diffusion.[16,17] Only with all these cutting-edge technologies was this level of high-density spotting of adhesive protein samples possible. Fluorescence immunostaining of our RPPM provided wide dynamic range and high reproducibility (Fig. 2). The linearity of fluorescence intensity was obtained in a wide range, and over 78% of quadruplicate showed a CV of < 0.1 (Fig. 2c). All these make our RPPM a reliable tool for biomarker validation.

The ninth component of complement (C9) is one of five component proteins (C5b, C6, C7, C8 and C9) of the membrane attack complex (MAC).[31] The MAC attaches to the surface of target cells and forms a pore across the cell membrane resulting in complement-dependent cytotoxicity (CDC). Aberrant activation of MAC has been implicated in the pathogenesis of various autoimmune and infectious diseases. We previously identified the significant increase of complement components C3 and C4A in the sera of endometrial cancer patients.[9] In the present study we found the plasma level of C9 was significantly elevated in colorectal cancer patients, including those with stage I and II diseases. The expression of membrane-bound CD46, CD55 and CD59 protects cancer cells from CDC,[32] but the precise role of complements and these modifiers in the process of carcinogenesis has not been fully established. Further efforts will be necessary to clarify the biological significance of increased circulating C9 in patients with colorectal cancer.

In the present study we identified and validated C9 as a plasma biomarker potentially useful for the detection of early stage colorectal cancer using the combination of innovative proteomic technologies. Although the clinical significance of C9 must be clarified in an independent clinical study, we were able to demonstrate the utility of the combination of 2DICAL and RPPM in biomarker discovery and validation. The combination is a rapid approach that could be applicable to the discovery of biomarkers for any types of human malignancy.

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Disclosure Statement

No potential conflict of interest relevant to this paper is declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Verification of reverse-phase plasma microarray (RPPM).

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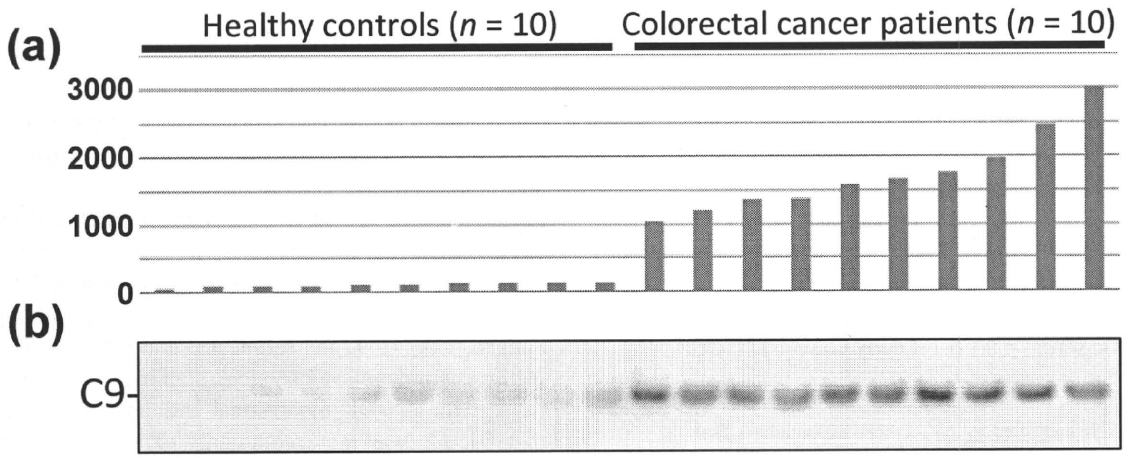

Supplementary Fig. S1.
Verification of reverse-phase plasma microarray (RPPM).
(a) Plasma complement component-9 (C9) level (in arbitrary unit) of 10 representative colorectal cancer patients and 10 healthy controls determined by RPPM.
(b) The same plasma samples were subjected to immunoblotting with anti-C9 antibody.

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ORIGINAL ARTICLE—ALIMENTARY TRACT

Dermokine as a novel biomarker for early-stage colorectal cancer

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Abstract
Background Colorectal cancer is a common disease that is usually detected at an advanced stage, because early-stage cancer is mostly asymptomatic and appropriate serologic biomarkers have not been established. We have previously identified dermokine (DK) as a peptide secreted by keratinocytes and we found that DK-β/γ was expressed in colorectal tumors. Therefore, we focused on DK-β/γ as a new candidate diagnostic serum marker for early colorectal cancer.

Methods DK-β/γ expression in human colorectal cancer cell lines and tissues was assessed by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) and immunohistochemistry. We established an experimental enzyme-linked immunosorbent assay (ELISA) to detect DK-β/γ in the serum of colorectal cancer patients, and we compared the sensitivities of common diagnostic markers, carcinoembryonic antigen (CEA), carbohydrate antigen (CA) 19-9, and serum p53 antibody (S-p53).
Results Immunohistochemical staining of colon tumor tissue with anti-DK monoclonal antibody (mAb) revealed that DK-β/γ was more commonly expressed in the early stages of colorectal cancer (Tis–T1; i.e., cancer in situ, intraepithelial or invasion of lamina propria [Tis]; tumor invades the submucosa [T1]) than in late-stage tumors (T2–T4; i.e., tumor invades the muscularis propria [T2];

This work is dedicated to the memory of Shoichiro Tsukita.

T. Tagi and T. Matsui contributed equally to this work.

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tumor invades through the muscularis propria into the subserosa, or into the nonperitonealized pericolic or perirectal tissues [T3]; tumor directly invades other organs or structures and/or perforates visceral peritoneum [T4]). Serum DK-β/γ levels were determined in 130 patients with colorectal cancer and 25 healthy volunteers. Serum DK-β/γ was detected in 33.3% of patients with early colorectal cancer (Tis–T1), which was higher than the rates for S-p53 (24.2%), CEA (9.1%), and CA19-9 (0%). The serum DK-β/γ test was complementary to the other marker tests. Therefore, when the combined four-marker test (DK/CEA/CA19-9/S-p53) was carried out, the diagnostic sensitivity for Tis and T1 tumors reached 60.6%.

Conclusions Serum DK-β/γ is the most promising of the existing tumor biomarkers for the diagnosis of early-stage colorectal cancer.

Keywords Serum marker · Dermokine · Colorectal cancer · Early stage

Abbreviations

DK	Dermokine
CEA	Carcinoembryonic antigen
CA19-9	Carbohydrate antigen 19-9
S-p53	Serum p53 antibody
ELISA	Enzyme-linked immunosorbent assay
SSE	Stratified squamous epithelium
Tis	Cancer in situ, intraepithelial or invasion of lamina propria
T1	Tumor invades the submucosa
T2	Tumor invades the muscularis propria
T3	Tumor invades through the muscularis propria into the subserosa, or into the nonperitonealized pericolic or perirectal tissues
T4	Tumor directly invades other organs or structures and/or perforates visceral peritoneum. We followed the International Union Against Cancer (UICC) classification

Introduction

If the tumor is diagnosed while it is limited to the mucosa or submucosa, colorectal cancer could be completely cured by endoscopic or surgical therapy; indeed, colorectal cancer is the third most common cancer, causing 49,960 deaths annually in the United States, and it was responsible for 8.8% of all cancer deaths in 2008 [1, 2]. Routine colonoscopy in normal adults often reveals a small benign tumor or adenoma of the gut epithelium, in the form of a protruding polyp. These adenomatous polyps are slow-growing tumors and are commonly believed to be the precursors of a large proportion of colorectal cancers. Unfortunately, currently, most patients diagnosed with colorectal cancer are already at an advanced stage. Therefore, early detection is the best and least expensive way to manage colorectal cancer patients.

Serologic biomarkers can be analyzed relatively noninvasively and economically compared with other diagnostic procedures. They have the potential to greatly enhance detection of the disease and can be used as markers for subsequent colonoscopy. Diagnostic blood tests based on the detection of carcinoembryonic antigen (CEA) are currently widely available, although the sensitivity of this marker in early-stage cancer is only 5–10% [3, 4]. Thus, new candidate molecules, particularly peptides secreted by cancers, for early-stage serodiagnosis are thought to be the most likely way to improve the cure rate and reduce medical costs.

Stratified epithelium is multilayered and confers physical protection against various mechanical stresses in areas such as the skin, esophagus, and vagina. Previously, we and Moffat et al. [5, 6] have identified dermokine (DK)-α/-β (sk30/89) as a novel keratinocyte-secreted peptide, as well as identifying the formation of a new stratified epithelium-secreted gene complex (SCC) with two other keratinocyte-secreted peptides, Kdap and suprabasin. DK has been reported to have several other isoforms, in addition to the α and β isoforms, including γ1 and 2, δ1–6, and ε1–3 (Fig. 1a) [7, 8]. The secreted forms DK-$\alpha/\beta/\gamma$ are highly expressed in the differentiated layer of stratified epithelia [6–8]. In addition to their expression in normal multilayered epithelia, DK-γ and -δ were also characterized in the human expressed sequenced tag database as carcinoma-expressing genes [8]. In this study, we showed that the secreted isoform of DK (DK-β/γ) was aberrantly expressed in colorectal cancer. We investigated the potential of serum DK as a novel biomarker and we also examined the benefits of a multimarker test to diagnose early colorectal cancer.

Materials and methods

Cell culture

A total of 15 colorectal cancer cell lines were used, which have been described previously [9]. We purchased 293/EBNA-1 cells from Invitrogen (Carlsbad, CA, USA) and the cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum. Transfection was performed with TransIT-LT1 (Mirus, Madison, WI, USA) as previously described [5].

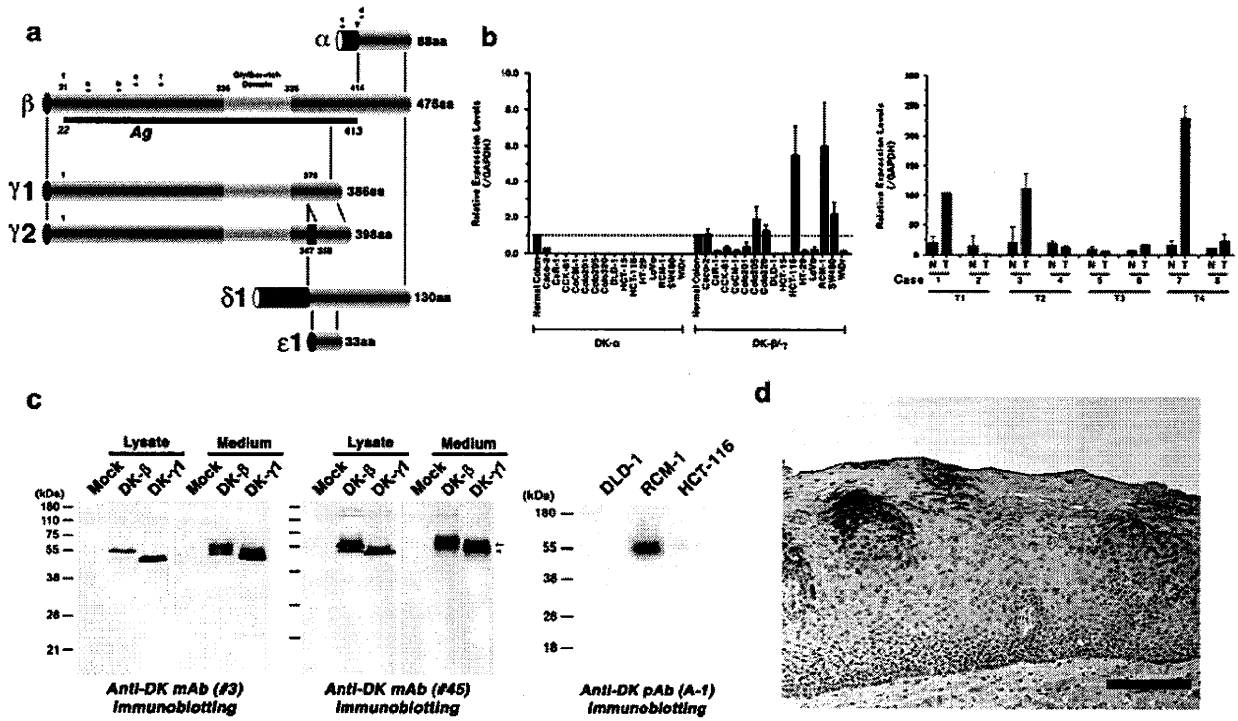


Fig. 1 Expression of dermokine (*DK*) isoforms in colorectal cancer cell lines and tissues. **a** Schematic representation of the structure of DK isoforms ($\alpha/\beta/\gamma1/\gamma2/\delta1/\varepsilon1$). *Arrows* indicate the primer pairs used for SYBR Green quantitative reverse transcription polymerase chain reaction (qRT-PCR) (*a*, *b* DK-β/γ; *c*, *d* DK-α) and TaqMan qRT-PCR (*e*, *f* DK-β/γ) in **b**. The *line* indicates the region used for the antigen (*Ag*). *Arrowheads* represent the signal peptidase cleavage site. **b** Relative expression levels of DK-α and DK-β/γ mRNAs in 15 colorectal cancer cell lines (*left*) and DK-β/γ mRNA in eight surgically resected primary colorectal cancers (*T*) and corresponding noncancerous colon mucosa (*N*) (*right*). The expression of the target mRNA was evaluated by quantitative real time RT-PCR. Results are presented as the ratios between each target mRNA and reference mRNA (glyceraldehyde-3-phosphate dehydrogenase [*GAPDH*]) expression. The relative expression level in colorectal cancer cell lines was normalized versus the level in one normal colon tissue sample. DK-β/γ was relatively highly expressed in several colorectal cancer cell lines, whereas DK-α was not. Similar to findings in the cell lines, DK-β/γ was relatively highly expressed in four of the eight colorectal cancer tissues. **c** Generation of DK-β/γ-specific monoclonal antibodies (*mAbs*). Cultured medium (*Medium*) and cell lysate (*Lysate*) of HEK293 cells transiently expressing DK-β and DK-γ were immunoblotted with anti-DK-β/γ mAb #3 (*left*) and #45 (*middle*), respectively. The anti-DK mAb specifically recognized the expressed and secreted DK-β (*arrow*) and DK-γ (*arrowhead*) proteins. Immunoblotting of conditioned medium with polyclonal Ab (*pAb*; A-1) is shown on the *left* (DLD-1, RCM-1, and HCT-116). **d** Immunohistological staining (*brown*) of human esophagus with anti-DK mAb #45. DK-β/γ was localized exclusively in the apical cytoplasm of the differentiated layer of the esophagus. ×100; *bar* 100 μm (color figure online)

Patients and samples

Serum samples and specimens of colorectal tumors were obtained from patients ($n = 130$) under a protocol approved by the Institutional Review Board of Kyoto Prefectural University of Medicine (KPU-M). The eligibility criteria of the cancer patients were: (1) histologically proven primary colorectal adenocarcinoma, (2) no active double cancer (synchronous and metachronous double cancer), (3) no synchronous colorectal neoplasm including adenoma, and (4) no prior treatment with chemotherapy or radiation therapy against any other malignancy. Also, serum samples from randomly selected volunteers were collected from KPU-M ($n = 25$). All patients gave written informed consent, and all aspects of these studies were approved by the ethics committees of KPU-M and Eisai Co., Ltd. (Tokyo, Japan). Blood was collected with the Vacutainer blood collection system (Kyokuto Pharmaceutical Industrial, Tokyo, Japan). The serum samples from postoperative patients were collected on the 7th day after surgical resection. All serum samples were aliquoted and stored at −80°C.

Quantitative real-time reverse transcription–polymerase chain reaction

For quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of colorectal cancer cell lines, total RNA was prepared from various colorectal cancer cell lines using TRIsure (Nippon Genetics, Tokyo,

Japan). Residual genomic DNA was removed by incubating samples with ribonuclease-free DNase (TAKARA Bio, Otsu, Japan). First-strand cDNA templates were prepared from the total RNA using Superscript II (Stratagene, La Jolla, CA, USA) with oligo-dT primers. Quantitative real-time PCR was performed in duplicate by monitoring the increase in fluorescence of SYBR Green I dye with a Power SYBR Green PCR Kit (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 7700 Sequence Detection System (Applied Biosystems) according to the manufacturer's protocol. All data were normalized to an internal standard [glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA]. The primer sets have been described previously [5].

Total RNA from primary colorectal cancer tissues was prepared using an RNeasy Midi kit (QIAGEN, Venlo, Netherlands). Residual genomic DNA was removed by incubating samples with RNase-Free DNase Set (QIAGEN). For the detection of DK-β/γ mRNA, first-strand cDNA synthesis and quantitative real-time RT-PCR was performed in duplicate with a SuperScript^{TM} III Platinum One-Step Quantitative RT-PCR system with ROX (Invitrogen) and an ABI Prism 7900 Sequence Detection System (Applied Biosystems) according to the manufacturers' protocols. All data were normalized as described above. TaqMan primers mixed with probes were purchased from Applied Biosystems. Primers for the experiment were as follows: DK forward 5′-ACAGGCAGAAGATGTCATTC GA-3′; reverse 5′-TGGGGACACCTTAGCATC-3′.

cDNA cloning and construction

cDNA of human DK-β and DK-γ was amplified by PCR using a 5′ *Sal*I-KOZAK-hDK-β primer (5′-AATTGTCGA CGCCACCATGAAGTTCCAGGGGCCCCTGG-3′) and a 3′ *Not*I-hDK-β primer (5′-AATTGCGGCCGCCTACCAA AACTTCACCCACTGCAGCAGG-3′) for DK-β and the 3′ *Not*I-hDK-γ primer (5′-AATTGCGGCCGCTCACGGG ATGCGAGAGCTTCTC-3′) for DK-γ. After digestion with *Sal*I and *Not*I, these cDNAs were cloned into *Sal*I–*Not*I sites of pcDNA3.1, in which the original *Sal*I site was deleted and a new *Sal*I site was introduced in the multicloning site (MCS). To produce a monoclonal antibody (mAb) that specifically recognized both human DK-β and -γ, we prepared antigen DK-$\beta\Delta$C, which comprised aa 22–413 of DK-β. Most of this sequence was overlapped by DK-β and -γ (Fig. 1a). DK-$\beta\Delta$C cDNA was amplified by PCR using a 5′ *Sal*I-KOZAK-hDK-β primer and a *Not*I-hDK-$\beta\Delta$C primer (5′-AATTGCGGCCGCCTCAATAATT GCTTTCCAGTTGAGG-3′). Human DK-$\beta\Delta$C cDNA was fused to the 5′-end of the cDNA that encoded secreted alkaline phosphatase (SEAP) tagged with $(His)_6$ to yield pcDNA3.1-hDK-$\beta\Delta$C-SEAP$(His)_6$, as described previously [5]. Transfection and purification of the antigen, hDK-$\beta\Delta$C-SEAP$(His)_6$ has also been described previously [5].

Antibody preparation

A polyclonal Ab (Anti-hDK pAb (A-1)) was raised against the recombinant protein, GST-hDKβ(22–167) (see Supplemental Material and Methods) and affinity-purified on maltose binding protein-hDKβ(22–167) covalently coupled to a Hitrap NHS-activated HP 1-ml column (GE Healthcare Japan, Tokyo, Japan). Monoclonal Ab generation was performed by Kojin Bio (Tokyo, Japan) using the antigen hDK-$\beta\Delta$C-SEAP $(His)_6$. Several mAbs (including #3 and #45) were raised, which specifically recognized recombinant human DK-β/γ by immunoblotting. These antibodies were purified by protein A Sepharose (GE Healthcare Japan) according to the manufacturer's instructions. To detect serum DK-β/γ in colorectal cancer patients, we established a sandwich ELISA with anti-DK-β/γ mAbs (Supplementary Fig. 2).

Immunohistochemistry

Paraffin sections (5 μm thick) of tumor tissues were subjected to immunohistochemical staining for the DK protein by the tyramine amplification method, which uses fluorescyl-tyramide. In brief, antigen retrieval was performed by heating the samples in Dako REAL Target Retrieval Solution (Dako), for 40 min at 98°C. Endogenous peroxidases were quenched by incubating the sections for 30 min in 3% H_2O_2. After a brief wash with phosphate buffered saline (PBS; pH 7.2) and 0.3% polyoxyethylene sorbitan monolaurate (Sigma-Aldrich), the sections were incubated for 45 min at room temperature with blocking reagent (Block Ace®; DS Pharma Biomedical, Osaka, Japan) to reduce the background signals. Each section was incubated at 4°C overnight with anti-DK mAb. CSAII (Dako, Glostrup, Denmark) was used for color development. The sections were counterstained with hematoxylin.

Detection of serum DK, CEA, CA19-9, and S-p53 concentration by enzyme-linked immunosorbent assay

We developed a DK-specific enzyme-linked immunosorbent assay (ELISA) that used an mAb against DK-β/γ. DK-β/γ was captured onto 96-well plates for ELISA as follows. First, the captured mAb anti-DK-β/γ (#45, IgG1) was added to each well of a 96-well plate and incubated overnight at 4°C. The wells were then washed with PBS and incubated with PBS containing 1% Block Ace (DS Pharmaceutical) to block nonspecific antibody binding. The serum samples (prepared as described before) were added to each well, and the plates were incubated overnight

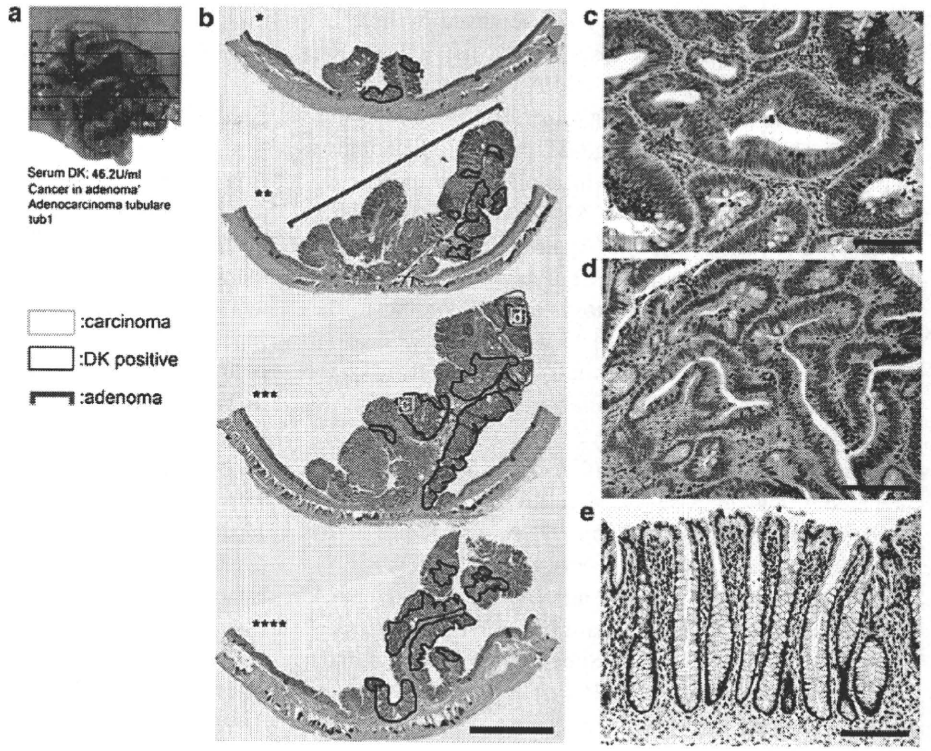


Fig. 2 Immunohistochemical staining of cancer in adenoma with anti-DK mAb. **a** Macroscopic view of noninvasive cancer in adenoma. **b** The DK-positive area (*outlined in black*) and cancer lesions (Tis, *outlined in red*) partially overlapped in noninvasive cancer, and DK was also expressed in the adjacent adenoma region. **c, d** DK was mainly located in the apical cytoplasm of adenoma cells (**c**), and it was located diffusely in the cancer (**d**). **e** DK was not expressed in the normal colon epithelium. ×5 (**b**), ×200 (**c–e**); *bars* 10 mm (**b**), 50 μm (**c–e**). Each *asterisk* indicated same specimen (**a** and **b**) (color figure online)

at 4°C. Horseradish peroxidase-conjugated anti-DK-β/γ mAb (#3, IgG2a) was added and the plates were incubated at room temperature for 1 h. DK was then detected with tetramethylbenzidine Liquid Substrate System for ELISA. The standard used in these assays was recombinant DK-β expressed in 293/EBNA-1 cells. Standard curves were prepared for each assay. Serum CEA, CA19-9 (Abbott Japan, Tokyo, Japan), and S-p53 (Medical and Biological Laboratories, Nagoya, Japan) were quantified according to the manufacturers' protocols.

Endoscopic examination of rectal cancer

The instruments used in this study were a magnifying videoendoscope system (CF-H260AZI; Olympus Optical, Tokyo, Japan) and a standard optical videoendoscopic system, 2 light sources, and a digital image filing system. One light source was for the standard optical filter (broadband), and the other was for the Narrow Band Imaging system.

Clinicopathological data and statistical analysis

Histological analysis was performed by two pathologists without any knowledge of the molecular and serum data. Patient data were collected retrospectively from the hospital database of KPU-M. The χ^2 test was performed to determine correlations among the various parameters, and Fisher's exact test was used as appropriate. Results are expressed as means ± SD. Differences were considered significant when the two-tailed P value was < 0.05. The data were analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

Results

DK mRNA was expressed in human colorectal cancer cell lines and tissue

We performed quantitative real-time RT-PCR analysis of DK-α and -β/γ in 15 colorectal cancer cell lines. DK-β/γ mRNAs were expressed more highly in Caco-2, Colo205, Colo320, HCT-116, RCM-1, and SW480 cells compared with normal colon tissue, but DK-α mRNA was rarely expressed (Fig. 1b, left). We then examined the expression levels of DK-β/γ in colorectal cancer tissues and corresponding noncancerous tissues (Fig. 1b, right). Similar to findings in the cell lines, DK-β/γ mRNAs were highly expressed in five of the eight colorectal cancer tissues.

DK was expressed in colorectal cancer and adenoma

To examine the protein expression of DK-β/γ in colorectal cancer tissues, we developed 12 mAbs against the 22–413 aa region of DK-β (Fig. 1a). Immunoblotting analysis

revealed that anti-DK mAbs #3 and #45 specifically recognized recombinant DK-β/γ (Fig. 1c). Epitope mapping revealed that mAb #45 recognized the 24-167aa of human DK-β/γ (Supplementary Fig. 1). Immunohistochemical staining with anti-DK mAb #45 revealed that DK-β/γ was localized in the apical cytoplasm of squamous cells in the spinous and granular layers of the esophagus, which is consistent with previous studies of human epidermis (Fig. 1d) [8]. Staining of Tis tumors (cancer in situ, intraepithelial or invasion of lamina propria), typical for 'cancer in adenoma', is shown in Fig. 2. DK-β/γ was widely expressed in almost half the total area of the adenomatous polyps. The DK-positive region had both cancer and adenoma. In DK-positive adenoma cells, there was strong staining for DK-β/γ in the apical cytoplasm of multilayered epithelia and weak staining on the basal side in glandular structures (Fig. 2c), whereas the DK-positive cancer cells showed a diffuse DK-β/γ pattern in the cytoplasm (Fig. 2d). Figure 3 shows small invasive cancer (size, 18 × 12 mm), which had slightly invaded the muscularis propria. Endoscopic examination revealed a shallow depressed lesion with a flat elevated area in the rectum (left upper panel). Magnifying colonoscopy showed irregular and disrupted vessels. The irregular and distorted crypts in the demarcated area, as observed in Kudo's V_I with irregular surface and partially V_N with amorphous surface, suggested deep submucosal invasion in the depressed lesion (right upper panel) [10, 11]. A DK-positive region was located in both the mucosa and submucosal invasion (Fig. 3b, right bottom panel). As shown in Fig. 3b (left bottom panel), intracellular accumulation of DK was definite in multilayer stratification lesions with hypercellularity.

Detection of serum DK concentration by enzyme-linked immunosorbent assay is promising for serodiagnosis of early-stage colorectal cancer

To detect serum DK-β/γ in colorectal cancer patients, we established a sandwich ELISA with anti-DK-β/γ mAbs #3 and #45. We measured serum DK-β/γ levels in 130 colorectal cancer patients and compared them with the levels in 25 randomly selected, healthy volunteers for whom there were full data on their medical condition. To simulate the diagnostic use of this test, we proposed a cutoff value (51 U/ml). The specificity of the serum DK-β/γ test was 92.0% in the 25 healthy volunteers. The median serum DK-β/γ level was slightly higher in the colorectal cancer patients [38.6 U/ml, interquartile range (IQR) 32.1–49.0 U/ml] than in the healthy volunteers [36.1 U/ml, interquartile range (IQR) 30.5–37.7 U/ml] (*Control* in Fig. 4a left). Interestingly, the median serum DK-β/γ level in early-stage cancer (Tis–T1 [tumor invades the submucosa])

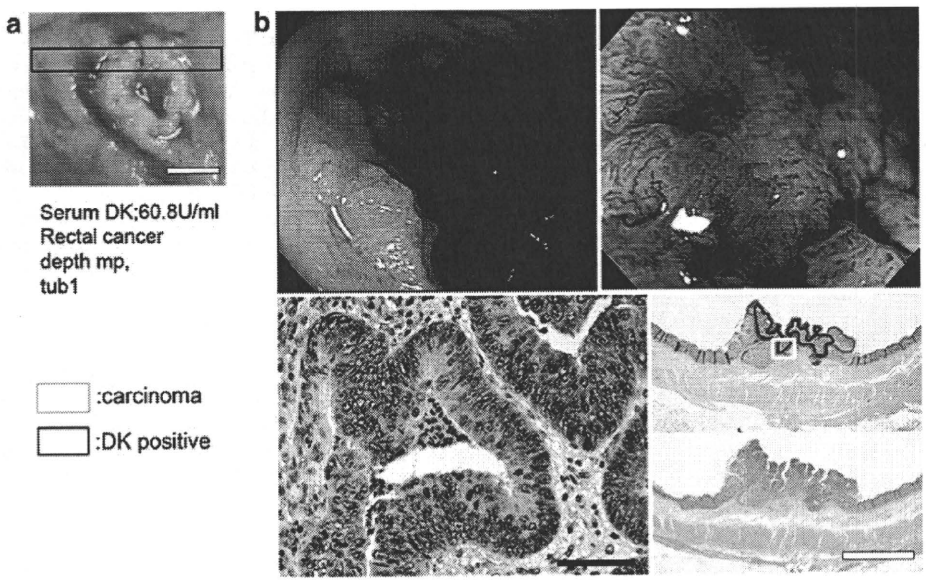
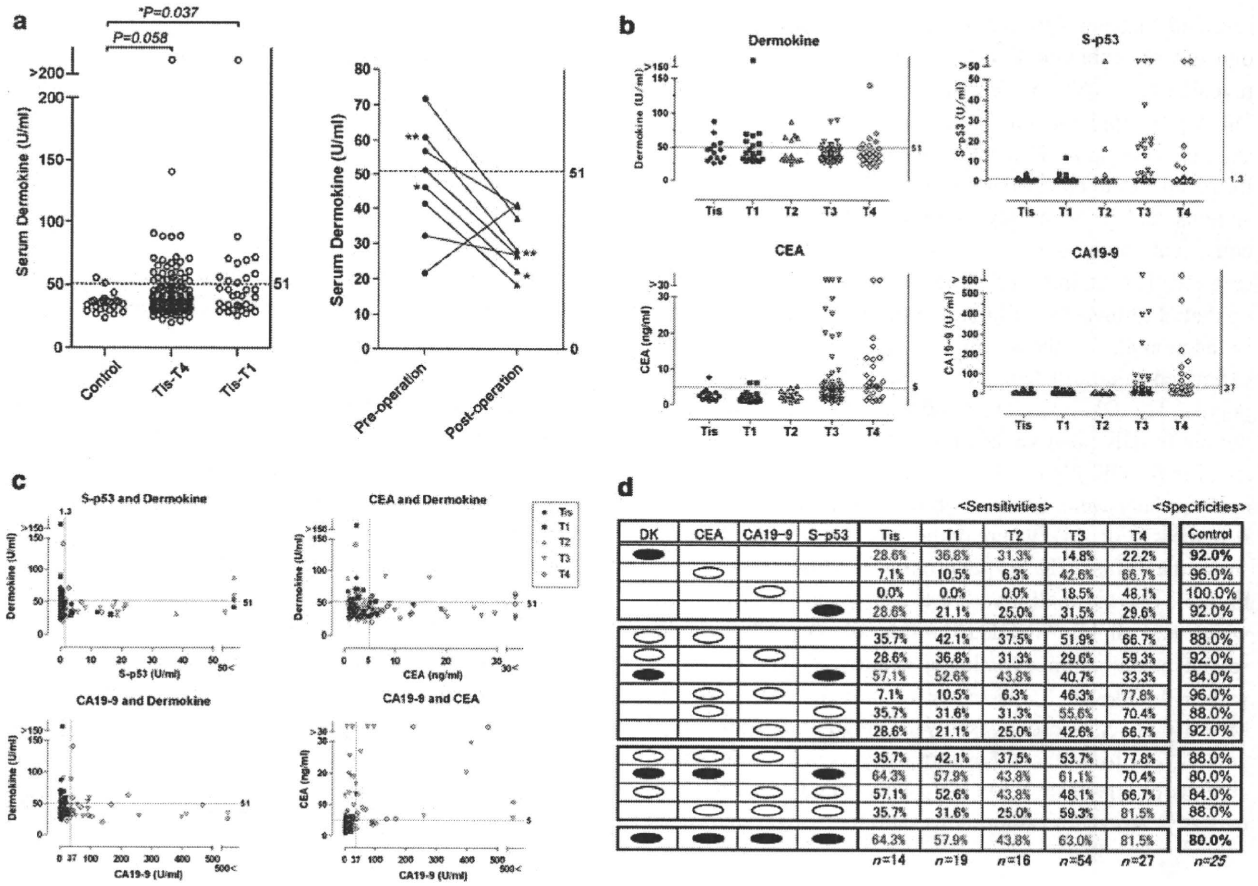


Fig. 3 Endoscopic features and immunohistochemical staining of cancer without adenoma with anti-DK mAb. **a** Macroscopic view of the rectal cancer. The size of the tumor was 18 × 12 mm. **b** Endoscopic examination revealed a shallow depressed lesion with a flat elevated area in the rectum (*left upper panel*). Magnifying colonoscopy showed irregular and disrupted vessels. The irregular and distorted crypts in the demarcated area, as observed in Kudo's V_I and partially V_N, suggested deep submucosal invasion in the depressed lesion (*right upper panel*). The DK-positive area (*right bottom panel, outlined in black*) was located in the deep region of carcinoma (*right bottom panel, outlined in red*). The staining pattern of DK was diffuse and disorganized in the cytoplasm. The staining of DK was distinctive in the region with high cellularity (*left bottom panel*). ×5 (**b** *right bottom panel*), ×200 (**b**); *white bar* 10 mm and *black bar* 50 μm. Microscopic picture (**b** *left bottom panel*) indicates the region of the *white square* in **b**, *right bottom panel* (color figure online)



DK	CEA	CA19-9	S-p53	Tis	T1	T2	T3	T4	Control
●				28.6%	36.8%	31.3%	14.8%	22.2%	92.0%
	○			7.1%	10.5%	6.3%	42.6%	66.7%	96.0%
		○		0.0%	0.0%	0.0%	18.5%	48.1%	100.0%
			●	28.6%	21.1%	25.0%	31.5%	29.6%	92.0%
○	○			35.7%	42.1%	37.5%	51.9%	66.7%	88.0%
○		○		28.6%	36.8%	31.3%	29.6%	59.3%	92.0%
●			●	57.1%	52.6%	43.8%	40.7%	33.3%	84.0%
	○	○		7.1%	10.5%	6.3%	46.3%	77.8%	96.0%
	○		○	35.7%	31.6%	31.3%	55.6%	70.4%	88.0%
		○	○	28.6%	21.1%	25.0%	42.6%	66.7%	92.0%
○	○	○		35.7%	42.1%	37.5%	53.7%	77.8%	88.0%
●	●		●	64.3%	57.9%	43.8%	61.1%	70.4%	80.0%
○		○	○	57.1%	52.6%	43.8%	48.1%	66.7%	84.0%
	○	○	○	35.7%	31.6%	25.0%	59.3%	81.5%	88.0%
●	●	●	●	64.3%	57.9%	43.8%	63.0%	81.5%	80.0%
				n=14	*n*=19	*n*=16	*n*=54	*n*=27	*n*=25

Fig. 4 DK enzyme-linked immunosorbent assay (ELISA). **a** Sera from 130 patients with colorectal cancer (Tis [cancer in situ, intraepithelial or invasion of lamina propria]; T1, tumor invades the submucosa; T2, tumor invades the muscularis propria; T3, tumor invades through the muscularis propria into the subserosa, or into the nonperitonealized pericolic or perirectal tissues; T4, tumor directly invades other organs or structures and/or perforates visceral peritoneum), and 25 randomly selected volunteers (*control*) were subjected to DK ELISA. The cutoff value was 51 U/ml. The serum DK level in early colorectal cancer (Tis–T1) was significantly higher than that in the control ($P = 0.037$). After surgical resection of primary carcinoma, serum DK decreased in seven of eight cases (**a** *right panel*). *Single asterisk*, the case shown in Fig. 2; *double asterisks*, the case shown in Fig. 3. **b** Comparison of the sensitivity of the diagnostic markers carcinoembryonic antigen (*CEA*), carbohydrate antigen 19-9 (*CA 19-9*), serum p53 antibody (*S-p53*), and DK according to depth of invasion. The cutoff values for each test are indicated by *dotted lines*. Early colorectal cancer (Tis–T1) is shown by *red symbols*. *Red circles*, Tis; *red squares*, T1; *white upward triangles*, T2; *white downward triangles*, T3; *white squares*, T4. DK was highly sensitive in Tis–T1, but less sensitive in T2–T4 tumors. In contrast, CEA, CA19-9, and S-p53 were sensitive in T2–T4 tumors. **c** Correlation of the sensitivity with that of other tumor markers shown in **b**. The cutoff values of each test are indicated by *dotted lines*. Serum DK was complementary to CEA, CA19-9, and S-p53, while serum CA19-9 was correlated with CEA. **d** Proposed multimarker analysis for the combination of CEA, CA19-9, S-p53, and DK. The serodiagnostic positive rates (%) of single, double, triple, and quadruple marker tests in Tis, T1, T2, T3, and T4. The highest positive rates of each multimarker test in each tumor are shown *in red text*. Tests for all four markers improved the diagnostic sensitivity for colorectal cancer to 43.8–81.5% for Tis–T1 tumors compared with the single, double, and triple marker tests. CEA is inadequate as a screening test for early-stage colorectal cancer, because its sensitivity is too low. In each multimarker test, the addition of the DK test improved the positive detection rate for early-stage tumors. The specificity of a single marker ranged from 92.0 to 100%, and that of the four-marker test was 80.0% (color figure online)

[41.5 U/ml, (IQR) 32.6–6.0 U/ml] was significantly higher than that in the *Controls* ($P = 0.037$), although there was no significant difference between levels in all colorectal cancer (Tis–T4; i.e., T2 [tumor invades the muscularis propria], T3 [tumor invades through the muscularis propria into the subserosa, or into the nonperitonealized pericolic or perirectal tissues], and T4 [tumor directly invades other organs or structures and/or perforates visceral peritoneum]) and levels in the *Controls* (Fig. 4a, left, $P = 0.058$). Generally, the diagnostic sensitivity of tumor markers increases as tumor invasion and metastasis proceed. The sensitivity of serum DK-β/γ was particularly high in Tis–T2 tumors. Relatively, the sensitivity of serum DK-β/γ in advanced cancer was lower than the sensitivities of CEA, CA19-9, and S-p53. Although there was no significant difference between Tis and T1–T4 ($P = 0.737$), or Tis–T1 and T2–T4

Table 1 Statistically significant differences between various combinations of serological tests with/without DK test

	Significance (*P*)							
	Tis ($n = 14$)	T1 ($n = 19$)	T2 ($n = 16$)	T3 ($n = 54$)	T4 ($n = 27$)	Tis–T1 ($n = 33$)	T2–T4 ($n = 97$)	Tis–T4 ($n = 130$)
DK/CEA vs. CEA	0.165	0.125	0.083	0.441	1.000	*0.008*	0.196	*0.017*
DK/CA19-9 vs. CA19-9	0.098	*0.008*	*0.043*	0.260	0.586	*P < 0.001*	*0.043*	*P < 0.001*
DK/S-p53 vs. S-p53	0.252	0.091	0.458	0.423	1.000	*0.023*	0.227	*0.020*
DK/CEA/CA19-9 vs. CEA/CA19-9	0.165	0.063	0.083	0.564	1.000	*0.008*	0.250	*0.025*
DK/CEA/S-p53 vs. CEA/S-p53	0.257	0.191	0.716	0.697	1.000	*0.048*	0.561	0.105
DK/CA19-9/S-p53 vs. CA19-9/S-p53	0.252	0.252	0.458	0.699	1.000	*0.023*	0.473	0.062
DK/CEA/CA19-9/S-p53 vs. CEA/CA19-9/S-p53	0.257	0.191	0.458	0.844	1.000	*0.048*	0.554	0.102

There were statistically significant differences between the results of single or variously combined serological tests using several tumor markers including DK. The DK test increased the sensitivities in various serological tests for Tis–T1 cases. Differences were considered significant when the two-tailed *P* value was <0.05

DK dermokine, *CEA* carcinoembryonic antigen, *CA* carbohydrate antigen, *S-p53* serum p53 antibody

Table 2 Comparison of clinicopathological features according to the serum concentrations of DK, CEA, CA19-9, and S-p53

	DK (U/ml)	CEA (ng/ml)	CA19-9 (U/ml)	S-p53 (U/ml)
Primary tumor				
Tis	43.8 (26.3–88.0)	2.6 (1.6–7.7)	3.6 (2.0–23.2)	0.5 (0.0–3.8)
T1	41.4 (29.5–460.7)	1.8 (0.9–6.2)	8.2 (2.0–26.7)	0.1 (0.0–12.0)
T2	35.0 (25.5–87.8)	2.6 (0.8–5.3)	7.2 (2.0–18.6)	0.4 (0.0–89.5)
T3	37.7 (26.5–90.5)	5.8 (0.5–212.8)	8.3 (2.0–1347.6)	0.3 (0.0–93.9)
T4	39.2 (20.4–140.7)	5.5 (1.1–2559.6)	36.7 (2.0–6446.8)	0.8 (0.0–93.3)
Lymph node metastasis				
None	38.7 (23.0–460.7)	2.8 (0.7–212.8)	7.8 (2.0–646.8)	0.4 (0.0–89.5)
Present	37.3 (20.4–90.5)	6.4 (0.5–2559.6)	10.4 (3.6–1347.6)	0.5 (0.0–93.9)
Distant metastasis				
None	37.9 (20.4–460.7)	3.3 (0.5–212.8)	8.2 (2.0–646.8)	0.3 (0.0–93.9)
Present	53.2 (31.7–88.3)	5.7 (3.5–2559.6)	26.6 (2.0–1347.6)	5.7 (0.6–93.3)

The serum concentration of DK was relatively high in Tis and T1 tumors, but it did not increase with tumor invasion, lymph node metastasis, and distant metastasis. Results were similar with S-p53, but CEA and CA19-9 increased dramatically in T3–T4 tumors. The serum concentrations of DK were not significant in regard to any T and N factors, but these concentrations were similar to those of S-p53 in our assay. The median DK value was slightly higher in cases of distant metastasis

Values are Median (range)

($P = 0.150$), the combination of DK and the other markers improved the sensitivities for detecting Tis–T1 tumor (Table 1). We compared the serum concentration of DK, CAE, CA19-9, and S-p53 in Tis–T4, with or without lymph node metastasis and distant metastasis. DK was relatively high in Tis-T1, but it did not increase with tumor invasion and lymph node metastasis. The results were similar to those of S-p53, but the serum concentrations of CEA and CA19-9 increased dramatically in T3-T4 tumors, lymph node metastasis, and distant metastasis. In the cases of distant metastasis, all markers including DK tended to be high (Table 2). Accordingly, our data indicate that DK is a unique and novel biomarker for early colorectal cancer. We confirmed that the serum DK level was decreased in seven of eight patients after surgical resection (Fig. 4a, right).

The relationship between clinicopathological features and multimarker test in colorectal cancer patients

We analyzed the clinicopathological features and serum DK concentrations of 130 patients, and we found that, in the noninvasive Tis–T1 group (Tis, 28.6%, $n = 4/14$; T1, 36.8%, $n = 7/19$; Tis–T1, 33.3%, $n = 11/33$), the serum DK-β/γ-positive rate was significantly higher than the positivity rates of CEA, CA19-9, and S-p53. These results suggest that the DK ELISA is more effective than the other ELISAs for the detection of early colorectal cancer (Table 2 and Supplementary Table 1).

To examine the correlation of DK with the other serum tumor markers, we analyzed the specificity and sensitivity of CEA, S-p53, and CA19-9. In most cases, DK-β/γ was

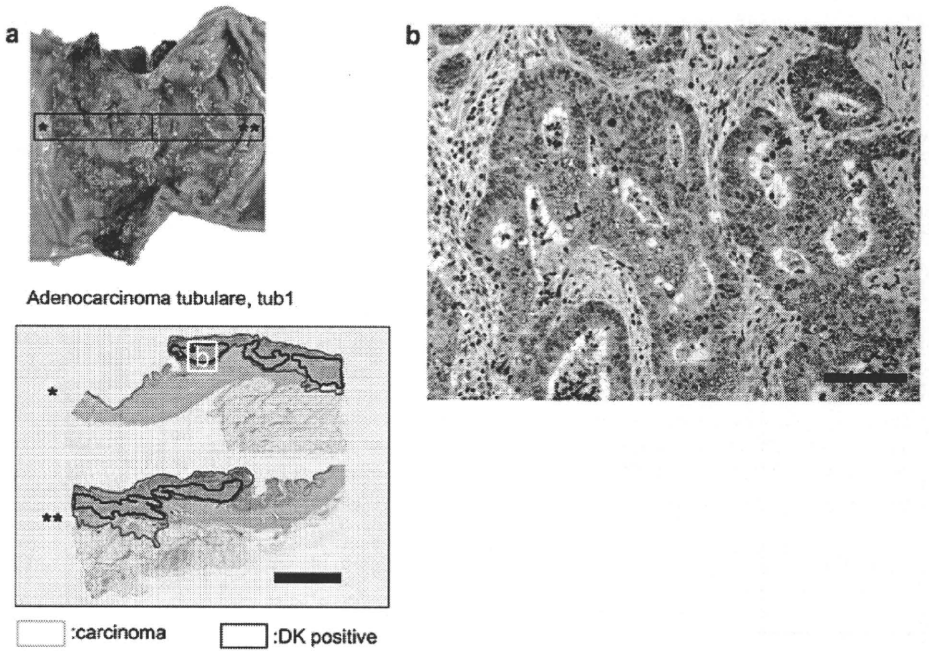


Fig. 5 Immunohistochemical staining of advanced cancer with anti-DK mAb. **a** Macroscopic view of advanced cancer. The DK-positive area (*outlined in black*) and cancer lesions (T3, *outlined in red*) are shown, and the DK-positive area was located in the deep region of carcinoma (*bottom panel*). *White square* (*b*) indicated microscopic picture in (**b**). **b** DK was mainly located diffusely in the cancer (*brown color*). ×5 (**a** *bottom panel*), ×200 (**b**); *bar* 10 mm (**a** *bottom panel*), 50 μm (**b**) (color figure online)

found to be a unique marker for the detection of early-stage cancer (Fig. 4c, d). Meanwhile, serum CEA-, CA19-9-, and S-p53-positive cases often overlapped in advanced cancer (Fig. 4c). These findings suggest that a multimarker assay that included DK-β/γ would improve the serodiagnostic sensitivity for early colorectal cancer. Consequently, the combination of the four tumor markers DK, CEA, CA19-9, and S-p53 correctly diagnosed 63.8% ($n = 83/130$) of the colorectal cancer patients. In Tis–T1 cancer, the diagnostic rate was improved to 57.9–64.3% using the three markers DK, CEA, and S-p53, compared with 7.1–10.5% when using the CEA test alone (Fig. 4d). Each marker had a different sensitivity with reference to tumor invasion.

Discussion

Stratified epithelium protects against various cytotoxic stresses in areas such as the skin, esophagus, and vagina. Glandular epithelium essentially has the function of secretion and absorption, and it differentiates to various types of cells with specific functions. The specialized functions of glandular epithelium include the maintaining of homeostasis. Chemical and mechanical cytotoxic stresses induce carcinoma in stratified epithelium, with ultraviolet light and smoking being well known as risk factors in squamous cell carcinoma. Although the occurrence of adenocarcinoma has not yet been sufficiently clarified, a few key genetic lesions are common to a large proportion of colorectal cancers. Adenomatous polyps are believed to be the precursors of a large proportion of colorectal cancers. However, as in the small invasive cancer shown in Fig. 3, we found that most invasive cancers did not always involve adenoma in part of the tumor. These findings suggest that there are certainly other different and unknown sequences of carcinogenesis. In spite of the high expression of DK-β/γ in stratified epithelium (esophagus), normal colon epithelium hardly expressed DK-β/γ (Figs. 1d, 2e). The data from our present experimental study suggested that some adenocarcinomas, especially Tis–T1 tumors, expressed DK-β/γ aberrantly. Although the molecular functions of DK-β/γ are still unknown, DK-β/γ has the feature of a secreted form, which can be detected in the serum and cell culture medium (Fig. 1c, right panel). Consequently, DK-β/γ may be a potential biomarker for a new serum test of colorectal cancer, though its molecular functions are still unclear. .

Although the genetic and epigenetic alterations that occur during the course of multistage colorectal carcinogenesis have been extensively studied, early-stage diagnosis by serum markers remains difficult [12–14]. For early-stage diagnosis, it is essential that cancer-secreting molecules can be detected in the serum, but no such candidates have been established yet. Our immunohistochemical data showed that DK-β/γ expression was found in some regions in early colorectal cancer (Fig. 2). On the other hand, small invasive carcinoma also expressed DK-β/γ (Fig. 3). We also showed that the serum concentration of DK-β/γ was relatively high (33.3%, $n = 11/33$) in early colorectal cancer patients. Thus, we speculate that the transient expression of DK-β/γ was an early event in carcinogenesis. In our data, the sensitivity of DK in advanced

cancer (T2–T4) was not so high, even though some cases of advanced cancer expressed DK. Invasive cancer showed immunohistochemical staining with anti-DK mAb (see Fig. 5). We have not yet found the molecular function of DK, and we do not know why adenocarcinoma expressed DK in the present study. We would like to carry out further study to clarify the molecular function of DK in advanced cancer and cancer cell lines in a future study.

In addition to our finding of the high positivity for DK in early-stage colorectal cancer, DK-β/γ was complementary to CEA/CA19-9/S-p53 in the sera of cancer patients. This suggested that the mechanism of the high expression of DK was different from the the mechanisms of CEA/CA19-9/S-p53 expression.

CEA is a glycoprotein that was originally found in 1965 in an extract from colon cancer tissue [15]. CEA is widely used as a tumor marker to detect adenocarcinomas, including colorectal cancer, and the American Society of Clinical Oncology (ASCO) 2006 recommendation for colorectal cancer is that the CEA test is beneficial for preoperative surgical staging and surgical treatment planning, and should also be performed every 3 months for at least 3 years for stage II and III postoperative patients [16]. CEA is produced in colorectal epithelium and is secreted into the lumen of the gut in adults. Therefore, it is normal for the serum CEA concentration to be relatively low and it has high specificity to detect colorectal carcinoma. However, ulcerative colitis, pancreatic diseases, liver cirrhosis, and smoking sometimes elevate the CEA concentration in the absence of a tumor. Furthermore, it is not an appropriate tumor marker for early diagnosis, because its sensitivity is not high enough. We found that the serum examinations of CEA and DK-β/γ can complement each other, and that the DK test can overcome the low sensitivity of CEA in early colorectal cancer (7.1–10.5%), when the DK and CEA tests are used in combination.

CA19-9 is glycoprotein modified by a sugar chain, and was originally found in 1981 in the sera of colorectal cancer and pancreatic cancer patients [17]. In our data the serum concentration of CA19-9 was elevated in T3–T4 invasive cancer (18.5–48.1%), although its sensitivity was very low for Tis–T1 (0%). Although a review of the literature published between 1999 and 2009 did not support the role of CA19-9 in the management of colorectal cancer, as for CEA, some CA19-9-positive sera were not positive for other markers, resulting in a slight increase in the overall positivity of the four-marker test.

The p53 protein has various biological activities to protect cells from genetic abnormalities. It regulates the cell cycle, and is involved in the activation of genetic repair enzymes and apoptosis [18–20]. Mutation of the p53 protein usually occurs in the core domain, and such mutations extend the half-life of the p53 protein and induce its abnormal accumulation in the cell. Crawford et al. [21] found autoantibodies to p53 protein in the sera of cancer patients. The sensitivity of S-p53 for Tis–T4 tumors ranged from 21.1 to 31.5% in our study. And the S-p53 test was complementary to not only the DK test but also to CEA/CA19-9, suggesting that the S-p53 test has additive effects to detect early colorectal cancer (Fig. 4d).

As shown in Fig. 4b–d, the diagnostic sensitivity of commercially available tumor markers (CEA, CA19-9, and S-p53) improves with tumor progression and invasion. It has been suggested that the increasing serum concentrations of these molecules result from the accumulation of irreversible genetic mutations and tissue disorganization. Mutations are most commonly observed in genes such as the protooncogene *K-Ras*, and the tumor suppressor genes *p53* and *Apc* in colorectal cancer developed from adenoma. Mutations in other genes have been observed in some colorectal cancers, and others still remain to be identified. Mutations in the *p53* gene allow many cancer cells to survive and proliferate despite DNA damage. Therefore, loss of *p53* function allows cancer cells to accumulate additional mutations and to avoid apoptosis and cell-cycle arrest. In our data, serum DK-β/γ and S-p53 were complementary markers in Tis–T1 tumors. Thus, it is possible that the aberrant expression of DK-β/γ may take part in different sequences of mutations, if it is directly concerned with carcinogenesis.

Although the highly sensitive multimarker test we have described here has low specificity, the serum-DK and S-p53 tests are new biomarkers. These two markers are useful to detect patients with early-stage colorectal cancer in opportunistic screening programs. At present, the four-marker test (CEA/CA19-9/S-p53/DK) would be insufficient for organized screening, because, according to our data, its specificity for colorectal cancer was limited to 80.0%. We speculate that the combination of other new candidates as serologic biomarkers may enable organized screening in the future.

In conclusion, a serum-based multimarker test, which includes DK-β/γ, may have promise for the serodiagnostic screening of early-stage colorectal cancer. Further analysis of the molecular function and mode of expression of DK isoforms might provide insight into the transcriptional changes involved in carcinogenesis.

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Anti-HuC and -HuD autoantibodies are differential sero-diagnostic markers for small cell carcinoma from large cell neuroendocrine carcinoma of the lung

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Abstract. Aiming to identify novel sero-diagnostic markers for neuroendocrine carcinomas of the lung, the two-dimensional gel electrophoresis-immunoblot method was used to analyze tumor-associated autoantibodies in patients with small cell lung carcinoma (SCLC) and large cell neuroendocrine carcinoma (LCNEC). Several autoantigens were revealed and anti-HuC autoantibody was detected only in sera of SCLC patients. Since Hu family proteins including HuC are well-known causes of paraneoplastic encephalomyelitis/sensory neuronopathy (PEM/SN), the expression of HuC as well as HuD mRNAs and their proteins was studied in 11 lung cancer cell lines. The expression of HuC and HuD mRNAs and proteins was only detected in SCLC- and LCNEC-derived cells. To validate the existence of anti-HuC and -HuD autoantibodies, we studied a large number of sera including those from lung cancer patients employing dot blot analysis. Anti-HuC and -HuD autoantibodies were detected only in SCLC cases with or without PEM/SN, and not in the sera of LCNEC patients. The mechanism leading to different anti-HuC and -HuD autoantibody production between SCLC and LCNEC is unclear; however, the results from the present and previous studies suggest that anti-HuC and -HuD autoantibodies are novel differential sero-diagnostic markers for SCLC from LCNEC.

Introduction

Lung cancer is the leading cause of cancer-related death worldwide. Based on the presence or absence of cellular neuroendocrine differentiation, lung cancer can be grouped into non-neuroendocrine or neuroendocrine tumors. The former is roughly equal to the non-small cell lung cancer (NSCLC) largely comprising squamous cell carcinoma (SCC) and adenocarcinoma (AD). The latter ranges from low-grade typical carcinoid (TC), intermediate-grade atypical carcinoid (AC), to high-grade small cell lung carcinoma (SCLC) and large cell neuroendocrine carcinoma (LCNEC) (1,2), and the 5-year survival rates for TC, AC, LCNEC, and SCLC were 87, 56, 27, and 9%, respectively (2). Similar results were also reported by Garcia-Yuste *et al*, and the 5-year survival rates for TC, AC, LCENC, and SCLC were 96, 72, 21, and 14%, respectively (3). These results show that both SCLC and LCNEC are highly malignant and have a similar poor prognosis. Pro-gastrin-releasing peptide (pro-GRP) is a well-known sero-diagnostic marker for SCLC; however, its positive rate is lower in stage I and II (35-45%) than in stage III (55-70%) and IV (70-80%). At present, no specific sero-diagnostic markers for distinguishing SCLC from LCNEC have been reported.

Autoantibodies are antibodies detected in the sera of patients with various autoimmune diseases. They are also frequently observed in the sera of patients with various malignancies even in the early stages, and, thus, the possibilities for them to be used as potential tumor markers have been suggested (4-10). Hanash (11) has described that harnessing the immune response to identify novel cancer biomarkers is an attractive strategy, because the immune system performs biological amplification which is equivalent to a PCR reaction by generating a detectable signal, with antigenic tumor proteins as templates, beginning at an early stage during tumor development when the tumor may be otherwise undetectable. Many tumor-related autoantibodies have been reported in pulmonary carcinomas (12-15). Thus, an exhaustive search for novel tumor-specific autoantibodies, which may serve as early sero-diagnostic markers for cancers, has commenced.

In this study, we detected tumor-associated autoantibodies by immunoblotting based on two-dimensional gel electrophoresis

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(2-DE) from the sera of patients with pulmonary neuroendocrine carcinomas. Identified antigens were further assessed to confirm their specific expressions in neuroendocrine carcinomas by RT-PCR and immunoblotting. Finally, the usefulness of the autoantigens was validated using the sera of patients with various types of pulmonary carcinoma, along with non-cancerous and healthy controls.

Materials and methods

Cell lines. Six SCLC (N230, N231, H69, H82, Lu130, and N417), two LCNEC (LCN1 and LCN2) (16), two AD (A549 and LC-2/ad), and one SCC (RERF LC-AI) cell line were used in this study. All cell lines were grown in RPMI-1640 (Sigma, Steinheim, Germany) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Auckland, NZ). After being washed with phosphate-buffered saline without bivalent ions (PBS-), harvested cells were separated into two gropus, one was fixed in 10% formalin and embedded in paraffin, and the other was stored at -80˚C until use for protein and total RNA extraction.

Sera. Sera from 80 pulmonary carcinoma patients (SCLC: 31, LCNEC: 7, AD: 21, and SCC: 21) and 21 non-neoplastic lung disease (interstitial pneumonia: 7, tuberculosis: 5, non-lung cancer diseases: 3, acute inflammation: 1, epithelioid granuloma: 1, cryptogenic organizing pneumonia: 1, nontuberculous mycobacteria: 1, aspergillosis: 1, inflammatory granuloma: i) patients treated at Kitasato University Hospital. Sera from 26 healthy volunteers were also used as a normal control. All sera were kept at -80˚C until use. The SCLCs was further divided into 15 limited and 12 extensive disease cases. This study was approved by the Ethics Committee of Kitasato University School of Medicine. All patients were informed of the aim of the study and gave consent to use their samples.

2D-immunoblotting (IB). Sample preparation and the two-dimensional gel electrophoresis (2-DE) used in this study were described in our previous study (17).

Proteins extracted from the mixture of two LCNECs (LCN1 and LCN2) or the mixture of three SCLCs (N231, H69, and Lu130) were separated by 2-DE. Two pieces of gel were prepared for each sample, one was transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp,. Bedford, MA, USA) for immunoblotting and the other was visualized by coomassie brilliant blue R-350 (CBB) staining (PhastGel Blue R, GE Healthcare, Uppsala, Sweden).

Blotting membranes were blocked with 0.05% casein/TBS (0.01 mol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl) for 30 min at room temperature (RT). Membranes prepared with LCNEC proteins were reacted with 100-times diluted pooled sera of five LCNEC patients and the membranes with prepared SCLC proteins were reacted with 100-times diluted pooled sera of five SCLC patients, respectively, for 1 h at RT. The dilution buffer was 0.0025% casein/TBS-T (TBS containing 0.1% Tween-20). Then, the membranes were incubated with 1,000-times diluted horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG polyclonal antibody (Dako, Glostrup, Denmark) for 30 min at RT. Finally, signals were developed by stable DAB solution (Invitrogen, Carlsbad, CA, USA).

Protein identification. In brief, protein spots that reacted with patients, sera were excised from 2-DE gels and destained with 50% (v/v) acetonitrile (ACN)/50 mM NH_4HCO_3, dehydrated with 100% (v/v) ACN, and then dried under vacuum conditions. Tryptic digestion was performed for 24 h at 37˚C in a minimum volume of digestion solution containing 0.5 ng/μl sequencing grade modified trypsin (Promega Corp., Madison, WI, USA) and 25 mM Tris-HCl buffer (pH 9.0). After incubation, digested protein fragments eluted in solutions were collected, and gels were washed once in 50% (v/v) ACN/5% trifluoroacetic acid (TFA) and collected in the same tube. Solutions containing digested protein fragments were measured by MALDI-TOF/TOF MS (autoflex-III; Bruker Daltonik GmbH, Bremen, Germany).

Fragment ion spectra from MS and MS/MS were submitted to MASCOT (http://www.matrixscience.com/) for a database search and the identification of corresponding proteins employing the following database: IPI human 20091026 (86379 sequences; 34740790 residues, http://www.ebi.ac.uk/IPI/IPIhuman.http://www.ebi.ac.uk/IPI/IPIhuman.html/).

RT-PCR. Total RNAs from the above-mentioned 11 lung cancer cell lines were extracted with Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Primers were designed with Oligo Primer Analysis Software, version 6.0 (Takara Bio Inc, Otsu, Japan) according to HuC and HuD mRNA sequences (18,19). HuC forward primer: 5'-TGCAAGTTGGTTCGGGACAAG-3' (582-602); reverse primer: 5'-GGCGGATGACTGGTAGAGG-3' (1031-1049). HuD forward primer: 5'-GTCTCTTCGGGAGCATTGGT-3' (415-434); reverse primer: 5'-CCTCTTATCAAAGCGGAT GAA-3' (753-773). PCR was performed with pretreatment at 94˚C for 2 min and 30 cycles of denaturation at 94˚C for 30 sec, annealing at 60˚C for 30 sec, and extension at 72˚C for 30 sec. Beta-2-microglobulin was used as an internal control. PCR products were electrophoresed on 3% agarose gel and stained with ethidium bromide.

1D-immunoblotting. Proteins were extracted from lung cancer cell lines with detergent lysis buffer (20) using an ultra-sonic homogenizer (UH-50; SMT Co., Tokyo, Japan). Each extracted protein (10 μg) was boiled and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a constant current at 15 mA. The immuno-blotting methods were generally the same to those used for 2D-IB with some modifications. Transferred membranes were blocked with 0.5% casein/TBS for 30 min at RT, followed by reaction with 200-times diluted HuC- and HuD-positive serum as anti-Hu protein antibody with dilution buffer for 1 h at RT, because purchased anti-HuC antibody did not show specific reactivity. Then, the membranes were incubated with 1,000-times diluted HRP-conjugated rabbit anti-human IgG polyclonal antibody (Dako) for 30 min at RT. Finally, signals were developed using Immobilon Western reagent (Millipore Corp.).

Micro-dot blot array. Anti-HuC and -HuD autoantibodies in sera were detected employing the automatic dot blot system, and the micro-dot blot array with a 256-solid pin configuration (Kakengeneqs Co., Ltd., Chiba, Japan) was used. In brief, 1 μl

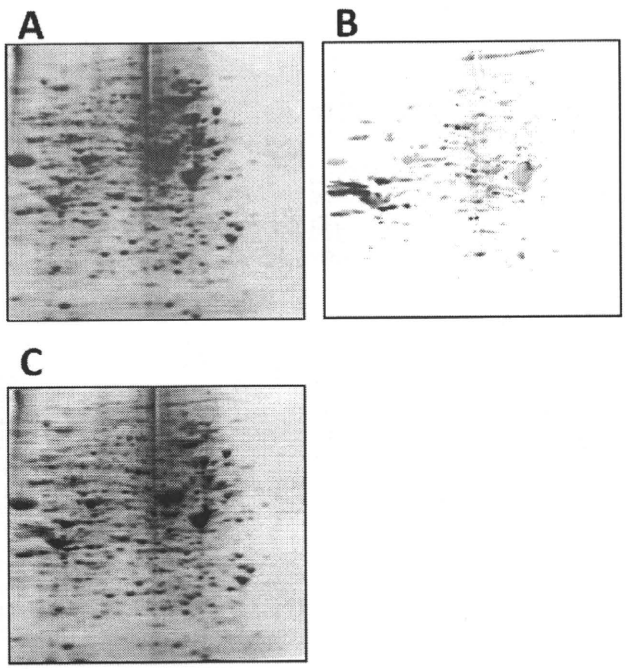


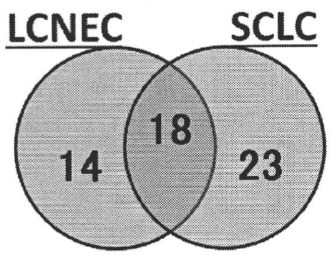
Figure 1. Detection of autoantibodies by 2D-immunoblotting. (A) Protein lysates from SCLC cell lines were separated by 2-DE and stained with CBB. (B) Immunoblot analysis was performed with mixed sera from patients with SCLC as primary antibodies, visualized with DAB solution. (C) A and B were merged.

each of proteins was spotted onto PVDF membranes, which were prepared by a wheat germ cell-free system (21). Then the membranes were blocked with 20% N101 (NOF Corp., Tokyo, Japan)/TBS for 1 h at RT. After being washed in TBS, the membranes were reacted with 100-times diluted sera with 1% N101/TBS for 30 min at RT. After TBS-T washing, the membranes were incubated with 1,000-times diluted HRP-conjugated rabbit anti-human IgG polyclonal antibody for 30 min at RT. Finally, signals were developed with Immobilon Western reagent. The data were analyzed using DotBlotChip-System software ver. 4.0 (Dynacom Co., Ltd., Chiba, Japan). Normalized signals are presented as the positive intensity minus background intensity around the spot. Statistical analysis was performed using the Mann-Whitney U test. The area under the curve (AUC) and best cut-off point were calculated employing receiver operating characteristic (ROC) analysis.

Results

Autoantigen identified by 2D-IB. The immunoreactivity of autoantibodies in sera was assessed by 2D-IB, and the representative positive protein spots on the membrane are shown in Fig. 1. Sixty-two and 63 positive spots were detected with sera from LCNEC and SCLC patients, respectively. In total, 32 proteins for LCNEC and 41 proteins for SCLC were identified as autoantigens. The identified proteins are summarized in Fig. 2. Twenty-three proteins including HuC

A



Classification	Spots	Antigens
LCNEC	62	32
SCLC	63	41

B

Symbol	Protein	MW	IPI	Molecular Function
ATP5A1	ATP synthase subunit alpha, mitochondrial	59714	IPI00440493	Transporter activity
DRG1	Developmentally regulated GTP-binding protein1	40517	IPI00031838	Unknown
EIF3I	Eukaryotic translation initiation factor 3 subunit I	36479	IPI00012795	Translation regulator activity
EIF4A1	Eukaryotic initiation factor 4A-I	46125	IPI00025491	Translation regulator activity
EIF4A2	Isoform 1 of eukaryotic initiation factor 4A-II	46373	IPI00328328	Translation regulator activity
EZR	Ezrin	69370	IPI00843975	Cytoskeletal anchoring activity
ELAVL3	Hu antigen C (HuC)	39547	IPI00031552	RNA binding
FKBP4	FK506-binding protein 4	51772	IPI00218005	Isomerase activity
HNRNPK	Isoform 1 of heterogeneous nuclear ribonucleoprotein K	50944	IPI00216049	Ribonucleoprotein
FSCN1	Fascin	54496	IPI00163187	Structural molecule activity
HNRPA3	Isoform 1 of heterogeneous nuclear ribonucleoprotein A3	39571	IPI00419373	RNA binding
HPRT1	Hypoxanthine-guanine phosphoribosyltransferase	24564	IPI00218493	Enzyme: Ribosyltransferase
HSP90AA1	Isoform 2 of heat shock protein HSP 90-alpha	98099	IPI00382470	Chaperone activity
HSPA1A	Heat shock 70 kDa protein 1	70009	IPI00304925	Chaperone activity
HSPA8	Isoform 1 of heat shock cognate 71 kDa protein	70854	IPI00003865	Heat shock protein activity
KRT8	Keratin, type II cytoskeletal 8	53671	IPI00554648	Structural molecule activity
HSPD1	60 kDa heat shock protein, mitochondrial	61016	IPI00784154	Heat shock protein activity
MDH2	Malate dehydrogenase, mitochondrial	35481	IPI00291006	Catalytic activity
PHB	Putative uncharacterized protein PHB	22723	IPI00793442	Receptor signaling complex scaffold activity
MSN	Putative uncharacterized protein MSN (Fragment)	67645	IPI00872814	Structural constituent of cytoskeleton
RPSAP15	RPSA 33 kDa protein (ribosomal protein SA)	33293	IPI00413108	Cell adhesion molecule activity
RUVBL2	RuvB-like 2	51125	IPI00009104	Transcription regulator activity
VTA1	Vacuolar protein sorting-associated protein VTA1(C6orf55)	33858	IPI00017180	Unknown

Figure 2. Identified autoantigens in LCNEC and SCLC patients. (A) Compared autoantigens identified in sera from patients with LCNEC or SCLC. Thirty-two and 41 autoantigens were identified from LCNEC and SCLC, respectively. The number of approved autoantigens identified both in LCNEC and SCLC was 18. (B) Twenty-three autoantigens including ELAVL3 (HuC) were identified only in SCLC patients.

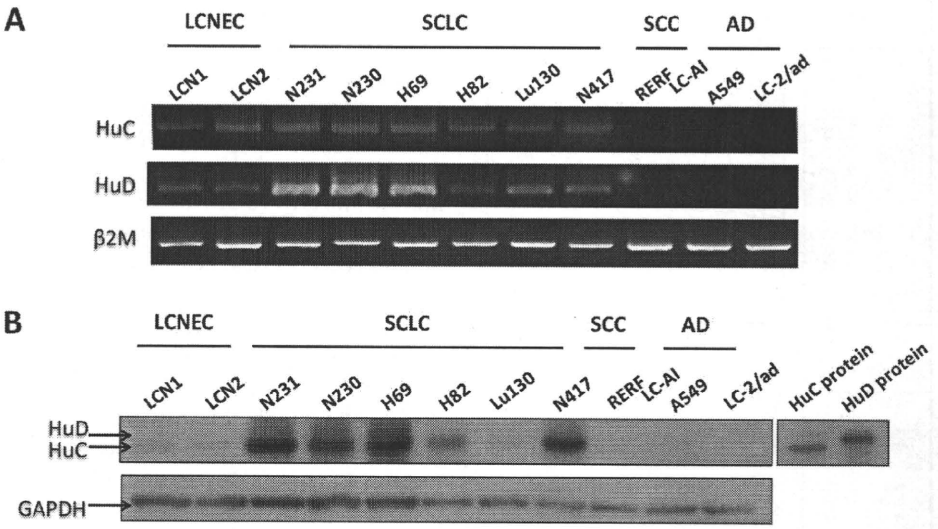


Figure 3. HuC and HuD expressions in lung cancer cell lines. (A) HuC and HuD mRNAs were detected by RT-PCR. Both mRNAs were expressed only in LCNEC and SCLC cell lines. The internal control was beta-2-microglobulin (β2M). (B) HuC protein levels were detected by immunoblot analysis. HuC and HuD recombinant proteins were used as a positive control and GAPDH was used as an internal control. HuC protein was also expressed only in the majority of SCLC and LCNEC cell lines, and HuD protein was expressed only in a part of SCLC cell lines.

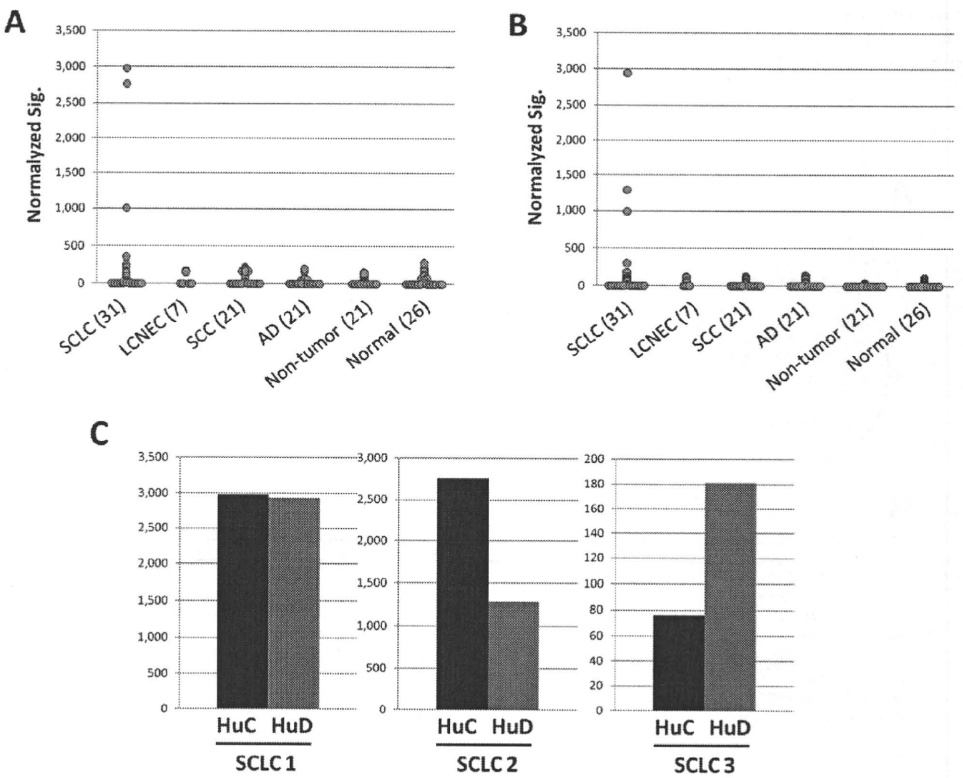


Figure 4. Anti-HuC and -HuD autoantibody levels in sera by dot blot analysis. Anti-HuC autoantibody was detected in 4/31 SCLC patients and not in the others (A). Anti-HuD autoantibody was also detected only in 6/31 SCLC patients (B). The quantitative ratio of anti-HuC and -HuD autoantibodies varied from case to case (C).

were detected only in SCLC, 14 only in LCNEC, and 18 were observed in both.

HuC and HuD expressions in lung cancer cell lines. To examine whether HuC and HuD are specifically expressed in neuroendocrine tumors of the lung, we performed RT-PCR using 11 lung cancer cell lines (Fig. 3A). The expressions of HuC and HuD mRNAs were detected in all neuroendocrine carcinoma-derived cell lines, but not in SCC or AD cell lines.

To confirm that HuC and HuD proteins were also specifically expressed in neuroendocrine tumors, we performed immunoblot analysis using the same 11 lung cancer cell lines (Fig. 3B).

Both proteins were also detected only in neuroendocrine carcinoma-derived cell lines. HuC protein was detected in 5 of 6 SCLC and the two LCNEC cell lines. HuD protein was also detected in 4 of 6 SCLC, but not in the two LCNEC cell lines. These results were generally in accordance with those of mRNA expression analyses by RT-PCR.

Validation of anti-HuC and -HuD autoantibodies. To confirm the utility of anti-HuC and -HuD autoantibodies as potential biomarkers in neuroendocrine carcinoma of the lung, we investigated their levels in patient and control sera by dot blot analysis. Anti-HuC autoantibody was detected in 4 of 31 SCLCs (Fig. 4A), but not in other lung cancer subtypes including LCNEC, non-neoplastic lesions, or healthy controls ($p=0.003$). At a cut-off point of 360, the sensitivity for SCLC was 12.9% (95% CI: 0.036-0.298). Anti-HuD autoantibody was also detected in 6 of 31 SCLCs (Fig. 4B), but not in the others ($p<0.001$). At a cut-off of 176, the sensitivity for SCLC was 19.6% (95% CI: 0.075-0.375). When compared with the others, both anti-HuC and -HuD autoantibodies showed 100% specificity for SCLC. The AUC-ROC levels were 0.577 and 0.602, respectively.

Anti-HuC and -HuD autoantibodies are detected in SCLC patients with and without PEM/SN. In this study, these autoantibodies were detected in patients without rather than with PEM/SN. Furthermore, the quantitative ratio of anti-HuC and -HuD autoantibodies varied from case to case (Fig. 4C).

Discussion

In this study, 55 autoantigens in total were identified employing 2D-immunoblotting. Our results confirmed the utility of this approach to identify tumor-associated antigens including HuC recognized by autoantibodies in sera from patients with lung cancer (12,13).

Hu proteins are a family consisting of four RNA-binding proteins, three of which are normally expressed in the nervous system (22). All four members have three RNA-interacting domains known as RRM (RNA recognition motif) (23). PEM/SN, which occurs in less than 1% of SCLC patients, is related to high titers of autoantibodies for neuronal Hu proteins (24-26). The mechanism by which the immune system identifies Hu proteins from tumor cells as foreign proteins and generates anti-Hu autoantibodies is still unknown. A few studies have focused on the genetic causes of ectopic neuronal Hu gene (ELAV) expression in neuroendocrine tumors and their roles in the onset and progression of such tumors (27,28). Dalmaou *et al* (29) and Graus *et al* (30) reported that approximately 16% of SCLC patients without PEM/SN have detectable levels of anti-Hu autoantibodies in their sera. In this study, both SCLC patients with and without PEM/SN were detected in 12.9% for anti-HuC and 19.4% for -HuD autoantibodies in their sera. This positive rate was almost the same as those reported previously. In a mouse model study, Kazarian *et al* found that anti-Hu reactivity appeared to arise prior to chemical evidence of cancer in these mice, suggesting the possibility of using anti-Hu for the early detection of SCLC (31). The present results on anti-HuC and -HuD autoantibodies supported this possibility, because these autoantibodies were found from limited to extensive diseases. Although the follow-up period was short and only a few controls were used, Tsou *et al* reported that SCLC patients with high levels of anti-Hu reactivity survived for longer than those with low levels ($p=0.08$) (32). In agreement with the study by Tsou *et al* and the present study, Verschuuren *et al* also reported that anti-Hu-positive SCLC cases survived longer, and Dalmau *et al* reported that anti-Hu-positive SCLC patients have relatively limited disease (29,33). Larger scale studies are need to classify the reason for the favorable prognosis of SCLC patients with anti-Hu autoantibodies.

In this study, anti-HuC and -HuD autoantibodies were detected only in SCLC patients, and not in those with other lung cancers, non-neoplastic disease, or healthy controls. Although the quantitative ratio of anti-HuC and -HuD antibodies varied from case to case, 23.3% of SCLC patients were positive for either anti-HuC or anti-HuD or both antibodies. The positive rate may rise using a more sensitive methodology. In spite of this, we detected HuC and HuD mRNAs and proteins in LCNECs, and failed to detect anti-HuC and -HuD autoantibodies in the sera of LCNEC patients, who share many biological features with SCLC patients. Although only a small number of cases were analyzed in a previous study, somatic mutations of the HuD gene were detected in a part of SCLC, TC, and AC cases of different neuroendocrine lung tumors including LCNEC (34). Thus, genetic mutations of HuC and HuD may contribute to the production of autoantibodies.

The present results suggest that anti-Hu autoantibodies are differently expressed between SCLC and LCNEC, and they may be used as novel sero-diagnostic and differential markers for these two tumor types.

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