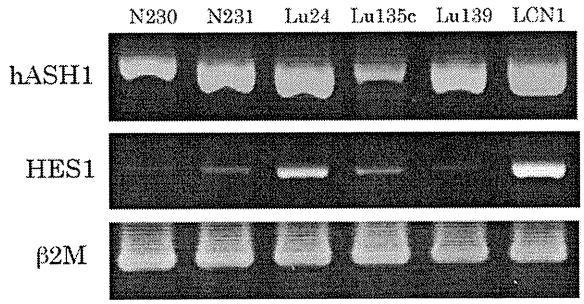

Fig. 1. Expression of (A) hASH1, (B) HES1, and (C) β2M mRNA in pulmonary neuroendocrine carcinoma cells five small cell lung carcinoma cell lines (N230, N231, Lu24, Lu135c, and Lu139) and a large cell neuroendocrine carcinoma cell line (LCN1). M, 100 bp ladder marker.

Hybridization with a β_2M antisense strand probe was used as an internal control to confirm the preservation of mRNA. Hybridization with the hASH1 and HES1 sense strand probes was also used as a negative control.

2.6. Evaluation of ISH

The evaluations of hASH1 and HES1 mRNA expression were performed by multiplying the percentage of positive tumor cells by the staining intensity. The percentage of positive tumor cells was scored as 0 (0%), 1+ (1–25%), 2+ (26–50%), 3+ (51–75%), or 4+ (76–100%). Staining intensity was also scored as 0 (negative), 1+ (weakly positive), 2+ (moderately positive), or 3+ (strongly positive). The Mann–Whitney *U*-test was used to statistically evaluate the ISH data. Statistical significance was assumed when $p < 0.05$.

3. Results

3.1. Detection of hASH1 and HES1 mRNA in cell lines using RT-PCR

The expression of both hASH1 and HES1, which produced bands of about 500 bp and 300 bp, respectively, was revealed in all fifteen cell lines. Representative RT-PCR results for six cell lines are shown in Fig. 1. Strong hASH1 expression signals were found in LCN1, N231, Lu24, and Lu139 cells, but its expression was weak in N230 and Lu135c cells. The LCN1 cells showed the strongest HES1 expression, followed by the Lu24 cells, and faint signals were observed in the other cell lines. As shown in the figure, the expression levels of β_2M, which was used as an internal control, were approximately the same in all cell lines. Both PCR product sequences completely corresponded to the established cDNA sequences of hASH1 [19] and HES1 [20], as confirmed using a cycle sequencing method.

Table 2
Expression of hASH1 and HES1 mRNA in pulmonary neuroendocrine carcinoma cells according to ISH.

	n	hASH1		HES1	
		Score	Positivity (%)	Score	Positivity (%)
SCLC	14	8.7	86	2.4	86
LCNEC	1	12.0	100	6.0	100

Score, mean staining score; LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma.

3.2. Detection of hASH1 and HES1 mRNA by ISH

hASH1 mRNA was detected by ISH and was observed as tiny brown granules in the cytoplasm. Twelve (86%) SCLC cell lines were positive for hASH1, and the mean staining score was 8.7. The LCN1 cells also showed strong hASH1 expression, with a staining score of 12.0 (Fig. 2 and Table 2). HES1 mRNA was observed in 12 (86%) SCLC cell lines, and the mean staining score was 2.4. The LCN1 cells also expressed HES1, and their staining score was 6.0 (Fig. 2 and Table 2). In brief, hASH1 mRNA was highly expressed in all pulmonary neuroendocrine carcinoma derived cell lines, except H82 and N417, and the obtained results generally correlated with the results of the RT-PCR analysis. The expression level of HES1 mRNA in the LCN1 cells was higher than those in the other cell lines, which was also approximately consistent with the results of the RT-PCR analysis. Within the SCLC cell lines, the expression of hASH1 mRNA was weaker or absent in the variant type cell lines compared with the classical cell lines. In addition, both of the HES1 negative SCLC cell lines belonged to the variant type, with one being negative for both hASH1 and HES1.

In lung cancer tissues, hASH1 mRNA expression was found in most SCLC and LCNEC as well as two AD. hASH1 mRNA expression was observed as fine brown granules in the cytoplasm of the tumor cells, but not in other cells, including inflammatory cells. On the other hand, various degrees of HES1 mRNA expression were observed in the cytoplasm of the tumor cells in all cases as well

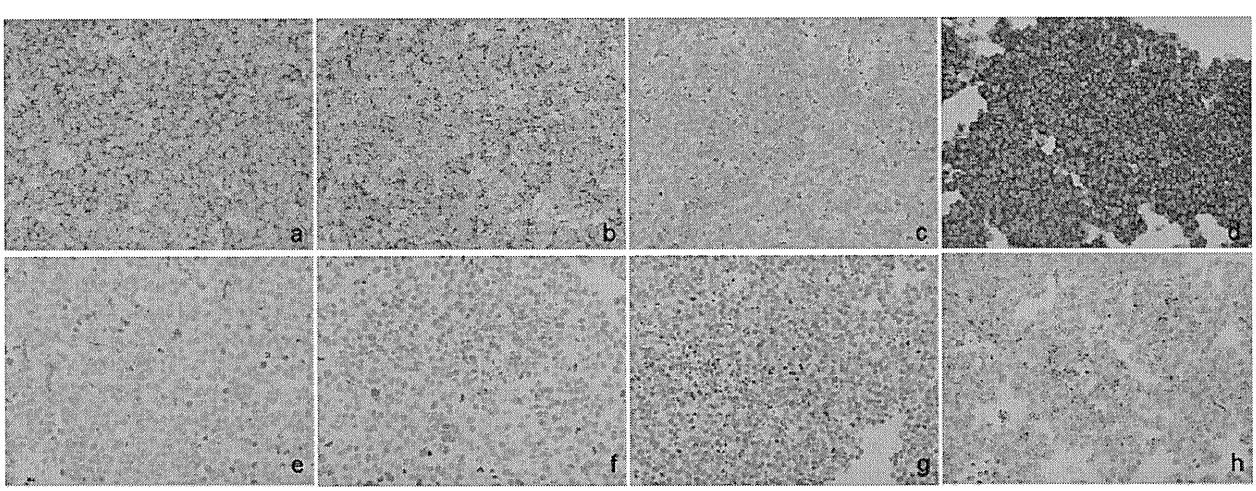

Fig. 2. Expression of (a–d) hASH1 and (e–h) HES1 mRNA in pulmonary neuroendocrine carcinoma cells according to *in situ* hybridization. (a and e) N231 cells; (b and f) N230 cells; (c and g) Lu135c cells; and (d and h) LCN1 cells. Various degrees of hASH1 mRNA expression were observed, and LCN1 showed a high level of hASH1 mRNA expression. The expression levels of HES1 mRNA were lower in SCLC than in LCNEC.

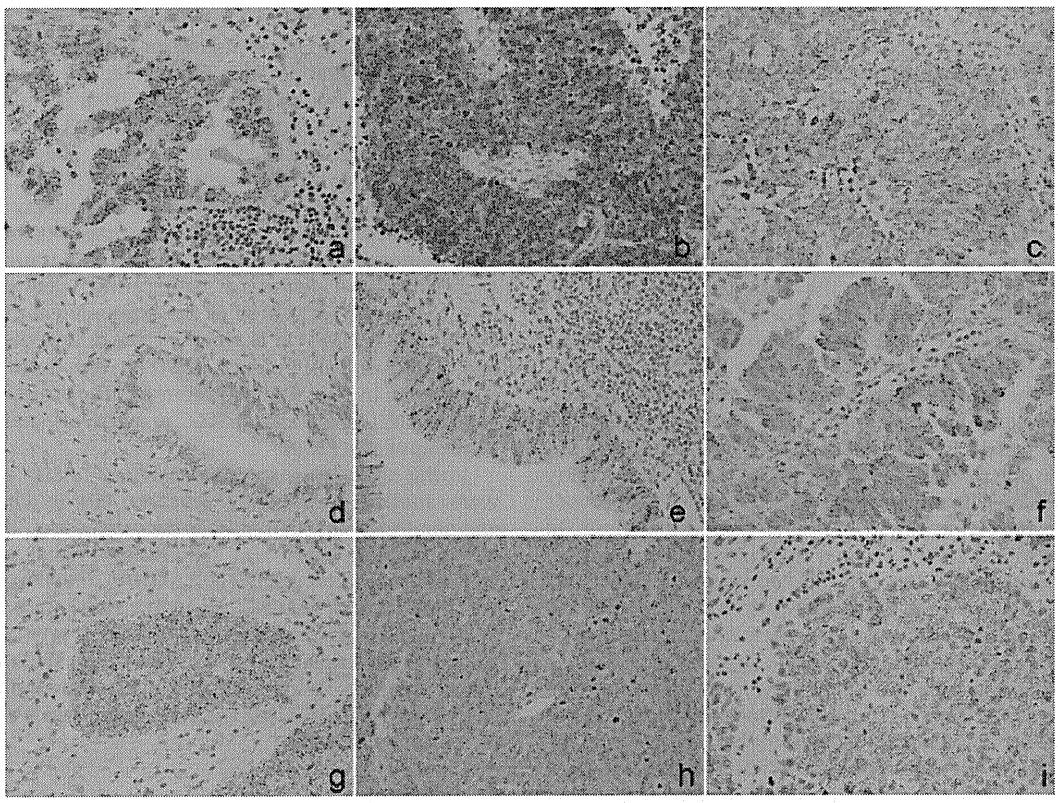

Fig. 3. Expression of (a–c) hASH1 and (d–i) HES1 mRNA in pulmonary carcinoma according to ISH. (a and f) adenocarcinomas; (b and h) small cell lung carcinomas; (c and i) large cell neuroendocrine carcinomas; (d and e) normal lung tissues; and (g) squamous cell carcinoma. For hASH1 mRNA expression, higher staining scores were recognized in SCLC than in LCNEC. For HES1 mRNA expression, higher staining scores were recognized in LCNEC than in SCLC.

as in non-neoplastic bronchial pseudostratified ciliated columnar epithelium cells (Fig. 3d and e). In total, hASH1 mRNA was observed in 24 of 32 (75%) SCLC cases and in 16 of 32 (50%) of LCNEC cases, and their mean staining scores were 8.6 and 4.2, respectively. Two of 14 (17%) cases of AD were also positive for hASH1 mRNA, and their mean staining score was 0.64 (Fig. 3a). hASH1 mRNA was not detected in the SCC cases (Table 3). In summary, hASH1 expression was detected in most (75%) SCLC, and the most of these cases showed high staining scores (Fig. 3b). On the other hand, hASH1 mRNA was only seen in about half of LCNEC cases, and their staining scores were significantly lower than those of SCLC ($p < 0.01$) (Fig. 3).

HES1 mRNA was detected in 19 of 32 of SCLC cases (59%) and 28 of 31 of LCNEC cases (87%), and their mean staining scores were 2.4 and 4.8, respectively. On the other hand, various degrees of HES1 mRNA expression were detected in all AD and SCC cases, and their mean staining scores were 5.6 and 6.2, respectively (Fig. 3f and g and Table 3). The expression levels of HES1 mRNA in the LCNEC cases were significantly higher than those in the SCLC cases ($p < 0.01$) (Fig. 3h and i). In addition, the expression of HES1 in SCC and AD was generally high.

Table 3
Expression of hASH1 and HES1 mRNA in resected pulmonary carcinomas according to ISH.

	n	hASH1		HES1	
		Score	Positivity (%)	Score	Positivity (%)
SCLC	32	8.6*	75	2.4*	59
LCNEC	32	4.2*	50	4.8*	87
AD	14	0.6	17	5.6	100
SCC	10	0	0	6.2	100

Score, mean staining score; AD, adenocarcinoma; LCNEC, large cell neuroendocrine carcinoma; SCC, squamous cell carcinoma; SCLC, small cell lung carcinoma.
* $p < 0.01$.

4. Discussion

In the present study, the expression of hASH1 and HES1, two bHLH type transcription factors that affect neuroendocrine differentiation, was examined using a highly sensitive ISH method [21] employing biotinylated tyramide in order to elucidate the biological properties of SCLC and LCNEC.

bHLH type transcription factors control cell differentiation in various tissues and are assigned into two distinct groups, activator and repressor genes [8]. hASH1, an activator gene, is important for the early development of neural and neuroendocrine progenitor cells in the central and peripheral nervous systems [11]. Guillemot et al. have reported that MASH1-dificient mice died at 12 h after birth due to hypoventilation and severe olfactory and central nervous system abnormalities [9]. Furthermore, Borges et al. reported that newborn mice displaying MASH1 gene disruption did not possess any detectable pulmonary neuroendocrine cells [11]. These results suggest that the differentiation of neuroendocrine cells in normal lungs is affected by the absence of the MASH1 gene. hASH1 is also selectively expressed in pulmonary neuroendocrine carcinomas as well as in a range of lung cancers with neuroendocrine features [11,22]. Furthermore, when the expression of hASH1 mRNA was abrogated with hASH1-specific antisense oligonucleotides in SCLC derived cell lines, hASH1 depletion was associated with an equally dramatic downregulation of neural marker expression, such as those of neuron-specific enolase and synaptophysin, relative to missense control cells [11]. These

data suggest that the expression of MASH1 or hASH1 in immature respiratory epithelial cells is essential for neuroendocrine cell differentiation [7,12]. In a previous study, we found that hASH1 was frequently expressed in pulmonary neuroendocrine carcinomas and that the expression of hASH1 was higher in SCLC than in LCNEC [14], suggesting that the constant expression of hASH1 is essential for establishing the characteristics of the neuroendocrine phenotype in both tumor types [14]. Moreover, the expression levels of hASH1 mRNA and neuroendocrine markers were higher in the classical type of SCLC derived cell lines than those belonging to the variant type [23], supporting the close correlation between neuroendocrine differentiation and hASH1 expression.

On the other hand, HES1, a major neuronal repressor gene, antagonizes the hASH1 complex and inhibits neuronal differentiation [13]. In the normal mammalian lung, HES1 positive cells are non-neuroendocrine cells that express Notch1 and Notch3 [12]. HES1 expression is upregulated in non-SCLC with non-neuroendocrine phenotypes [13], and HES1 is not or is faintly expressed in SCLC cell lines displaying neuroendocrine phenotypes and expressing hASH1, as detected by Western blot analysis. In the present study, our results showed that most SCLC and LCNEC expressed not only hASH1 but also HES1 to various degrees. In a previous study, disruption of the HES-1 gene led to upregulated expression of proneural basic helix–loop–helix factors and premature neurogenesis in mouse embryos [24], suggesting that HES1 acts as a negative regulator of neurogenesis and that a reduction in HES1 expression is essential for progenitor cells to proceed to neuroendocrine differentiation [25]. Thus, the balance between the expression levels of the two reciprocal bHLH type transcription factors hASH1 and HES1 is important for the timing of neuronal differentiation [8].

In the present study, we detected hASH1 and HES1 expression in most SCLC and LCNEC cases. The expression levels of hASH1 mRNA tended to be significantly higher in SCLC than in LCNEC, whereas HES1 mRNA expression was significantly higher in LCNEC than in SCLC. We and others have reported that LCNEC shares more characteristics with adenocarcinomas than SCLC. It has been reported that the expression of hASH1 mRNA is limited to lung cancers with neuroendocrine characteristics and that not all neuroendocrine tumors express it [11,14,19,26]. We also found that hASH1 expression is strongly correlated with the expression levels of neuroendocrine markers, such as CgA, GRP, and CT, and that the hASH1 expression in lung cancers mimicked its temporary expression in the fetal period because hASH1 was not expressed in fully differentiated TC but was expressed in less differentiated ATC similar to its expression in SCLC and LCNEC [14]. The higher expression of hASH1 in SCLC detected in the present study may indicate that it differentiates more predominantly in the neuroendocrine direction. On the other hand, LCNEC may predominantly differentiate toward the bronchial epithelium phenotype since it expresses HES1 more strongly, even though it has neuroendocrine features.

We have examined the differences between SCLC and LCNEC using various proteomic techniques [27,28]. We used sera from patients with adenocarcinoma or small cell lung carcinoma (n = one each) as a source of primary antibodies and 2D immunoblotting to screen cell lysates from lung cancer cell lines. Cytokeratin (CK) 18 and villin1 were identified as tumor associated antigens, and this was validated in an immunohistochemical study of pulmonary carcinomas of various histologic types. We demonstrated that the CK18 staining score was significantly higher in AD and LCNEC and that villin1 expression was generally limited to AD and LCNEC. These findings indicated that both CK18 and villin1 could be used to differentiate adenocarcinomas and/or LCNEC from SCLC and SCC [27]. Moreover, we conducted proteome analysis using cell lines derived from SCLC and LCNEC and the agarose two-dimensional electrophoresis method. The expression levels of twenty-five proteins were found to differ between the SCLC and LCNEC cell lines. As cellular size differs greatly between SCLC and LCNEC, we focused on the cytoskeletal proteins CK7, 8, 18, and 19 and studied various types of pulmonary carcinomas using immunohistochemical techniques. The expressions levels of CK7, 8, 18, and 19 were significantly higher in LCNEC than in SCLC, suggesting that LCNEC and SCLC can be differentiated using CK7, 8, 18, and 19 staining [28]. Nitadori et al. performed tissue microarray analysis of surgically resected LCNEC and SCLC specimens using 48 antibodies. They demonstrated that the expression of four proteins, CK7, 8, E-cadherin and β-catenin, was significantly higher in LCNEC than in SCLC [29]. Sturm et al. studied the expression of thyroid transcription factor-1 (TTF-1) in neuroendocrine tumors of the lung on IHC, and higher expression levels of TTF-1 were detected in SCLC and LCNEC than in TC and AC. Furthermore, the positivity of TTF-1 was 49% in LCNEC and 85.5% in SCLC, respectively, and the differences were significant [30].

In this study, the expression levels of hASH1 and HES1 mRNA were studied with highly sensitive ISH in 88 formalin-fixed and paraffin-embedded pulmonary carcinomas. The mean hASH1 mRNA staining score was significantly higher in SCLC than in LCNEC ($p < 0.01$). Inversely, HES1 mRNA was expressed significantly less in SCLC than in LCNEC ($p < 0.01$). These findings suggest that SCLC bends toward neuroendocrine differentiation, while LCNEC shows characteristics more associated with the bronchial epithelium phenotype. Taken together with previous findings, the present data strongly suggest that the biological characteristics of the two tumors are different.

5. Conclusion

To clarify the biological differences between SCLC and LCNEC, we investigated the expression of hASH1 and HES1 using a highly sensitive ISH method involving digoxigenin-labeled cRNA probes and biotinylated tyramide. The expression of hASH1 mRNA was significantly higher in SCLC than in LCNEC ($p < 0.01$), whereas that of HES1 mRNA was lower in SCLC than in LCNEC ($p < 0.01$). These findings reveal that SCLC more strongly expresses the neuroendocrine phenotype, while LCNEC shows characteristics more similar to the bronchial epithelium phenotype, suggesting that the biological characteristics of these two tumors are different.

Conflict of interest statement

None declared.

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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].

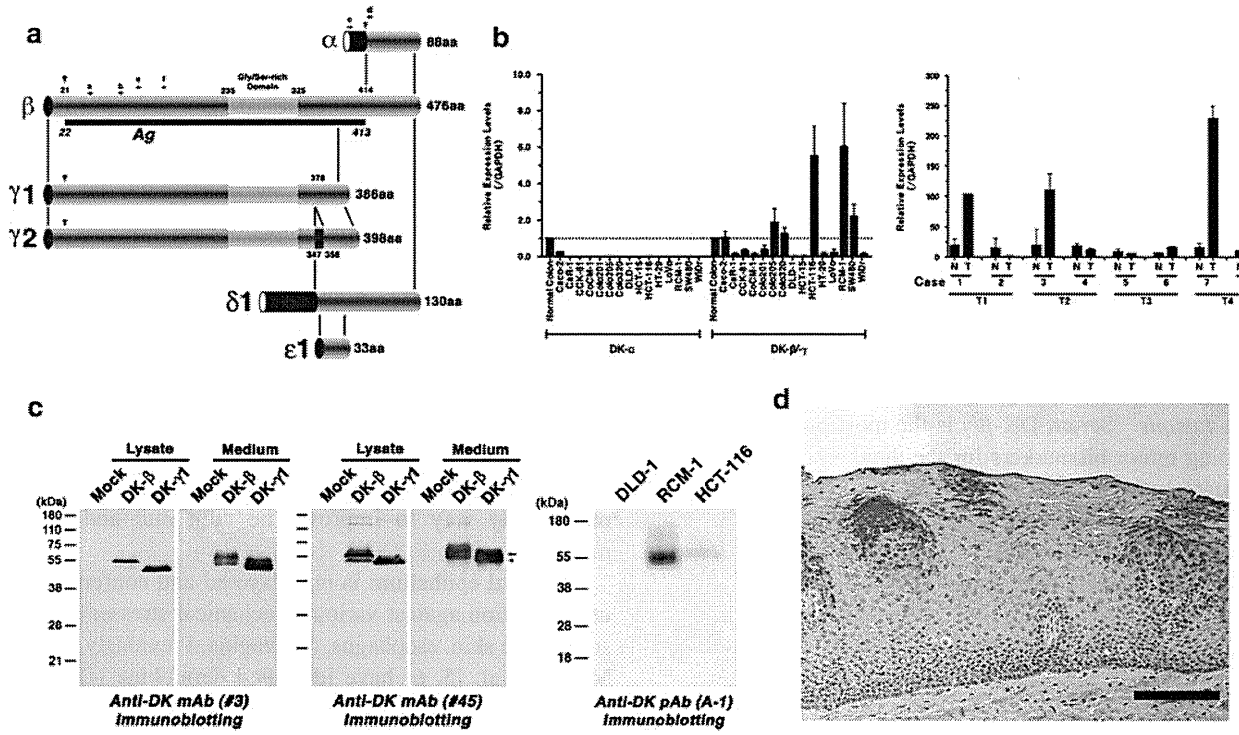

Fig. 1 Expression of dermokine (*DK*) isoforms in colorectal cancer cell lines and tissues. **a** Schematic representation of the structure of DK isoforms (α/β/γ1/γ2/δ1/ε1). *Arrows* indicate the primer pairs used for SYBR Green quantitative reverse transcription polymerase chain reaction (qRT-PCR) (*a*, *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

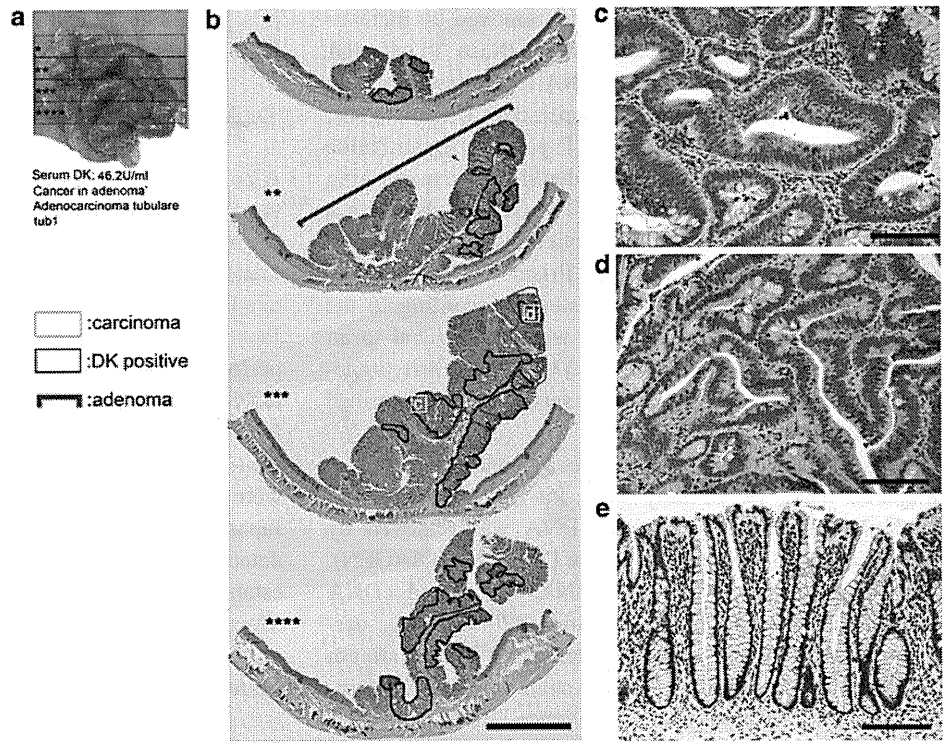

Fig. 2 Immunohistochemical staining of cancer in adenoma with anti-DK mAb. **a** Macroscopic view of noninvasive cancer in adenoma. **b** The DK-positive area (*outlined in black*) and cancer lesions (Tis, *outlined in red*) partially overlapped in noninvasive cancer, and DK was also expressed in the 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])

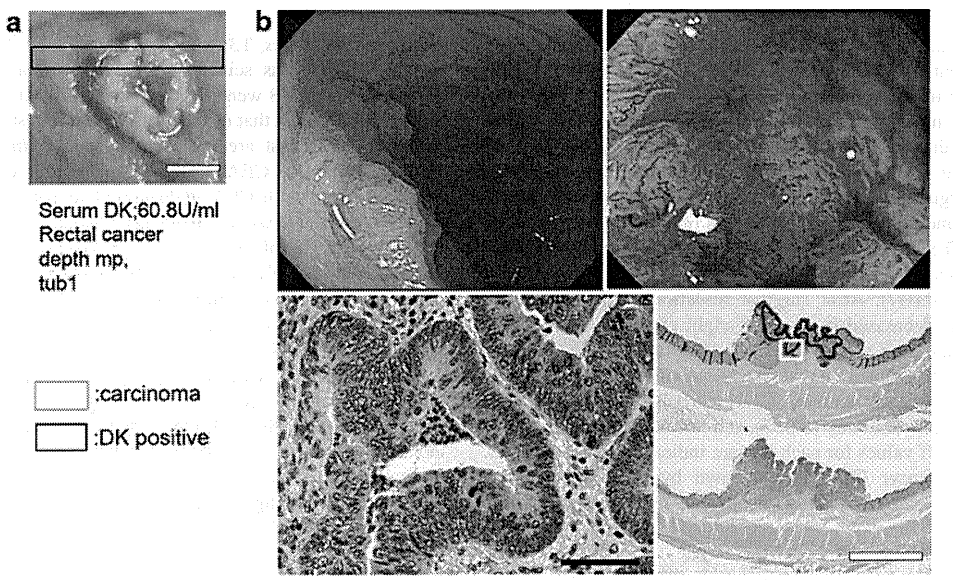

Fig. 3 Endoscopic features and immunohistochemical staining of cancer without adenoma with anti-DK mAb. **a** Macroscopic view of the rectal cancer. The size of the tumor was 18 × 12 mm. **b** Endoscopic examination revealed a shallow depressed lesion with a flat elevated area in the rectum (*left upper 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)

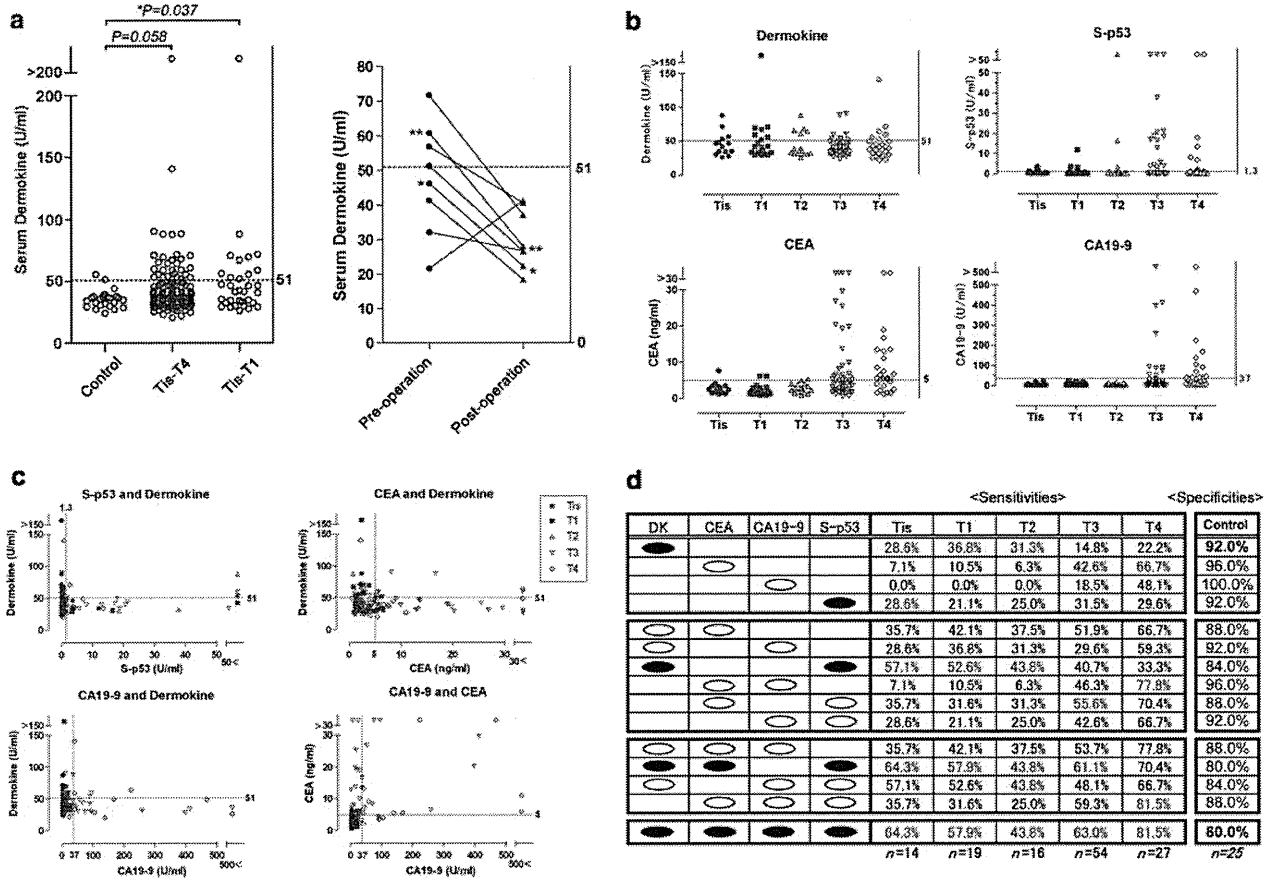

Fig. 4 DK enzyme-linked immunosorbent assay (ELISA). **a** Sera from 130 patients with colorectal cancer (Tis [cancer in situ, intraepithelial or invasion of lamina propria]; T1, tumor invades the submucosa; T2, tumor invades the muscularis propria; T3, tumor invades through the muscularis propria into 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

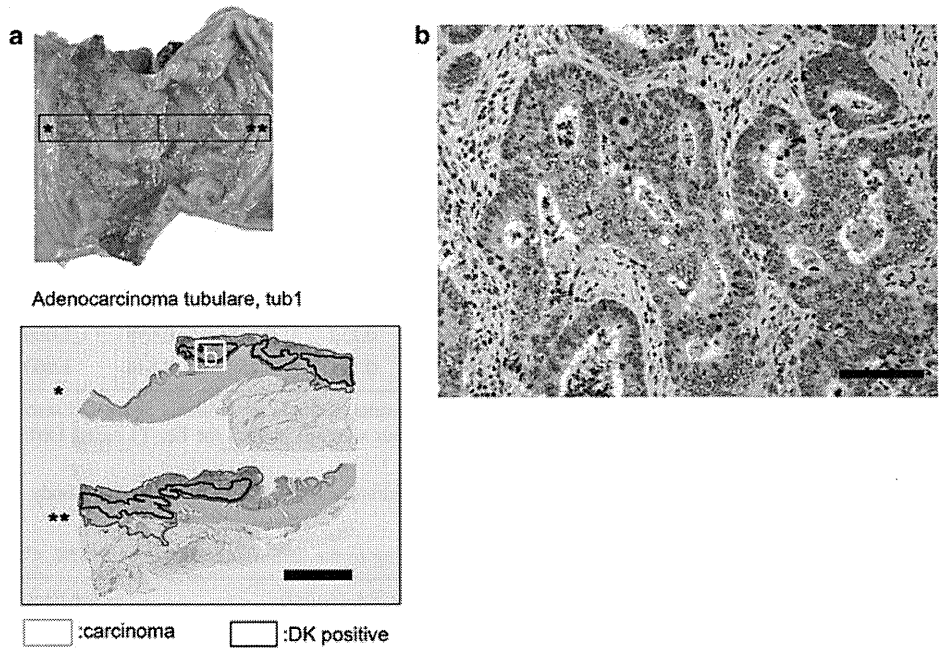

Fig. 5 Immunohistochemical staining of advanced cancer with anti-DK mAb. **a** Macroscopic view of advanced cancer. The DK-positive area (*outlined in black*) and cancer lesions (T3, *outlined in red*) are shown, and the DK-positive area was located in the deep region of carcinoma (*bottom panel*). *White 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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