A-bomb survivor (Fig. 3). This is the initial study of gene expression in solid cancer among A-bomb survivors which has not been possible using archival tissue samples. We could identify 10 genes whose expression levels in the tumors were significantly different between A-bomb survivors (LSS cohort members (>0 dose)) and control subjects (non-LSS members) by one-way ANOVA ($p<0.05$). Those included two genes selected by SAGE analysis and one gene related to DNA damage response. They might be candidate genes which participate in radiation-induced carcinogenesis and possible genetic markers for radiation-induced gastric cancer.

Thus, our project is the powerful way to approach the mechanism of radiation-induced carcinogenesis and identify genetic markers for radiation-induced cancer. This must contribute to not only an improvement of medical care for A-bomb survivors but also risk assessment of cancer in radiation exposure by medicine and occupation and novel strategy for effective cancer prevention based on molecular mechanism.

5. Project members

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Serum Concentration of Reg IV in Patients with Colorectal Cancer: Overexpression and High Serum Levels of Reg IV Are Associated with Liver Metastasis

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Key Words
Colorectal cancer · Reg IV · Serial analysis of gene expression · Serum tumor marker

Abstract
Objective: Regenerating islet-derived family, member 4 (regenerating gene type IV, Reg IV) is overexpressed in colorectal cancer (CRC). The aim of this study was to investigate the diagnostic utility of Reg IV determination in sera from patients with CRC. ***Methods:*** We examined the expression and distribution of Reg IV in CRC by immunohistochemistry and determined Reg IV levels in sera from patients with CRC by enzyme-linked immunosorbent assay. ***Results:*** Immunostaining revealed that 23 of 80 (29%) CRC cases were positive for Reg IV. CRC cases with metastatic recurrence in the liver showed more frequently Reg IV staining than those without ($p = 0.0102$). Patients with CRC showing Reg IV staining had a significantly worse survival than those without Reg IV staining ($p = 0.0117$). Preoperatively, serum Reg IV concentrations were not elevated in CRC patients at stage 0–III, being in contrast to the significantly increased preoperative levels in stage IV CRC patients with liver metastasis. ***Conclusion:*** These results suggest that Reg IV is a prognosticator for poor survival. Serum Reg IV concentration may predict CRC recurrence in the liver.

Introduction

It is generally accepted that cancer develops as a result of multiple genetic and epigenetic alterations. Better knowledge of changes in gene expression that occur during carcinogenesis may lead to improvements in the diagnosis, treatment and prevention. Identification of novel biomarkers for cancer diagnosis and novel targets for treatment is a major goal [1]. Genes encoding transmem-

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brane/secretory proteins expressed specifically in cancers may be ideal biomarkers for cancer diagnosis [2]. In addition, if the function of the gene product is involved in the neoplastic process, this gene may constitute a therapeutic target. To identify potential molecular markers for cancer and to better understand the development of cancer at the molecular level, comprehensive gene expression analysis may be useful. Among the comprehensive methods used to analyze transcript expression levels, array-based hybridization [3] and serial analysis of gene expression (SAGE) [4] are currently the most common approaches. We previously performed SAGE in four primary gastric cancers (GCs) [5] and identified several GC-specific genes [6]. Of these genes, *Regenerating islet-derived family, member 4* (*REG4*, which encodes Reg IV) is a candidate gene for cancer-specific expression, at least in patients with GC. *REG4* was originally identified by high-throughput sequencing of a cDNA library derived from inflammatory bowel disease patients [7]. Reg IV is a member of the Reg gene family, which includes three other genes (Reg Iα, Reg Iβ and Reg III) [7]. In GC, expression of Reg Iα has been reported [8, 9]. Reg Iα expression is enhanced in advanced T grade GCs and in GCs that were not well differentiated. Overall and disease-free survival are poor for patients with Reg Iα-positive GCs. Quantitative reverse transcription (RT)-polymerase chain reaction (PCR) analysis revealed that approximately 50% of GCs overexpress the *REG4* gene [5]. Although various normal tissues express *REG4*, expression levels are much lower in normal tissues than in cancerous tissues [5]. Our previous immunohistochemical analysis revealed that Reg IV was expressed in 30% of GC tissues and was associated with both the intestinal mucin phenotype and neuroendocrine differentiation [10]. In addition, Reg IV is a secreted protein, and we showed that serum Reg IV represents a novel biomarker for GC [11]. The diagnostic sensitivity of serum Reg IV was superior to that of serum carcinoembryonic antigen (CEA) or carbohydrate antigen 19-9 (CA19-9).

Overexpression of Reg IV has been reported in colorectal cancer (CRC) [10, 12, 13]. In the normal colon, little or no Reg IV immunostaining is observed in epithelial cells, whereas strong Reg IV immunostaining is detected in neuroendocrine cells [10, 12, 14]. In our previous immunohistochemical analysis, Reg IV staining was identified in 36% of CRC cases [10]. Real-time RT-PCR analysis revealed that although more than 70% of human CRC samples showed increased *REG4* expression [12, 13], the other REG mRNAs are present at very low or undetectable levels in most samples [12]. Therefore, Reg IV may be a serum biomarker for CRC; however, serum Reg IV concentrations have not been investigated in CRC patients.

In the present study, we examined the expression and distribution of Reg IV in human CRC by immunohistochemistry, and the relationship between Reg IV staining and clinicopathological characteristics. We have reported two Reg IV staining patterns (mucin-like staining and strong perinuclear staining) [10]. Mucin-like staining is observed in goblet cells and goblet cell-like vesicles of cancer cells. These cells are positive for MUC2 (a marker of goblet cells). In contrast, strong perinuclear staining is detected in neuroendocrine cells. These cells are positive for chromogranin A (a marker of neuroendocrine cells). Therefore, we examined the coexpression of Reg IV and chromogranin A or MUC2 by double-immunofluorescence staining. We also assessed serum Reg IV levels in CRC patients by enzyme-linked immunosorbent assay (ELISA) to investigate the potential diagnostic utility of Reg IV determination.

Patients and Methods

Tissue Samples

Primary tumor samples were collected from 80 patients with CRC (35 women and 45 men; age range, 46–93 years; mean, 68 years) and serum samples from 78 patients with CRC (28 women and 50 men; age range, 46–84 years; mean, 67 years). Patients were treated at the Hiroshima University Hospital or an affiliated hospital. For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 80 patients with CRC who underwent surgical excision. Information on patient survival was available for 30 of the 80 CRC cases and was not available for the remaining 50 CRC cases. Among the serum samples from the 78 patients with CRC, primary CRC tissue samples were available for immunohistochemical analysis from 50 cases. The remaining 28 primary CRC tissue samples were not available because of lack of tumor tissue samples. Serum samples from 78 patients with CRC were obtained before surgery and before initiation of therapy. Control serum samples were obtained from 50 healthy individuals (14 women and 36 men; age range, 61–86 years; mean, 71 years). Serum samples were stored at −80°C until analysis. Tumor staging was performed according to the TNM classification system [15]. Because written informed consent was not obtained, for strict privacy protection, identifying information was removed from all samples before analysis. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government.

Immunohistochemistry

Formalin-fixed, paraffin-embedded samples were sectioned, deparaffinized and stained with hematoxylin and eosin to ensure that the sectioned block contained tumor cells. Adjacent sections

were then stained immunohistochemically. Sections were pretreated by microwave irradiation in citrate buffer for 30 min to retrieve antigenicity. After blocking peroxidase activity with 3% H_2O_2-methanol for 10 min, sections were incubated with normal goat serum (Dako, Carpinteria, Calif., USA) for 20 min to block nonspecific antibody binding. Sections were incubated with goat anti-Reg IV antibody (diluted 1:50; R & D Systems, Abingdon, UK) for 60 min at room temperature followed by incubation with peroxidase-labeled anti-goat IgG for 60 min. Staining was completed with a 5-min incubation with the substrate-chromogen solution (Dako). Sections were counterstained with 0.1% hematoxylin. Reg IV staining was classified according to the percentage of stained cancer cells, as described previously [10]. When >10% of cancer cells were stained, immunostaining was considered positive.

Double-immunofluorescence staining was performed as described previously [10]. Alexa Fluor 546-conjugated anti-goat IgG (Molecular Probes, Eugene, Oreg., USA) and Alexa Fluor 488-conjugated anti-mouse IgG (Molecular Probes) were used as secondary antibodies.

ELISA

For measurement of the serum Reg IV concentration, a sandwich ELISA method was developed as described previously [11]. First, polystyrene microtiter plates were coated with mouse monoclonal anti-Reg IV antibody (R & D Systems) by overnight incubation of 50 μl antibody diluted in 125 ng Tris buffer/well (pH 7.4). The plates were then washed 3 times with washing buffer. After the plates were blocked with 1% milk in phosphate-buffered saline, 50 μl of recombinant Reg IV standard or sample were added to each well and incubated overnight at 4°C. After 3 washes, 50 μl of biotinylated goat polyclonal anti-Reg IV antibody (R & D Systems) in assay buffer (1% bovine serum albumin, Tris buffer, pH 7.4, 0.05% normal goat serum) were added to each well (75 ng antibody/well). The mixture was then incubated for 1 h with shaking at 37°C and washed 3 times with washing buffer. The plates were incubated with 50 μl alkaline phosphatase-conjugated streptavidin (Dako) diluted 1:2,000 in diluent containing 1% BSA and Tris buffer (pH 7.4) per well for 1 h at 37°C and washed 3 times. Color development was performed with the addition of pNPP chromogenic substrate (Sigma-Aldrich, St. Louis, Mo., USA) followed by incubation at 37°C for 1 h. Absorbance at 405 nm was measured with an ELISA plate reader. As a reference standard, known concentrations of human recombinant Reg IV [10] (0–0 ng/ml) were tested in triplicate.

Measurement of CEA and CA19-9

CEA and CA19-9 were measured with a commercially available automated immunoassay method (Modular Analytics, Roche Diagnostics, Indianapolis, Ind., USA). The upper limits of normal for this method are 5.0 ng/ml for CEA and 37 U/ml for CA19-9.

Statistical Methods

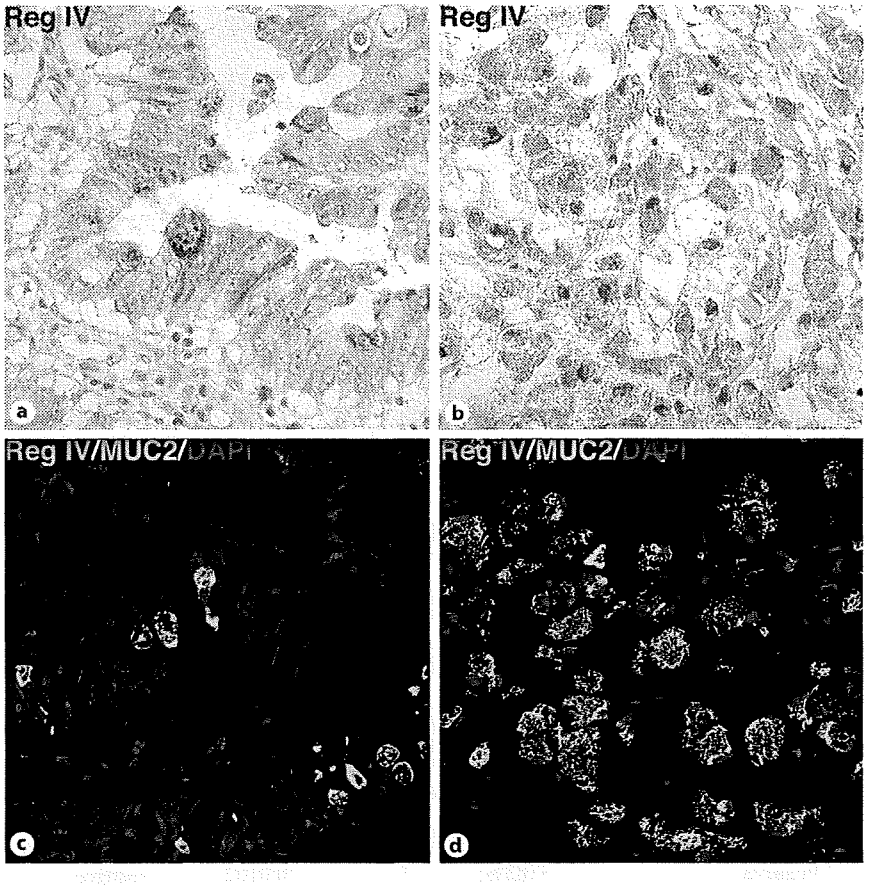
Associations between clinicopathological parameters and Reg IV staining were analyzed by χ^2 test. Kaplan-Meier survival curves were constructed for Reg IV-positive and Reg IV-negative patients to compare survival between both groups. Differences between survival curves were tested for statistical significance by log-rank test [16]. Differences in the serum Reg IV concentration between two groups were tested by nonparametric Mann-Whitney U test. Correlations between the serum Reg IV concentration and the serum concentration of CEA or CA19-9 were assessed by Spearman's rank correlation test. A p value <0.05 was considered statistically significant.

Results

Immunohistochemical Analysis of Reg IV in CRC and Peritumoral Mucosa

We performed immunohistochemical analysis of Reg IV in 80 human CRC samples. Representative results of Reg IV immunostaining in CRC are shown in figure 1. In CRC tissues, Reg IV staining was observed in goblet cell-like vesicles of both well-differentiated (fig. 1a) and poorly differentiated adenocarcinoma cells (fig. 1b). We confirmed that tumor cells showing mucin-like staining of Reg IV were positive for MUC2 by double-immunofluorescence staining (fig. 1c, d). In total, 23 of 80 (29%) CRC cases were positive for Reg IV staining, and all Reg IV-positive cases showed mucin-like staining. We analyzed the relationship between Reg IV staining and clinicopathological characteristics. No correlation was found between Reg IV staining and depth of invasion, lymph node metastasis or tumor stage (table 1). We next analyzed the relationship between Reg IV staining and clinicopathological characteristics in 30 CRC cases followed up at the hospital (table 2). CRC cases with metastatic recurrence in the liver showed Reg IV staining more frequently than those without ($p = 0.0102$, χ^2 test, table 2). In addition, patients with CRC showing Reg IV staining had a significantly worse survival rate than patients without Reg IV staining ($p = 0.0117$, log-rank test, fig. 1e). Reg IV staining was observed more frequently in poorly differentiated/mucinous adenocarcinoma (6/9, 67%) than in well-/moderately differentiated adenocarcinomas (17/71, 24%, $p = 0.0228$, χ^2 test).

We then focused on the peritumoral mucosa of CRC because overexpression of *REG4* mRNA has been reported in peritumoral mucosa of CRC by Northern blot analysis [12]. Interestingly, extensive Reg IV staining was observed, and all peritumoral mucosa samples of the 80 CRC cases were positive for Reg IV regardless of Reg IV staining in tumor cells. Reg IV staining gradually decreased with increasing distance from the tumor tissue (fig. 2a). In the mucosa closest to the tumor tissue, almost all goblet cells showed Reg IV staining (fig. 2b, c), whereas only few goblet cells showed Reg IV staining in the mucosa distant from the tumor tissue (fig. 2d). We confirmed that goblet cells showing Reg IV staining were



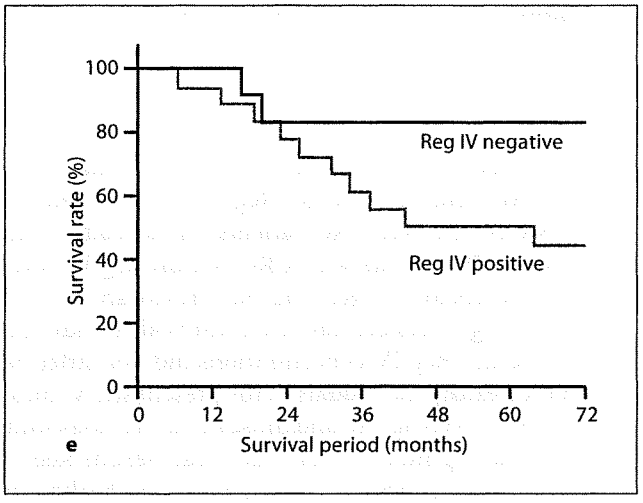


Fig. 1. Immunostaining of Reg IV in CRC tissues. **a**, **b** Immunostaining of Reg IV (brown) in well-differentiated (**a**) and poorly differentiated adenocarcinoma (**b**; original magnification, ×400). Reg IV staining was observed in goblet cell-like vesicles. **c**, **d** Double-immunofluorescence staining of Reg IV (red) and MUC2 (green) in well-differentiated (**c**) and poorly differentiated adenocarcinoma (**d**; original magnification, ×400). Goblet cell-like vesicles of tumor cells were positive for both Reg IV and MUC2 staining. **e** Prognostic value of Reg IV staining. CRC patients with Reg IV immunostaining (n = 18) revealed a significantly worse survival rate than those showing no Reg IV staining (n = 12; p = 0.0117, log-rank test). p = 0.0117.

positive for MUC2 by double-immunofluorescence staining (fig. 2e, f). Strong perinuclear Reg IV staining was detected in both the mucosa closest to the tumor tissue and the mucosa distant from the tumor tissue (fig. 2b–d). We confirmed that cells showing strong perinuclear Reg IV staining were positive for chromogranin A by double-immunofluorescence staining (fig. 2g, h), indicating that these cells were neuroendocrine cells.

Table 1. Association of Reg IV expression with clinicopathological features of 80 CRC cases

Characteristics	Reg IV expression, n		p value χ^2 test
	positive	negative	
Age			
≤65 years	5 (19%)	22	0.2372
>65 years	18 (34%)	35	
Sex			
Male	15 (33%)	30	0.4365
Female	8 (23%)	27	
Tumor location			
Right/transverse	4 (17%)	20	0.1958
Left/sigmoid/rectum	19 (34%)	37	
T grade			
Tis/T1/T2	6 (43%)	8	0.3376
T3/T4	17 (26%)	49	
N grade			
N0	12 (27%)	32	0.9406
N1/N2	11 (31%)	25	
Stage			
0	1 (50%)	1	0.7331
I	3 (30%)	7	
II	7 (23%)	24	
III	11 (31%)	25	
IV	1 (100%)	0	
Histologic type			
Well/moderately	17 (24%)	54	0.0228
Poorly/mucinous	6 (67%)	3	
Metastatic recurrence in the liver (n = 30)			
Positive	5 (63%)	3	0.0102
Negative	2 (9%)	20	

Table 2. Association of Reg IV expression with clinicopathological features of 30 CRC cases followed-up at the hospital

Characteristics	Reg IV expression, n		p value χ^2 test
	positive	negative	
Age			
≤65 years	2 (18%)	9	0.9524
>65 years	5 (26%)	14	
Sex			
Male	4 (22%)	14	0.8601
Female	3 (25%)	9	
Tumor location			
Right/transverse	2 (22%)	7	0.9250
Left/sigmoid/rectum	5 (24%)	16	
T grade			
Tis/T1/T2	0	0	–
T3/T4	7 (23%)	23	
N grade			
N0	4 (20%)	16	0.8787
N1/N2	3 (30%)	7	
Stage			
0	0	0	0.8787
I	0	0	
II	4 (20%)	16	
III	3 (30%)	7	
IV	0	0	
Histologic type			
Well/moderately	6 (21%)	22	0.9540
Poorly/mucinous	1 (50%)	1	
Metastatic recurrence in the liver (n = 30)			
Positive	5 (63%)	3	0.0102
Negative	2 (9%)	20	

Serum Reg IV Concentrations in Patients with CRC

We next examined whether Reg IV could be detected by ELISA in sera from CRC patients. In 50 healthy individuals and 78 patients with CRC, serum Reg IV levels were assessed before surgery (fig. 3a). Previously, we studied serum Reg IV concentrations in 101 healthy individuals [11]. Serum Reg IV concentrations did not differ between the healthy individuals in the present study (mean ± SE, 0.59 ± 0.08 ng/ml) and those in the previous study (0.52 ± 0.05 ng/ml). In total, the mean serum Reg IV concentration was 0.54 ± 0.04 ng/ml in 151 healthy individuals (the previous 101 samples plus the present 50 samples). Serum Reg IV concentrations were not significantly elevated in presurgical CRC patients regarding stage: stage 0 (n = 3) 0.58 ± 0.01 ng/ml; stage I (n = 17) 1.07 ± 0.25 ng/ml; stage II (n = 15) 1.20 ± 0.28 ng/ml, and stage III (n = 31) 0.70 ± 0.09 ng/ml (fig. 3a). In contrast, the serum Reg IV concentration in presurgical CRC patients at stage IV was significantly elevated (n = 12, 3.54 ± 0.47 ng/ml, $p < 0.0001$, Mann-Whitney U test). Because metastatic recurrence in the liver was frequently found in Reg IV-positive CRC by immunohistochemistry, the serum concentration of Reg IV was compared in patients with CRC with and without liver metastasis. The serum Reg IV concentration was significantly higher in CRC patients with liver metastasis (n = 6, 4.58 ± 0.48 ng/ml) than in those without liver metastasis (n = 6, 2.48 ± 0.56 ng/ml, $p = 0.0260$, Mann-Whitney U test; fig. 3a). Among the serum samples from the 78 CRC patients, primary CRC tissue samples were available for immunohistochemical analysis of Reg IV from 50 cases. In case 12, which showed a high serum Reg IV concentration, strong and extensive Reg IV staining was observed in the primary CRC sample (fig. 3b). In contrast, in case 25, in which the serum Reg IV concentration was very low, no Reg IV staining was observed in the primary CRC sample

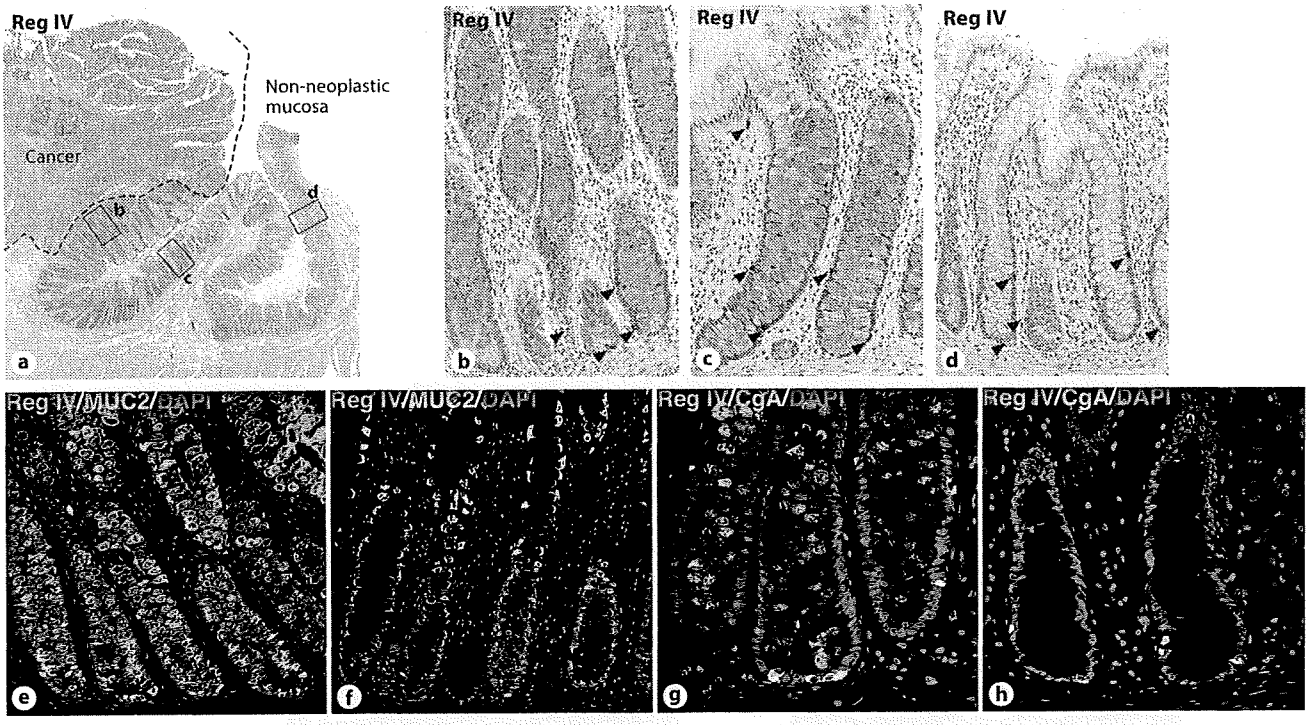


Fig. 2. Immunohistochemistry of Reg IV in the peritumoral mucosa of CRC. **a** Immunostaining of Reg IV (brown) in the peritumoral mucosa of CRC (original magnification, ×5). Reg IV staining decreased gradually with increasing distance from the tumor tissue. **b–d** High-magnification images of the fields indicated by boxes in **a**. **b**, **c** Immunostaining of Reg IV (brown) in the mucosa closest to the tumor tissue. Almost all goblet cells demonstrated Reg IV staining. Strong perinuclear staining of Reg IV (arrowheads) was also present (original magnification, ×200). **d** Immunostaining of Reg IV (brown) in the mucosa distant from the tumor tissue. Few goblet cells showed Reg IV staining, whereas strong perinuclear Reg IV staining (arrowheads) was present (original magnification, ×200). **e**, **f** Double-immunofluorescence staining of Reg IV (red) and MUC2 (green) in the mucosa closest to the tumor tissue (**e**) and distant from the tumor tissue (**f**; original magnification, ×200). Goblet cells (MUC2-positive cells) were positive for Reg IV in the mucosa closest to the tumor tissue (**e**), but not in the mucosa distant from the tumor tissue (**f**). **g**, **h** Double-immunofluorescence staining of Reg IV (red) and chromogranin A (CgA; green) in the mucosa closest to the tumor tissue (**g**) and in the mucosa distant from the tumor tissue (**h**; original magnification, ×400). Neuroendocrine cells (chromogranin A-positive cells) were positive for Reg IV in both the mucosa closest to the tumor tissue (**g**) and the mucosa distant from the tumor tissue (**h**).

(fig. 3b). In serum samples from CRC patients showing Reg IV-positive immunostaining (n = 16, 1.12 ± 0.20 ng/ml), the Reg IV concentration was significantly higher than that in those showing no Reg IV immunostaining (n = 34, 0.47 ± 0.07 ng/ml; p = 0.0052, Mann-Whitney U test). However, all CRC cases with high serum concentrations of Reg IV did not necessarily show Reg IV immunostaining in the primary tumor. In serum samples from patients with CRC showing Tis (intraepithelial or invasion of the lamina propria, n = 3, 0.58 ± 0.01 ng/ml), the Reg IV concentration was not elevated compared to that from healthy individuals (p = 0.7576, Mann-Whitney U test). The Reg IV concentrations were significantly elevated in serum samples from patients with CRC showing T1/2 (T1: tumor invading the submucosa, T2: tumor invading muscularis propria, n = 22, 1.22 ± 0.25 ng/ml) and T3/4 (tumor invading beyond the subserosa, n = 53, 1.42 ± 0.20 ng/ml) compared to that from healthy individuals (p = 0.0456 and 0.0026, respectively, Mann-Whitney U test) and therefore detectable in serum samples from patients with CRC invading the submucosa. The Reg IV concentration was significantly higher in serum samples from patients with CRC showing poorly differentiated/mucinous adenocarcinoma (n = 13, 2.25 ± 0.39 ng/ml) than that in those showing well/moderately differentiated adenocarcinoma (n = 65, 1.14 ± 0.16 ng/ml;

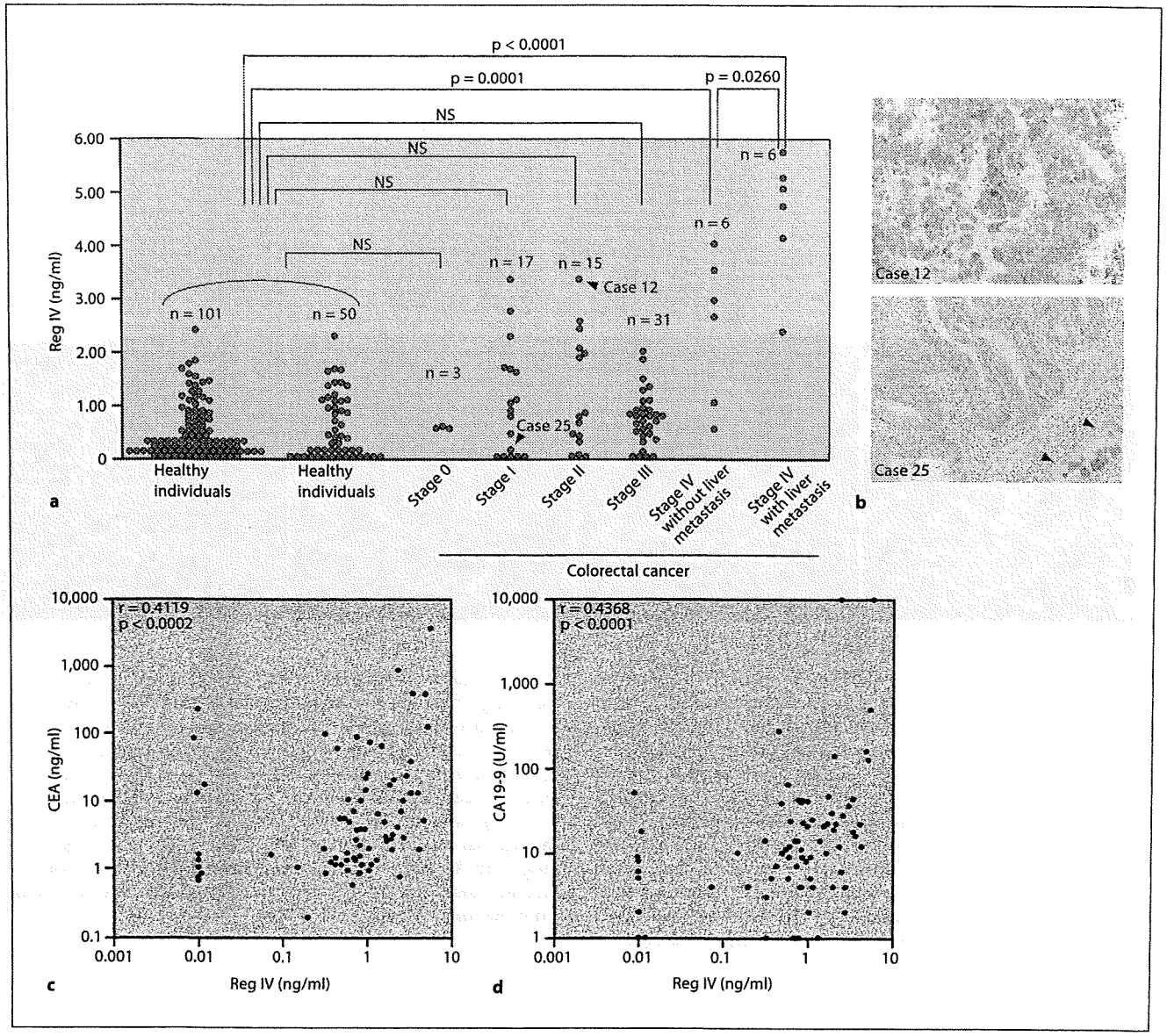


Fig. 3. ELISA of serum samples from the 50 healthy individuals and the 78 patients with CRC. **a** Detection of Reg IV protein in serum samples by ELISA. Yellow bar indicates the cutoff levels defined on the basis of the previous study (2.00 ng/ml [11]). Differences in the serum Reg IV concentrations between both groups were tested by Mann-Whitney U test. Serum Reg IV concentrations of 101 healthy individuals were measured in our previous study [11]. NS = Nonsignificant. **b** Immunostaining of Reg IV in primary CRC samples. Strong and extensive Reg IV staining was observed in case 12, which also showed a high serum concentration of Reg IV. In case 25, no Reg IV staining was observed in tumor tissue despite Reg IV staining in peritumoral tissue (arrowheads). The serum Reg IV concentration was low. **c**, **d** Relation between serum concentrations of Reg IV and CEA and CA19-9 (**d**). Correlations were examined using Spearman's rank correlation test.

p = 0.0058, Mann-Whitney U test). In our previous study, the cutoff level for Reg IV was set at 2.00 ng/ml [11]. The sensitivity and specificity to detect CRC were 24 (19/78) and 99% (149/151), respectively.

CEA and CA19-9 levels were also measured in the same serum samples. The sensitivity and specificity of CEA to detect CRC were 44 (34/78) and 100% (151/151), respectively, and 21 (16/78) and 100% (151/151) for CA19-

Table 3. Diagnostic sensitivity of serum Reg IV, CEA and CA19-9 with respect to tumor stage

	Reg IV %	CEA %	p value	CA19-9 %	p value
Stage					
0 (n = 3)	0	33	0.3496	0	1.0000
I (n = 17)	18	29	0.8076	18	1.0000
II (n = 15)	33	47	0.8835	40	0.7964
III (n = 31)	3	42	0.0084	6	0.5728
IV (n = 12)	83	67	0.9644	42	0.4906
Specificity	99	100		100	

Significant differences were assessed by χ^2 test (Reg IV vs. CEA and Reg IV vs. 19-9).

9, respectively. Spearman's rank correlation test revealed significant correlations between serum Reg IV and CEA ($r = 0.4119$, $p = 0.0002$) and CA19-9 levels ($r = 0.4368$, $p < 0.0001$; fig. 3c, d). Of 44 CRC patients with normal serum CEA values, 6 patients (14%) were found to express Reg IV at 99% specificity, and of 62 CRC patients with normal serum CA19-9 values, 12 patients (19%) were found to express Reg IV at 99% specificity. The sensitivities of serum Reg IV, CEA and CA19-9 with respect to tumor stage are listed in table 3. The sensitivity and specificity of serum Reg IV combined with CEA and CA19-9 for CRC detection were 56 (44/78) and 99% (149/151), respectively.

Discussion

Although overexpression of Reg IV has been reported in several human cancers, the biologic function of Reg IV is poorly understood. In recent studies, Reg IV overexpression has been ascribed a role in the pathogenesis of GC and CRC. Reg IV is a potent activator of the epidermal growth factor receptor (EGFR)/Akt/AP-1 signaling pathway, and CRC cell lines treated with recombinant Reg IV showed increased expression of Bcl-2, Bcl-xl and survivin, which are proteins associated with the inhibition of apoptosis [17]. In fact, EGFR activation modulates apoptotic susceptibility [reviewed in ref. 18]. We previously demonstrated that forced expression of Reg IV inhibits apoptosis induced by 5-fluorouracil. Forced Reg IV expression inhibited cytochrome C release, activation of caspases-9 and -3 and PARP cleavage in 5-fluorouracil-treated GC cells, and induced phosphorylation of the EGFR at Tyr^{992} and expression of Bcl-2 [11]. Adenomas from the intestines of 14-week-old $APC^{min/+}$ mice showed significantly increased Reg IV expression and an associated increase in Bcl-2 expression [13]. Thus, overexpression of Reg IV may lead to a tumor phenotype displaying increased resistance to apoptosis. Nanakin et al. reported that Reg IV enhances proliferation of the DLD-1 cell line [19]. In the present study, 29% of the CRC cases were positive for Reg IV staining. CRC cases with metastatic recurrence in the liver showed Reg IV staining more frequently than those without. In addition, patients with CRC showing Reg IV staining had a significantly worse survival rate than those showing no Reg IV staining. Because an increase in the resistance to apoptosis is part of the metastatic evolution of CRC cells [20], inhibition of apoptosis by Reg IV may participate in liver metastasis. It has also been reported that Reg IV induces the expression of matrix metalloproteinase-7 (MMP-7, also known as matrilysin) [17] which promotes liver metastases [21]. Overexpression of Reg IV may contribute to liver metastasis through induction of MMP-7.

Interestingly, extensive Reg IV staining was observed in peritumoral mucosa of CRC, and Reg IV staining decreased gradually with increasing distance from the tumor tissue. This tendency was observed in goblet cells but not in neuroendocrine cells. It is well known that the peritumoral mucosa of CRC is often hyperplastic [22]. Whether the hyperplastic mucosa adjacent to CRC is a precancerous lesion or is a response to the growing cancer is unclear. We reported that EGFR is induced in the peritumoral mucosa of CRC [22], suggesting that inhibition of apoptosis may contribute to hyperplastic changes in the peritumoral mucosa of CRC. Enhancement of proliferative activity by Reg IV may also account for hyperplastic changes in peritumoral mucosa. Because Reg IV is a secreted protein, Reg IV from the peritumoral mucosa may assist in the proliferation of cancer cells. It has been reported that various growth factors, e.g. transforming growth factor-α and basic fibroblast growth factor, are increased in the peritumoral mucosa of CRC [22], and these growth factors induce Reg IV expression. Thus, Reg IV protein expression induced by growth factors may function as a growth-promoting and/or an antiapoptotic factor in the peritumoral mucosa of CRC.

In the present study, we measured Reg IV levels in the sera of patients with CRC by ELISA. Although expression of Reg IV was detected in CRC by immunohistochemistry, preoperative levels of Reg IV were increased in a small number of serum samples from CRC patients at stage 0–

III, indicating that serum Reg IV is unsuitable for early detection of CRC. The sensitivity of serum Reg IV was lower than that of CEA. In addition, Spearman's rank correlation test revealed a significant correlation between serum Reg IV and CEA levels, suggesting that measuring both markers in serum may not improve overall sensitivity for the detection of CRC. The sensitivity of a combination of serum Reg IV, CEA and CA19-9 for CRC detection (56%) was slightly higher than that of serum CEA alone (44%). In the present study, Reg IV staining was observed in the peritumoral mucosa of all CRC cases examined, suggesting that Reg IV protein from intramucosal cells may flow into the intestinal lumen but not into blood vessels. In fact, high serum concentrations of Reg IV (>2.00 ng/ml) were not detected in CRC cases showing Tis. Although we confirmed that Reg IV levels in serum samples from CRC patients showing Reg IV staining were significantly higher than those in patients showing no Reg IV staining, high serum concentrations of Reg IV did not necessarily correspond with Reg IV immunostaining of the primary tumor in all CRC cases. This discrepancy between immunostaining and ELISA results may be due to methodological differences. Immunohistochemical results of Reg IV were evaluated as the percentage of stained cancer cells; the intensity of immunostaining was not evaluated because we had no suitable internal control for the intensity of immunostaining. More detailed quantitative methods for the measurement of Reg IV protein, such as intratumor Reg IV concentration, are needed to clarify the relation between Reg IV protein levels in sera and Reg IV levels in primary CRC samples. In GC tissues, both mucin-like staining and strong perinuclear staining of Reg IV were observed [10], whereas only mucin-like staining was observed in our CRC patients. Because the intensity of perinuclear staining was higher than that of mucin-like staining, it is possible that tumor cells showing strong perinuclear staining of Reg IV may be the main source of serum Reg IV.

In contrast, in CRC patients at stage IV the serum Reg IV concentration was significantly elevated preoperatively and significantly higher in CRC patients with liver metastases than in those without liver metastasis. This result is consistent with our immunohistochemical results showing that Reg IV-positive CRC cases frequently showed metastatic recurrence in the liver. The effect of secreted Reg IV on liver metastases should be investigated.

In summary, we showed that Reg IV is a good marker for poor survival in patients with CRC. Although the serum Reg IV concentration is unsuitable for early detection of CRC, it may predict metastatic recurrence in the liver. The mechanism by which Reg IV promotes liver metastasis remains unclear. Identification of a cell surface receptor for Reg IV will further improve our understanding of the basic mechanisms of action of Reg IV.

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Cancer-Testis Antigen Lymphocyte Antigen 6 Complex Locus K Is a Serologic Biomarker and a Therapeutic Target for Lung and Esophageal Carcinomas

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Abstract

Gene expression profile analyses of non–small cell lung carcinomas (NSCLC) and esophageal squamous cell carcinomas (ESCC) revealed that lymphocyte antigen 6 complex locus K (*LY6K*) was specifically expressed in testis and transactivated in a majority of NSCLCs and ESCCs. Immunohistochemical staining using 406 NSCLC and 265 ESCC specimens confirmed that LY6K overexpression was associated with poor prognosis for patients with NSCLC ($P = 0.0003$), as well as ESCC ($P = 0.0278$), and multivariate analysis confirmed its independent prognostic value for NSCLC ($P = 0.0035$). We established an ELISA to measure serum LY6K and found that the proportion of the serum LY6K-positive cases was 38 of 112 (33.9%) NSCLC and 26 of 81 (32.1%) ESCC, whereas only 3 of 74 (4.1%) healthy volunteers were falsely diagnosed. In most cases, there was no correlation between serum LY6K and conventional tumor markers of carcinoembryonic antigen (CEA) and cytokeratin 19-fragment (CYFRA 21-1) values. A combined ELISA for both LY6K and CEA classified 64.7% of lung adenocarcinoma patients as positive, and the use of both LY6K and CYFRA 21-1 increased sensitivity in the detection of lung squamous cell carcinomas and ESCCs up to 70.4% and 52.5%, respectively, whereas the false positive rate was 6.8% to 9.5%. In addition, knocked down of LY6K expression with small interfering RNAs resulted in growth suppression of the lung and esophageal cancer cells. Our data imply that a cancer-testis antigen, LY6K, should be useful as a new type of tumor biomarker and probably as a target for the development of new molecular therapies for cancer treatment. [Cancer Res 2007;67(24):11601–11]

Introduction

Aerodigestive tract cancer (including carcinomas of lung, esophagus, oral cavity, pharynx, and larynx) accounts for one third of all cancer deaths in the United States and is the most common cancer in some areas of the world (1). Lung cancer is one of the most common malignant tumors in the world, and non–small cell lung cancer (NSCLC) accounts for nearly 80% of those cases (2). Esophageal squamous cell carcinoma (ESCC) is one of the most lethal malignancies of the digestive tract, and at the time of diagnosis, most of the patients are at advanced stages (3). In spite of the use of modern surgical techniques combined with various adjuvant treatment modalities, such as radiotherapy and chemotherapy, the overall 5-year survival rate of ESCC patients still remains at 40% to 60% (4) and that of lung cancer patients is only 15% (5, 6), although a reduced quality of life remains in those who do survive. Several tumor markers, such as progastrin-releasing peptide, neuron-specific enolase, cytokeratin 19-fragment (CYFRA 21-1), squamous cell carcinoma antigen (SCC), and carcinoembryonic antigen (CEA) are shown to be elevated in serum of lung cancer patients (7), whereas SCC, CEA, and CYFRA 21-1 are elevated in the serum of advanced ESCC patients (8). However, their sensitivity remains at 20% to 50% and no tumor marker has been sufficiently useful for detection of lung cancer and ESCC at potentially curative stage, and a limited number of practical prognostic biomarker is presently available for selection of treatment modalities for individual patients. Therefore, new diagnostic tools and therapeutic strategies, such as development of molecular targeted agents, antibody therapy, and cancer vaccines, are urgently required (6).

Cancer-testis antigens are defined to be proteins that are highly expressed in cancer cells but not in normal cells, except for cells in reproductive tissues, such as testis, ovary, and placenta (9, 10). Because the cells from these tissues do not express MHC class I molecules, cancer-testis antigens are considered to be a promising target for immunotherapy, such as cancer vaccines, and also a good biomarker for diagnosis of cancer and monitoring of relapse.

Systematic analysis of expression levels of thousands of genes using a cDNA microarray technology is an effective approach for identifying molecules involved in pathways of carcinogenesis or those associated with efficacy to anticancer therapy (11–16); some of such genes or their gene products may be good target molecules for development of novel therapies and/or cancer biomarkers. To identify such molecules, particularly for cancer-testis antigens, we had performed genome-wide expression profile analysis of 101 lung cancer and 19 ESCC patients, coupled with enrichment of tumor cells by laser capture microdissection (12–16) and then compared with the expression profile data of 31 normal human tissues (27 adult and 4 fetal organs; refs. 17, 18). To verify the biomedical and clinicopathologic significance of the respective gene products, we have established a screening system by a combination of the

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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tumor-tissue microarray analysis of clinical lung and esophageal cancer materials and RNA interference technique (19–33). This systematic approach revealed that lymphocyte antigen 6 complex locus K (*LY6K;* alias HSJ001348, cDNA for differentially expressed CO16 gene), a member of LY6 family, is likely to be a novel cancer-testis antigen that was overexpressed commonly in primary NSCLCs and ESCCs and was essential for growth/survival of cancer cells.

LY6K was initially identified as an unannotated transcript by several groups (accession no. AJ001348; AB105187), and recent analysis by Bioinformatics classified it as a member belonging to the LY6 family that shows a high homology to the low-molecular weight GPI-anchored molecule (34). A previous study showed the elevated expression of *LY6K* mRNA in human head and neck squamous cell carcinomas and breast cancers (34, 35); however, no report has clarified the significance of activation of LY6K in human cancer progression and its potential as a therapeutic target, as well as serologic/prognostic biomarker.

Here, we report evidence that LY6K, a member of cancer-testis antigen, plays a significant role in pulmonary and esophageal carcinogenesis, and suggest that LY6K could be a new type of diagnostic and prognostic biomarker that could detect certain proportion of cancer patients that may not be diagnosed by conventional tumor makers in the clinic.

Materials and Methods

Cell lines and tissue samples. The 18 human NSCLC cell lines, 14 esophageal carcinoma cell lines, and human small airway epithelial cells were used as described elsewhere (19, 30). Primary NSCLC and ESCC samples had been obtained earlier with informed consent (refs. 15, 16, 25, 30; see Supplementary Materials and Methods). A total of 406 formalin-fixed samples of primary NSCLCs and adjacent normal lung tissues had been obtained earlier along with clinicopathologic data from patients undergoing surgery at Saitama Cancer Center. These NSCLC patients received resection of their primary cancers without preoperative chemotherapy and/or radiotherapy, and among them, only patients with positive lymph-node metastasis were treated with cisplatin-based adjuvant chemotherapies after their surgery. A total of 265 formalin-fixed primary ESCCs and adjacent normal esophageal tissue samples had also been obtained from patients undergoing curative surgery at Keiyukai Sapporo Hospital. These ESCC patients received resection of their primary cancers without preoperative chemotherapy and/or radiotherapy. NSCLC specimen and five tissues (heart, liver, lung, kidney, and testis) from postmortem materials (two individuals with SCC) were also obtained from Hiroshima University. The pathologic stage was determined according to the classification of the Union Internationale Contre le Cancer (36). This study and the use of all clinical materials mentioned were approved by individual institutional ethical committees.

Serum samples. Serum samples were obtained with informed consent from 74 healthy individuals as controls and from 65 nonneoplastic lung disease patients with chronic obstructive pulmonary disease (COPD) enrolled as a part of the Japanese Project for Personalized Medicine (BioBank Japan) or admitted to Hiroshima University Hospital. All of these COPD patients were current and/or former smokers. [The mean (±1 SD) of pack-year index was 55.6 ± 50.1 SD; pack-year index was defined as the number of cigarette packs (20 cigarettes per pack) consumed a day multiplied by years]. Serum samples were also obtained with informed consent from 112 NSCLC patients admitted to Hiroshima University Hospital, as well as Kanagawa Cancer Center Hospital, and 81 esophageal cancer patients, who were admitted to Keiyukai Sapporo Hospital or who were registered in the BioBank Japan. These 112 NSCLC cases included 85 adenocarcinomas and 27 SCCs (see Supplementary Materials and Methods). Samples were selected for the study on the basis of the following criteria: (*a*) patients were newly diagnosed and previously untreated and (*n*) their tumors were pathologically diagnosed as lung or esophageal cancers (stages I-IV). Serum was obtained at the time of diagnosis and stored at −150°C.

Semiquantitative reverse transcription–PCR and Northern blot analyses. We performed semiquantitative reverse transcription–PCR (RT-PCR) and Northern blot analyses as described in Supplementary Materials and Methods.

Preparation of anti-LY6K polyclonal antibody. Two types of rabbit antibodies termed TM38 and MB44 specific for LY6K were raised by immunizing rabbits with 6-histidine fused human LY6K protein (codons 23-109 and 71-204, respectively) and purified with standard protocols using affinity columns (Affi-gel 10; Bio-Rad Laboratories) conjugated with the 6-histidine fused protein. On Western blots, we confirmed that the antibodies were specific for LY6K, using lysates from NSCLC tissues and cell lines, as well as normal lung tissues.

Western blot analysis. We used an enhanced chemiluminescence Western blotting analysis system (GE Healthcare Biosciences). SDS-PAGE was performed in 7.5% polyacrylamide gels. PAGE-separated proteins were electroblotted onto nitrocellulose membranes (GE Healthcare Biosciences) and incubated with a rabbit polyclonal antihuman LY6K antibody. A goat antirabbit IgG–horseradish peroxidase (HRP) antibody (GE Healthcare Biosciences) was served as the secondary antibodies for these experiments.

Immunohistochemistry and tissue microarray. Tumor-tissue microarrays were constructed using 406 formalin-fixed primary NSCLCs and 265 ESCCs, as published previously (see Supplementary Materials and Methods; refs. 37–39).

To investigate the status of the LY6K protein in clinical lung-cancer samples that had been embedded in paraffin blocks, we stained the sections in the following manner. Briefly, a rabbit polyclonal antihuman LY6K antibody (TM38) was added after blocking of endogenous peroxidase and proteins. The sections were incubated with HRP-labeled antirabbit IgG as the secondary antibody. Substrate-chromogen was added, and the specimens were counterstained with hematoxylin.

Three independent investigators assessed LY6K positivity semiquantitatively without prior knowledge of clinicopathologic data. The intensity of LY6K staining was evaluated using the following criteria: strong positive (2+), dark brown staining in >50% of tumor cells completely obscuring cytoplasm; weak positive (1+), any lesser degree of brown staining appreciable in tumor cells; absent (scored as 0), no appreciable staining in tumor cells. Cases were accepted only as strongly positive if reviewers independently defined them as such.

Statistical analysis. We used contingency tables to analyze the relationship of LY6K expression levels and clinicopathologic variables of NSCLC patients. Tumor-specific survival curves were calculated from the date of surgery to the time of death related to NSCLC or to the last follow-up observation. Kaplan-Meier curves were calculated for each relevant variable and for LY6K expression; differences in survival times among patient subgroups were analyzed using the log-rank test. Univariate and multivariate analyses were performed with the Cox proportional hazard regression model to determine associations between clinicopathologic variables and cancer-related mortality (see Supplementary Materials and Methods).

ELISA. Serum levels of LY6K were measured by sandwich-type ELISA, which had been originally constructed. In brief, for detection of soluble LY6K in serum, 96-well flexible microtiter plates (Nalge Nunc International) were coated with 2 ng/mL of capturing polyclonal antibody to LY6K (TM38) overnight. Wells were blocked with 200 μL PBS (pH 7.4) containing 1% bovine serum albumin (BSA), 5% sucrose, and 0.05% NaN_3 for 2 h and then incubated for 2 h with 3-fold diluted serum samples in PBS (pH 7.4) containing 1% BSA. After washing with PBS (pH 7.4) containing 0.05% Tween 20, the wells were incubated for 2 h with 200 ng/mL of biotin-conjugated polyclonal anti-LY6K antibody (MB44), followed by reaction with avidin-conjugated peroxidase (P347; Dako Cytomation) for 30 min using a substrate reagent (R&D Systems). To prepare biotinylating rabbit polyclonal antibodies to LY6K (MB44), we used Biotin Labeling Kit-NH2 (LK03) according to the supplier's protocol (Dojindo Laboratories). Levels of CEA in serum were measured by ELISA with a commercially available enzyme test kit (Hope Laboratories, Belmont, CA), according to the

supplier's recommendations. Levels of CYFRA 21-1 in serum were measured by ELISA with a commercially available kit (DRG; see Supplementary Materials and Methods).

RNA interference assay. We had previously established a vector-based RNA interference system, psiH1BX3.0, to direct the synthesis of small interfering RNAs (siRNA) in mammalian cells (see Supplementary Materials and Methods; refs. 19, 21).

Results

LY6K expression in lung and esophageal tumors, cell lines, and normal tissues. To identify novel molecules, such as cancer-testis antigens to serve as diagnostic biomarkers and/or targets for development of therapeutic agents for lung and esophageal cancers, we had applied cDNA microarray analysis to search for genes that were highly transactivated in a large proportion of these tumors, but scarcely expressed in normal tissues. Among 27,648 genes screened, we identified the *LY6K* transcript to show >3-fold expression in ~ 80% of NSCLCs and ESCCs compared with normal lung or esophageal tissue (control). Moreover, *LY6K* showed testis-specific expression in normal tissues. Therefore, we determined to select LY6K as a good candidate for further analyses. We confirmed its transactivation by semiquantitative RT-PCR experiments in 9 of 10 additional NSCLC tissues, in 10 of 18 lung-cancer cell lines, in 8 of 8 ESCC tissues, and in 12 of 14 esophageal cancer cell lines (Fig. 1*A* and *B*).

We subsequently generated rabbit polyclonal antibodies specific for human LY6K and confirmed by Western blot analysis an expression of LY6K protein in NSCLC samples in four representative pairs of NSCLC tissues and in four lung-cancer cell lines (two LY6K-positive and two LY6K-negative cell lines; Fig. 1*C*). We performed immunofluorescence analysis to examine the subcellular localization of endogenous LY6K in the four lung-cancer cell lines (LC319, NCI-H1373, NCI-H226, and A427) and found that LY6K was mainly located at cytoplasm of tumor cells with granular appearance (Fig. 1*D, left*). Because LY6K encodes GPI-anchored protein and some of GPI-anchored proteins were known to be secreted into extracellular space (40), we applied ELISA to examine its presence in the culture media of the lung-cancer cell lines. The amounts of detectable LY6K in the culture media was concordant to the expression levels of LY6K detected with semiquantitative RT-PCR and Western blot analyses (Fig. 1*D, right*).

Northern blot analysis using a *LY6K* cDNA fragment as the probe identified a transcript of ~ 1.8 kb that was highly and exclusively expressed in testis among 23 normal human tissues examined (Fig. 2*A*). We subsequently examined expression of LY6K protein in five normal tissues (heart, liver, lung, kidney, and testis), as well as lung cancers using anti-LY6K antibody, and found that it was hardly detectable in the former four tissues while positive LY6K staining appeared in testis and lung tumor tissues (Fig. 2*B*).

Association of LY6K overexpression with poor clinical outcomes for NSCLC and ESCC patients. To verify the biological and clinicopathologic significance of LY6K, we examined the expression of LY6K protein by means of tissue microarrays consisting of 406 NSCLC and 265 ESCC cases that underwent surgical resection. LY6K staining was observed mainly in the cell membrane and cytoplasm of tumor cells, but was hardly detectable in surrounding normal tissues (Fig. 2*C, top* and *bottom*). We classified a pattern of LY6K expression on the tissue array ranging from absent/weak (scored as 0-1+) to strong (2+). Positive staining was observed in 219 of 254 (86.2%) lung adenocarcinoma cases, 103 of 112 (92.0%) lung SCCs, 24 of 28 (85.7%) lung large cell carcinomas, and 12 of 12 (100%) lung adenosquamous carcinomas, whereas no staining was observed in any of the normal portions of the same tissues. Of the 406 NSCLC cases examined, LY6K was strongly stained in 139 cases (34.2%; score 2+), weakly stained in 219 cases (54.0%; score 1+), and not stained in 48 cases (11.8%; score 0; details were shown in Table 1*A*). NSCLC patients whose tumors showed strong LY6K expression revealed shorter tumor-specific survival compared with those with absent/weak LY6K expression (P = 0.0003 by log-rank test; Fig. 2*D, left*). We also applied univariate analysis to evaluate associations between patient prognosis and other factors, including age (<65 versus ≥65), gender (female versus male), histologic type (adenocarcinoma versus nonadenocarcinoma), pT classification (T1, T2 versus T3, T4), pN classification (N0 versus N1, N2), and LY6K status (0, 1+ versus 2+). Among those variables, LY6K status (P = 0.0004), elderly (P = 0.0093), male (P = 0.0050), nonadenocarcinoma histologic classification (P = 0.0108), advanced pT stage (P < 0.0001), and advanced pN stage (P < 0.0001) were significantly associated with poor prognosis (Table 1*B*). In multivariate analysis of the prognostic factors, strong LY6K expression, elderly, higher pT stage, and higher pN stage were indicated to be independent prognostic factors (P = 0.0035, <0.0001, <0.0001, and <0.0001, respectively; Table 1*B*).

Positive staining was observed in 252 of 265 (95.1%) esophageal cancers, whereas no staining was observed in any of the normal portions of the same tissues. LY6K was strongly stained in 175 cases (66.0%; score 2+), weakly stained in 77 cases (29.1%; score 1+), and not stained in 13 cases (4.9%; score 0; details were shown in Table 2*A*). The median survival time of ESCC patients was significantly shorter in accordance with the higher expression levels of LY6K (P = 0.0278 by log-rank test; Fig. 2*D, right*). We also applied univariate analysis to evaluate associations between ESCC patient prognosis and several factors including age (<65 versus ≥65), gender (female versus male), pT stage (tumor depth; T1, T2 versus T3, T4), pN stage (node status; N0 versus N1), and LY6K status (score; 0, 1+ versus 2+). Among those variables, LY6K status (P = 0.0289), male (P = 0.0029), advanced pT stage (P < 0.0001), and advanced pN stage (P < 0.0001) were significantly associated with poor prognosis (Table 2*B*). In multivariate analysis, LY6K status did not reach the statistically significant level as an independent prognostic factor for surgically treated ESCC patients enrolled in this study (P = 0.2266), whereas pT and pN stages, as well as male gender, did, suggesting the relevance of LY6K expression to these clinicopathologic factors in esophageal cancer (P = 0.0149, 0.0047, and <0.0001, respectively; Table 2*B*).

Serum levels of LY6K in patients with NSCLC or ESCC. Because the *in vitro* findings had suggested that LY6K could be secreted into extracellular space (Fig. 1*D, right*), we examined whether LY6K was secreted into serum from patients with NSCLC or ESCC to validate its potential as a novel serum biomarker. ELISA experiments detected LY6K in serologic samples from the great majority of the 193 patients with lung or esophageal cancer. The mean (±1 SD) of serum LY6K in 112 lung cancer patients was 331.3 ± 739.3 pg/mL and those in 81 ESCC patients were 209.3 ± 427.4 pg/mL. In contrast, the mean (±1 SD) serum levels of LY6K in 74 healthy individuals were 34.2 ± 65.3 pg/mL, and those in 65 patients with COPD, who were current and/or former smokers, were 54.4 ± 233.8 pg/mL. The levels of serum LY6K protein were significantly higher in lung or esophageal cancer patients than in healthy donors (between lung adenocarcinoma patients and healthy individuals, P < 0.0001; between lung SCCs and healthy

individuals, P = 0.0145; between ESCCs and healthy individuals, P < 0.0001; Mann-Whitney U test), whereas the difference between healthy individuals and COPD patients was not significant (P = 0.5325; Fig. 3*A*). According to histologic types of lung cancer, the mean (±1 SD) serum levels of LY6K were 324.1 ± 737.4 pg/mL in 85 adenocarcinoma patients and 354.1 ± 758.8 pg/mL in 27 SCC patients; the differences between the two histologic types were not significant. High levels of serum LY6K were detected even in

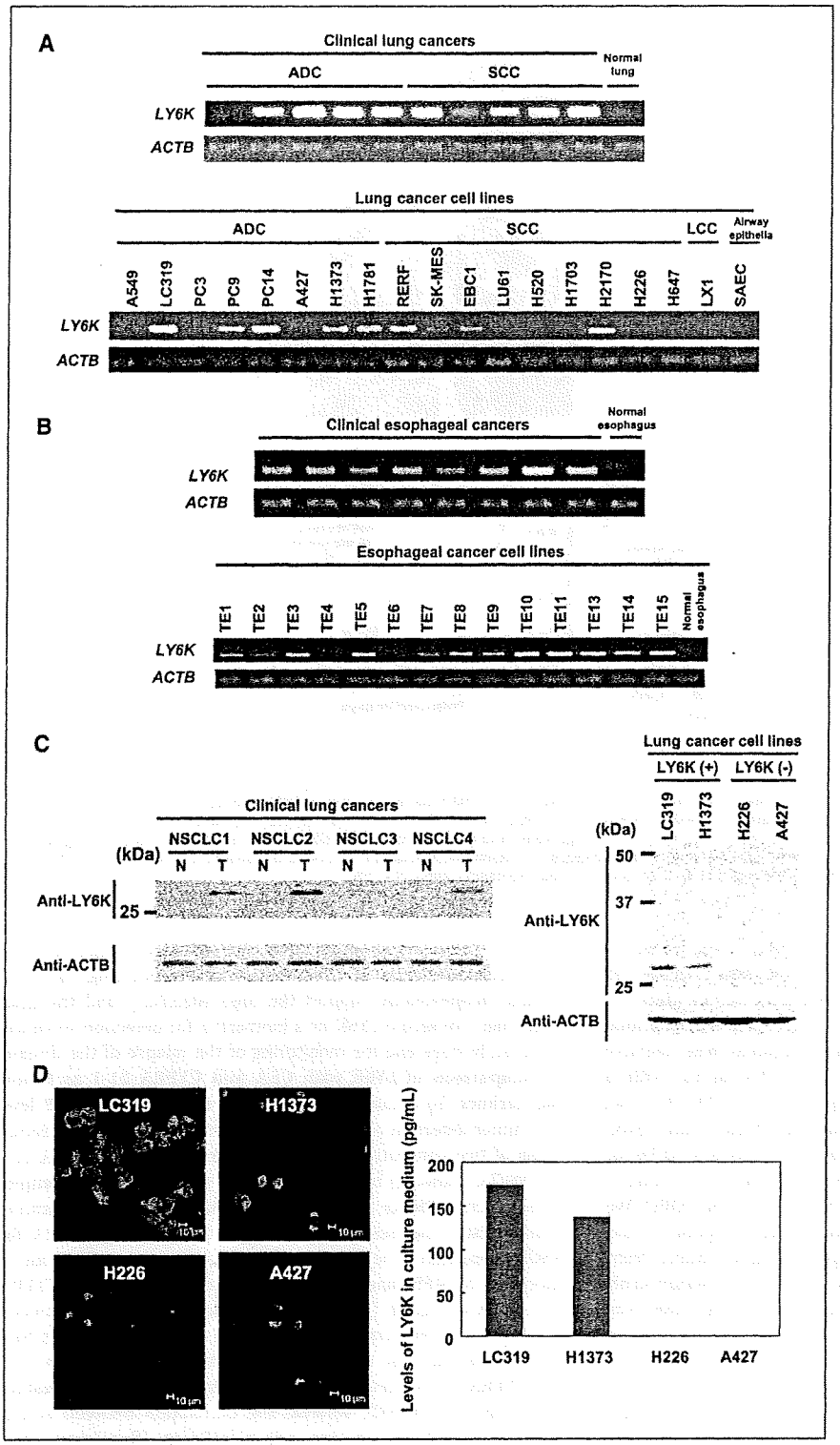

Figure 1. Expression and subcellular localization of LY6K in NSCLCs and ESCCs. *A*, expression of *LY6K* in 10 clinical NSCLC samples (*top*) and 18 lung cancer cell lines (*bottom*) examined by semiquantitative RT-PCR analysis. *B*, expression of *LY6K* in eight clinical ESCC samples (*top*) and 14 esophageal cancer cell lines (*bottom*), as detected by semiquantitative RT-PCR analysis. *C*, expression of LY6K protein in four representative pairs of NSCLC samples (*left*) and four lung cancer cell lines (*right*) examined by Western blot analysis. *D*, subcellular localization of endogenous LY6K protein in lung cancer cells (*left*). LY6K is stained at the cytoplasm of the cell with granular appearance in LC319 and NCI-H1373 cells, but not in NCI-H226 and A427 cells (*right*). Measurement of secreted LY6K levels with ELISA in culture medium of LY6K-expressing LC319 and NCI-H1373 cells and nonexpressing NCI-H226 and A427 cells.

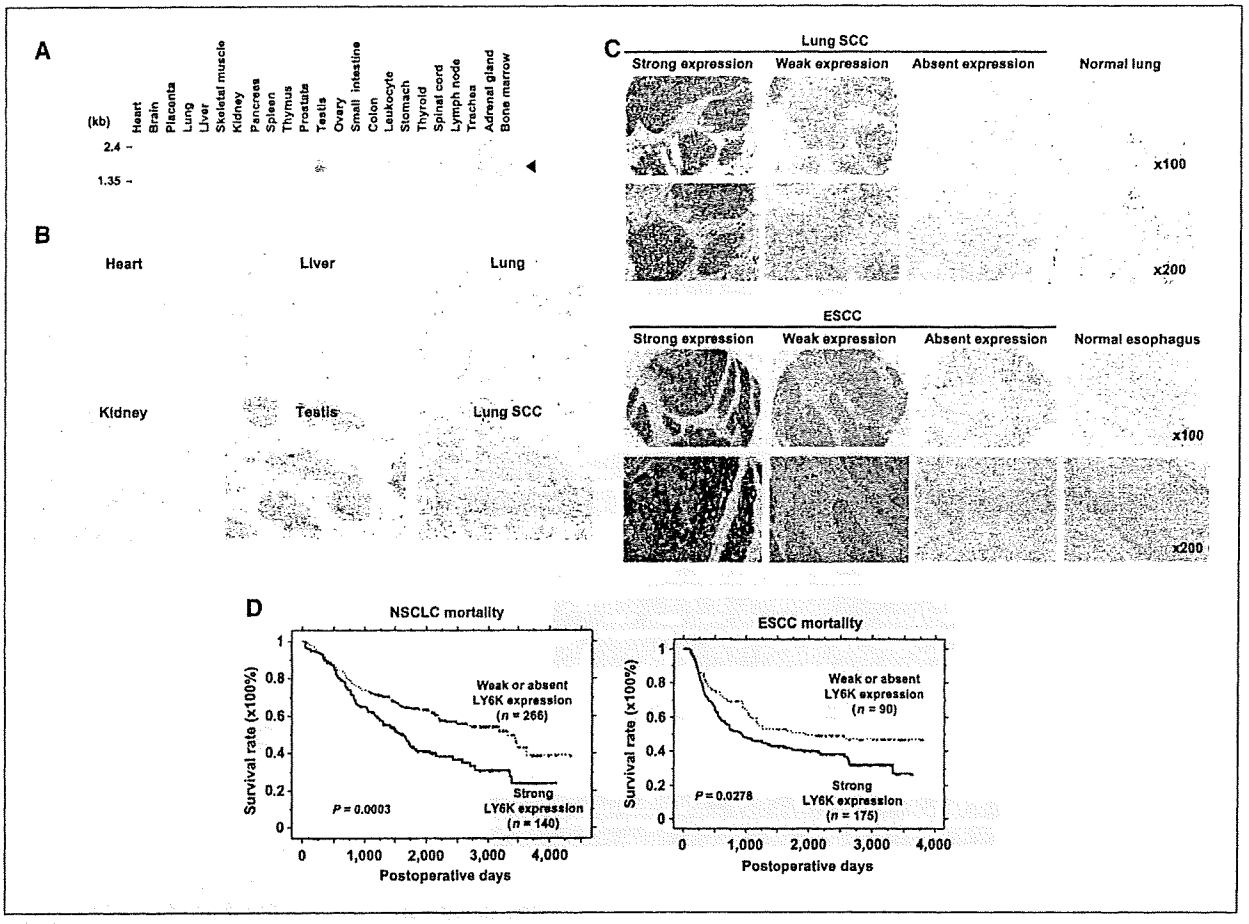

Figure 2. Expression of LY6K in normal tissues, as well as NSCLC and ESCC tissues, and association of LY6K overexpression with poor clinical outcomes for NSCLC and ESCC patients. *A*, Northern blot analysis of the *LY6K* transcript in 23 normal adult human tissues. *B*, immunohistochemical evaluation of LY6K protein in representative normal tissues: adult heart, liver, lung, kidney, and testis, as well as lung SCC tissues. *C*, immunohistochemical evaluation of LY6K expression on tumor tissue microarrays (*top*, ×100; *bottom*, ×200). Examples are shown of strong, weak, and absent LY6K expressions in cancer tissues and of no expression in normal tissues. *Top*, lung SCC and normal lung; *bottom*, ESCC and normal esophagus. *D*, Kaplan-Meier analysis of survival of patients with NSCLC (*left*, $P = 0.0003$ by the log-rank test) and ESCC (*right*, $P = 0.0278$ by the log-rank test) according to the expression levels of LY6K.

patients with earlier-stage tumors (Fig. 3*B*). Using receiver-operating characteristic curves drawn with the data of these 193 lung or esophageal cancer patients and 74 healthy donors (data not shown), the cutoff level in this assay was set to provide optimal diagnostic accuracy and likelihood ratios (minimal false negative and false positive results) for LY6K, i.e., 157.0 pg/mL with a sensitivity of 33.2% (64 of 193) and a specificity of 4.1% (3 of 74). According to tumor histology, the proportions of the serum LY6K-positive cases were 31.8% for adenocarcinoma (27 of 85), 40.7% for SCC (11 of 27), and 32.1% for ESCC (26 of 81). The proportions of the serum LY6K-positive cases were 9.2% (6 of 65) for COPD. We then performed ELISA experiments using paired preoperative and postoperative (2 months after the surgery) serum samples from lung cancer and ESCC patients to monitor the levels of serum LY6K in the same patients. The concentration of serum LY6K was significantly reduced after surgical resection of primary tumors (Fig. 3*C*). We further compared the serum LY6K values with the expression levels of LY6K in primary tumors in the same set of 16 NSCLC cases whose serum had been collected before surgery (eight patients with LY6K-positive tumors and eight with LY6K-negative tumors). The levels of serum LY6K showed good correlation with the expression levels of LY6K in primary tumor (Fig. 3*D*). The results independently support the high specificity and the great potentiality of serum LY6K as a biomarker for detection of cancer at an early stage and for monitoring of the relapse of the disease.

Comparison of LY6K with CEA and CYFRA 21-1 as tumor biomarkers. To evaluate the feasibility of using serum LY6K level as a tumor detection biomarker, we also measured by ELISA serum levels of two conventional tumor markers (CEA and CYFRA 21-1 for NSCLC patients) in the same set of available serum samples from cancer patients and control individuals. Receiver-operating characteristic analyses determined the cutoff value of CEA for NSCLC detection to be 2.5 ng/mL (with a sensitivity of 39.3% and a specificity of 94.6%) and also determined the cutoff value of CYFRA 21-1 to be 2.0 pg/mL (with a sensitivity of 33.9% and a specificity of 97.2%; data not shown). As shown in Supplementary Fig. S1*A* (*left* and *middle*), the correlation coefficient between serum CEA and CYFRA 21-1 values was significant (Spearman rank correlation $\rho = 0.355$, $P = 0.0002$), whereas the correlation between serum LY6K and CEA values was not significant (Spearman rank

Table 1.

A. Association between LY6K positivity in NSCLC tissues and patients' characteristics (n = 406)

		LY6K strong positive	LY6K weak positive	LY6K absent	P (strong versus weak/absent)
	n = 406	n = 139	n = 219	n = 48	
Gender					
Male	280	104	144	32	0.0707
Female	126	35	75	16	
Age (y)					
<65	198	70	107	21	0.6022
≥65	208	69	112	27	
Histologic type					
ADC	254	70	149	35	0.0002*,†
SCC	112	51	52	9	
Others	40	18	18	4	
pT factor					
$T_1 + T_2$	294	100	159	35	>0.9999
$T_3 + T_4$	112	39	60	13	
pN factor					
N_0	253	78	142	33	0.0526
$N_1 + N_2$	153	61	77	15	

B. Cox's proportional hazards model analysis of prognostic factors in patients with NSCLCs

Variables	Hazards ratio (95% CI)	Unfavorable/favorable	P
Univariate analysis			
LY6K	1.684 (1.265-2.240)	Strong (+)/weak (+) or (−)	0.0004 †
Age (y)	1.467 (1.099-1.958)	≥65/<65	0.0093 †
Gender	1.597 (1.151-2.216)	Male/female	0.0050 †
Histologic type	1.450 (1.090-1.929)	Others/ADC[1]	0.0108 †
pT factor	2.039 (1.515-2.745)	$T_3 + T_4/T_1 + T_2$	<0.0001 †
pN factor	2.430 (1.827-3.231)	$N_1 + N_2/N_0$	<0.0001 †
Multivariate analysis			
LY6K	1.554 (1.156-2.088)	Strong (+)/weak (+) or (−)	0.0035 †
Age (y)	1.838 (1.369-2.468)	≥65/<65	<0.0001 †
Gender	1.368 (0.953-1.965)	Male/female	0.0892
Histologic type	1.053 (0.762-1.456)	Others/ADC[1]	0.7530
pT factor	1.947 (1.439-2.636)	$T_3 + T_4/T_1 + T_2$	<0.0001 †
pN factor	2.369 (1.763-3.184)	$N_1 + N_2/N_0$	<0.0001 †

Abbreviations: 95% CI, 95% confidence interval; ADC, adenocarcinoma; others, large cell carcinoma plus adenosquamous cell carcinoma.
*Adenocarcinoma versus other histology.
† $P < 0.05$ (Fisher's exact test).

correlation ρ = 0.021, P = 0.8275), indicating that measuring both markers in serum can improve overall sensitivity for detection of NSCLC to 61.6%. False-positive results for either of the two tumor markers among normal volunteers (control group) accounted for 9.5%, whereas the false-positive rates for CEA and LY6K in the same control group were 4.1% and 5.4%, respectively. According to tumor histology, the sensitivity of the combination of serum LY6K and CEA as a tumor detection marker was 64.7% for adenocarcinoma and 51.6% for SCC, suggesting the usefulness of this combination for adenocarcinoma detection.

The correlation coefficient between serum LY6K and CYFRA 21-1 values for NSCLC patients was not significant (Spearman rank correlation ρ = 0.119, P = 0.2114; Supplementary Fig. S1*A, right panel*), also indicating that measurement of serum levels of both markers can improve overall sensitivity for detection of NSCLC to 59.8%; for diagnosing NSCLC, the sensitivity of CYFRA 21-1 alone was 33.9%. False-positive cases for either of the two tumor markers among normal volunteers (control group) were 6.8%, although the false-positive rates for CYFRA 21-1 in the same control group were 2.7%. According to tumor histology, the sensitivity of the combination of serum LY6K and CYFRA 21-1 for the detection of tumors was 56.5% for adenocarcinoma and 70.4% for SCC, indicating the usefulness of this combination for SCC detection. Combination of LY6K with both CEA and CYFRA 21-1 indicated

that 21 of 54 (38.9%) NSCLC patients who were negative for both CEA and CYFRA 21-1 were diagnosed as LY6K-positive (Supplementary Fig. S1*B*).

We further measured by ELISA serum levels of CEA and CYFRA 21-1 in the same set of serum samples from ESCC patients (Supplementary Fig. S1C). The correlation coefficient between serum LY6K and CEA values for ESCC patients was not significant (Spearman rank correlation ρ = 0.153, P = 0.0781; Supplementary Fig. S1*C*, *middle*), indicating that measuring both markers in serum can improve overall sensitivity for detection of ESCC to 44.3% whereas the sensitivity of CEA alone was 18.0%. The correlation between serum LY6K and CYFRA 21-1 values for ESCC patients was also not significant (Spearman rank correlation ρ = 0.034, P = 0.6989; Supplementary Fig. S1*C*, *right*). A combined assay for both LY6K and CYFRA 21-1 classified 52.5% of ESCC patients as positive, whereas the sensitivity of CYFRA 21-1 alone was 23.0%. Combination of LY6K with both CEA and CYFRA 21-1 indicated that 16 of 40 (40.0%) ESCC patients who were negative for both CEA and CYFRA 21-1 were diagnosed as LY6K-positive (Supplementary Fig. S1*D*). The data clearly suggest that serum LY6K levels were also high in certain proportion of cancer patients that could not be diagnosed by the combination of CEA and CYFRA 21-1.

Effect of LY6K-siRNAs on growth of NSCLC cells. To assess whether LY6K plays a role in growth or survival of lung cancer cells, we designed and constructed plasmids to express siRNA against *LY6K* (si-*LY6K*-1 and si-*LY6K*-2), along with two different control plasmids (siRNAs for EGFP and SCR), and transfected them into lung cancer (RERF-LC-AI and LC319) and esophageal cancer (TE8) cells to suppress expression of endogenous LY6K (representative data of RERF-LC-AI and TE8 was shown in Fig. 4 and Supplementary Fig. S2). The amount of LY6K protein in the cells transfected with si-*LY6K*-2 was significantly decreased in comparison with cells transfected with any of the two control siRNAs or si-*LY6K*-1 (Fig. 4 and Supplementary Fig. S2). In accordance with its suppressive effect on protein levels of LY6K, transfected si-*LY6K*-2 caused significant decreases in the numbers of viable cells (Fig. 4 and Supplementary Fig. S2).

Table 2.

A. Association between LY6K positivity in esophageal cancer tissues and patients' characteristics (n = 265)

	Total	LY6K strong positive	LY6K weak positive	LY6K absent	P (strong versus weak/absent)
	n = 265	n = 175	n = 77	n = 13	
Gender					
Male	239	159	70	10	0.6645
Female	26	16	7	3	
Age (y)					
<65	171	110	55	6	0.4982
≥65	94	65	22	7	
pT factor					
$T_1 + T_2$	124	72	42	10	0.0134*
$T_3 + T_4$	141	103	35	3	
pN factor					
N_0	99	60	32	7	0.1800
$N_1 + N_2$	166	115	45	6	

B. Cox's proportional hazards model analysis of prognostic factors in patients with esophageal cancers

Variables	Hazards ratio (95% CI)	Unfavorable/favorable	P
Univariate analysis			
LY6K	1.480 (1.041-2.105)	Strong (+)/weak(+) or (−)	0.0289*
Age (y)	1.024 (0.733-1.429)	≥65/<65	0.8924
Gender	3.165 (1.485-6.765)	Male/female	0.0029*
pT factor	2.660 (1.883-3.745)	$T_3 + T_4/T_1 + T_2$	<0.0001*
pN factor	3.984 (2.653-5.988)	$N_1 + N_2/N_0$	<0.0001*
Multivariate analysis			
LY6K	1.246 (0.872-1.781)	Strong (+)/weak(+) or (−)	0.2266
Gender	2.575 (1.202-5.514)	Male/female	0.0149*
pT factor	1.866 (1.211-2.882)	$T_3 + T_4/T_1 + T_2$	0.0047*
pN factor	3.096 (2.020-4.739)	$N_1 + N_2/N_0$	<0.0001*

*P < 0.05 (Fisher's exact test).

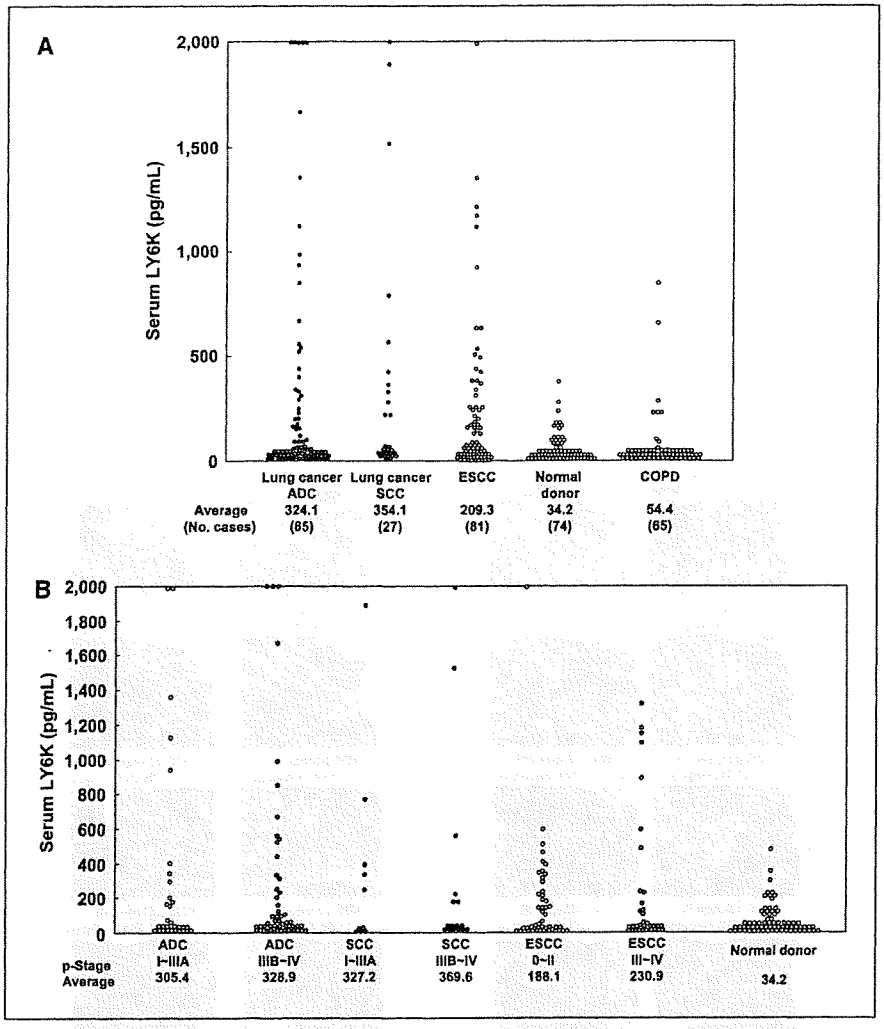

Figure 3. Serologic concentration of LY6K determined by ELISA in serum of patients with lung cancers or esophageal cancers and in healthy controls or nonneoplastic lung disease patients with COPD. *A*, distribution of LY6K in sera from patients with lung adenocarcinoma (*ADC*), lung SCC, and ESCC. Averaged serum levels are shown under the panel. Differences were significant between lung adenocarcinoma patients and healthy individuals ($P < 0.0001$, Mann-Whitney *U* test), between lung SCC patients and healthy individuals ($P = 0.0145$), and between ESCC patients and healthy individuals ($P < 0.0001$). *B*, distribution of LY6K in sera from patients at various clinical stages of lung cancers.

Discussion

Recent acceleration in identification and characterization of novel molecular targets for cancer therapy has enhanced development of new types of anticancer agents or antibodies and cancer vaccines (8). Molecular targeted drugs are expected to be highly specific to malignant cells, with minimal adverse effects due to their well-defined mechanisms of action. As an approach to such goal, one promising strategy is to combine the power of genome-wide expression analysis to effectively screen genes that are overexpressed in cancer cells but scarcely expressed in normal organs, with high throughput screening of their protein expression related to clinical outcome by means of tissue microarray, as well as with examining loss of function phenotypes by RNA interference systems (19–33). Using this combination of approaches for the study, we have shown that LY6K is a novel cancer-testis antigen, whose overexpression shows unfavorable prognostic significance in NSCLC patients and affects on growth of the cancer cells.

Like other LY6 antigens, LY6K has 10 cysteine residues in a conserved position and harbors the sequence structure that theoretically determines GPI anchoring. Members of the LY6 family are suspected to have functions related to cell signaling and/or cell adhesion (41), although the precise role of LY6K in lung carcinogenesis or the physiologic function in the normal cells is unknown. Because *LY6K* gene is located at chromosome 8q24, a region of allelic gain in more than half of lung cancers (42), its overexpression might be partly explained by amplification or chromosomal aberration at this locus.

There are several known GPI-anchored proteins that are applicable to diagnosis of human cancer in certain clinical or preclinical settings (43). Human CEA is supposed to be a GPI-anchored protein (43) and expressed highly in a significant proportion of relatively advanced adenocarcinomas, particularly those from the colon, pancreas, breast, and lung (44). Its presence in serum in cancer patients has been used for disease staging and as an indicator of residual disease and/or tumor recurrences (44). In addition, some tumor-specific markers and prognostic markers, such as CD109, glypican-3, CEA-related cell adhesion molecule 6, and prostate stem cell antigen, are categorized as the GPI-anchored protein (40, 45–47). Among them, CD109 and glypican-3 are also known to be the cancer-testis antigens. On the other hand, there are several reports on GPI-anchored proteins as

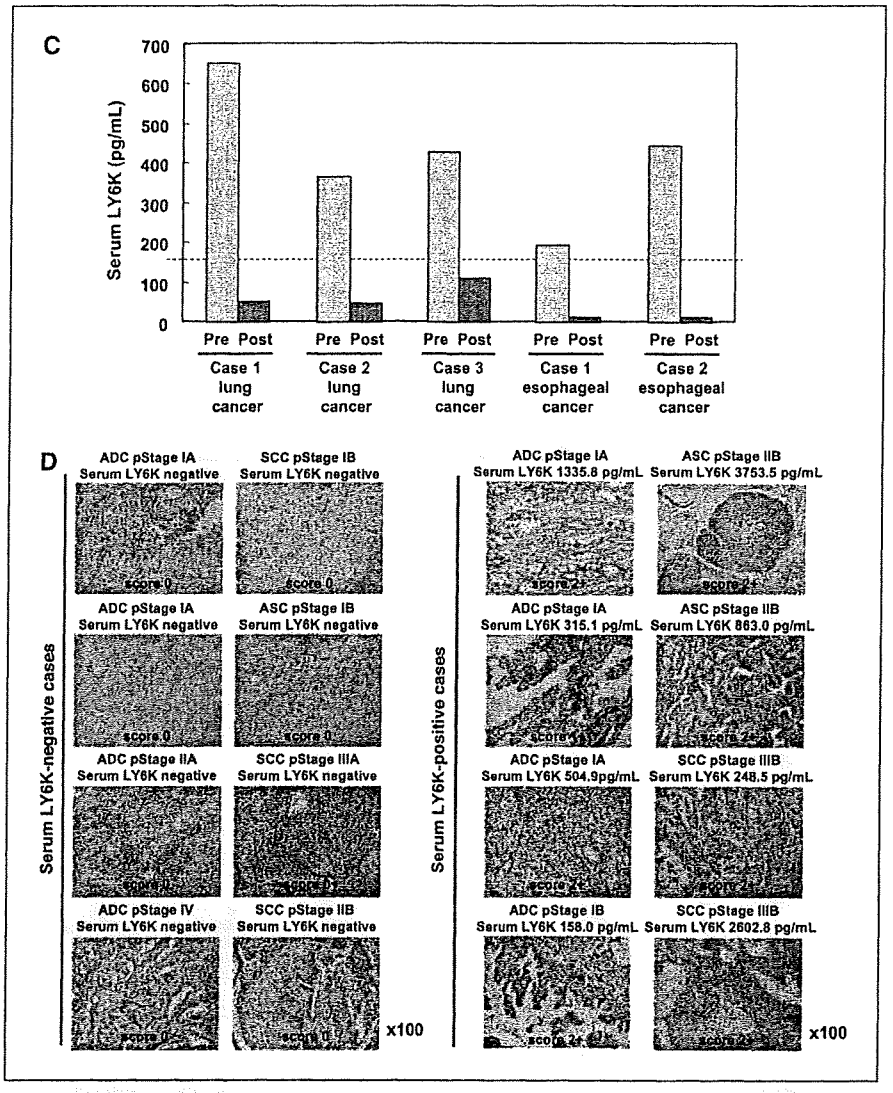

Figure 3 *Continued. C,* serologic concentration of LY6K before and after surgery (postoperative days at 2 mo) in patients with NSCLC and ESCC. *Dotted line,* cutoff level for LY6K (157.0 pg/mL). *D,* serum LY6K levels (pg/mL) and the expression levels of LY6K in primary tumor tissues in the same NSCLC patients. *Score,* intensity of LY6K staining that was evaluated using the criteria described in Materials and Methods.

immunotherapeutic targets for human cancer. The CEA-TRICOM (three T-cell costimulatory molecules: B7-1, intercellular adhesion molecule (ICAM)-1, and LFA-3, which were designated TRICOM) vaccines has been shown to safely generate significant CEA-specific immune responses against advanced cancer in the phase I clinical trial (48). Recently, two independent studies showed that a passive immunotherapy approach using anti-prostate stem cell antigen monoclonal antibody inhibited prostate tumor growth and metastasis formation and further prolonged survival times of mice bearing human prostate cancer xenografts (49, 50).

In this study, we showed that LY6K was expressed only in testis among the normal tissues examined and was highly expressed in 88.2% of surgically resected samples from NSCLC patients and in 95.1% of those from ESCC patients. The LY6K overexpression was associated with the shorter cancer-specific survival period. Suppression of LY6K expression with siRNA effectively suppressed growth of lung and esophageal cancer cells that expressed LY6K. The combined results strongly suggest that LY6K is likely to be associated with highly malignant phenotype of those tumors. Because LY6K is considered to be the cancer-testis antigens, LY6K might be a good target for cancer immunotherapy.

We also found that LY6K protein was secreted into serum from patients with lung cancer or esophageal cancer that strongly expressed LY6K. Positivity of serum LY6K seems to be considerably correlated with the presence of primary tumors, because the concentration of serum LY6K was dramatically reduced after surgical resection of primary tumors and the levels of serum LY6K showed good correlation with the expression levels of LY6K in primary tumor tissue in the same patients. Interestingly, the correlation coefficient between serum LY6K and CEA or CYFRA 21-1 values was not significant, whereas the correlation coefficient between serum CEA and CYFRA 21-1 values was significant. In fact, 38.9% to 40.0% of NSCLC and ESCC patients who were negative for both CEA and CYFRA 21-1 were diagnosed to be positive for LY6K (Supplementary Fig. S1*B* and *D*). An assay combining both LY6K and CEA/CYFRA 21-1 increased the sensitivity, such that 64.7% to 70.4% of the patients