Serum concentration and expression of Reg IV in patients with esophageal cancer: Age-related elevation of serum Reg IV concentration

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Abstract. *Regenerating islet-derived family, member 4* (*REG4*, which encodes Reg IV) is a marker for cancer and inflammatory bowel disease. This study aimed to investigate the diagnostic utility of Reg IV measurement in sera from esophageal cancer patients. Reg IV expression was examined in 269 esophageal cancer samples by immunostaining and the Reg IV levels in sera were measured from 65 patients with esophageal squamous cell carcinoma (SCC) by enzyme-linked immunosorbent assay. No Reg IV staining was detected in 255 SCC and 4 small cell carcinoma samples, whereas Reg IV was stained in 4 of 10 (40%) adenocarcinoma samples. Serum Reg IV concentration in esophageal SCC patients was significantly higher compared to that of the control subjects (P=0.0003). A significant correlation between serum Reg IV concentration and age was found in control subjects (P<0.0001). When serum Reg IV concentration was analyzed according to age, the distribution of serum Reg IV concentration in patients with esophageal SCC was similar to that of the control subjects. These results suggest that Reg IV expression is highly specific for adenocarcinoma of the esophagus. Further investigation is required to clarify whether Reg IV serves as a serum tumor marker for esophageal cancer.

Introduction

Regenerating islet-derived family, member 4 (*REG4*, which encodes Reg IV) is a member of the *REG* gene family, which constitutes a multi-gene family belonging to the calcium-dependent lectin superfamily. *REG4* was originally identified by high-throughput sequence analysis of a large inflammatory bowel disease cDNA library (1). Previously a serial analysis of gene expression in four primary gastric cancer tissues was performed and a number of gastric cancer-specific genes were identified (2,3). Of these genes, *REG4* is a candidate gene for cancer-specific expression in patients with gastric cancer. Our previous immunohistochemical analysis showed that Reg IV was expressed in 30% of gastric cancers and was associated with intestinal mucin phenotype and neuroendocrine differentiation (4). A number of immunohistochemical analyses of Reg IV have been reported in human cancers, including lung, breast, pancreas, colorectal, prostate, salivary gland, kidney, urinary bladder and gallbladder cancer (4-11). These analyses indicate that Reg IV is expressed in adenocarcinoma cells showing intestinal mucin differentiation. Reg IV staining also aids in the diagnosis of gastrointestinal signet ring cell carcinoma, a unique subtype of adenocarcinoma (12). By contrast, little is known about Reg IV expression in other histological types of cancer, such as squamous cell carcinoma (SCC).

In addition to adenocarcinoma, Reg IV expression has been reported in neuroendocrine neoplasms, including gastrointestinal and renal carcinoids (4,10,13). Reg IV expression is also found in small cell carcinoma of the lung (14). However, Reg IV expression in small cell carcinoma of the esophagus has yet to be investigated.

Reg IV is a secreted protein and a novel biomarker for gastric cancer (15). The diagnostic sensitivity of serum Reg IV was superior to that of serum carcinoembryonic antigen or carbohydrate antigen 19-9. Serum Reg IV serves as a tumor marker for colorectal, pancreatic and prostate cancer (5,7,10). The data support the hypothesis that Reg IV protein is a potentially novel serum tumor marker for a wide range of malignancies. However, the serum concentration of Reg IV in esophageal cancer has yet to be measured.

In the present study, the expression and distribution of Reg IV in human esophageal cancers, including SCC, adenocarcinoma and small cell carcinoma, was examined by immunohistochemistry. Previously two Reg IV staining patterns, i.e., mucin-like and strong perinuclear staining were reported (4). Mucin-like staining is observed in goblet cells

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and goblet cell-like vesicles of cancer cells. These cells are positive for MUC2 (a marker of goblet cells). By contrast, strong perinuclear staining is detected in neuroendocrine cells. These cells are positive for chromogranin A (a marker of neuroendocrine cells). Therefore, the coexpression of Reg IV and MUC2 or chromogranin A was examined. Additionally, the Reg IV levels in sera from patients with esophageal cancer were measured using an enzyme-linked immunosorbent assay (ELISA) to investigate the potential diagnostic utility of Reg IV measurement.

Materials and methods

Tissue samples. Primary tumor samples from 279 patients with esophageal cancer (35 females and 244 males; age range 36-84 years, mean 65) and serum samples from 65 patients with esophageal cancer (8 females and 57 males; age range 49-82 years, mean 65) were collected. The patients had undergone curative resection between 1990 and 2002 at Oita University Hospital, Oita, Japan. Only patients without pre-operative radio- or chemotherapy and without clinical evidence of distant metastasis were enrolled in the study. The histologic classification was based on the World Health Organization system. Tumor staging was performed according to the TNM stage grouping system. For strict privacy protection, identifying information for the samples was removed prior to being analyzed in accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government.

For quantitative reverse transcription-polymerase chain reaction (RT-PCR), 10 primary esophageal cancer tissue samples and their corresponding non-neoplastic mucosa samples were used. The 10 esophageal cancer samples were all SCC. The samples were obtained at the time of resection, immediately frozen in liquid nitrogen and stored at -80˚C until use. It was microscopically confirmed that the tumor specimens consisted mainly (>50%) of carcinoma tissue.

The 269 primary esophageal cancer tissue samples were used for immunohistochemical analysis. The samples were archival formalin-fixed, paraffin-embedded tissues. These 269 esophageal cancer samples were classified histologically as SCC (n=255), adenocarcinoma (n=10) or small cell carcinoma (n=4).

Serum samples were used to measure Reg IV levels using ELISA. All 65 serum samples were obtained from patients with esophageal SCC prior to surgery and before initiation of therapy. Primary esophageal SCC tissue samples from all 65 patients with esophageal cancer were available for immunohistochemical analysis. The control serum samples were obtained from 133 healthy individuals (92 females and 41 males; age range 21-80 years, mean 51). Control subjects were randomly selected from individuals visiting hospitals for regular health checks or due to certain symptoms, such as appetite loss or epigastralgia. The control subjects were confirmed to be free of malignancy by gastrointestinal endoscopy and biopsy. The serum samples were stored at -80˚C until analysis.

Quantitative RT-PCR. Total RNA was extracted with an RNeasy mini kit (Qiagen, Valencia, CA, USA) and 1 μg of total RNA was converted to cDNA with a First Strand cDNA synthesis kit (Amersham Biosciences, Piscataway, NJ, USA). Quantitation of *REG4* mRNA levels was performed by real-time fluorescence detection as previously described (2). In brief, PCR was performed with a SYBR-Green PCR Core Reagents kit (Applied Biosystems, Foster City, CA, USA). Real-time detection of the emission intensity of SYBR-Green bound to double-stranded DNA was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as previously described (16). ACTB-specific PCR products were amplified from the same RNA samples and served as an internal control.

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. The sections were pre-treated by microwaving in citrate buffer for 30 min to retrieve antigenicity. After peroxidase activity was blocked with 3% H_2O_2-methanol for 10 min, the sections were incubated with normal goat serum (Dako, Carpinteria, CA, USA) for 20 min to block non-specific antibody binding. The sections were incubated with a primary antibody against Reg IV (rabbit polyclonal antibody, diluted 1:50; anti-Reg IV antibody was raised and characterized in our laboratory) (4), MUC2 (1:50; Novocastra, Newcastle, UK) or chromogranin A (1:50; Novocastra) for 1 h at room temperature, followed by incubation with peroxidase-labeled anti-rabbit or anti-mouse IgG for 1 h. Staining was completed with a 10-min incubation in a substrate-chromogen solution. The sections were counterstained with 0.1% hematoxylin. The specificity of the Reg IV antibody was previously characterized (4). Staining of each antibody was considered positive if any tumor cells were stained.

Enzyme-linked immunosorbent assay. For the measurement of serum Reg IV concentration, a sandwich ELISA method was developed as previously described (15). First, polystyrene microtiter plates were coated with mouse monoclonal anti-Reg IV antibody (R&D Systems, Abingdon, UK) by overnight incubation of 50 μl/125 ng/well antibody diluted in Tris buffer (pH 7.4). The plates were then washed three times with wash buffer. After the plates were blocked with 1% milk in phosphate-buffered saline, 50 μl of recombinant Reg IV standard or sample was added to each well and incubated overnight at 4˚C. After three washes, 50 μl of biotinylated goat polyclonal anti-Reg IV antibody (R&D Systems) in assay buffer [1% bovine serum albumin (BSA), Tris buffer (pH 7.4) and 0.05% normal goat serum] was added to each well (75 ng antibody/well). The mixture was then incubated for 1 h with agitation at 37˚C and washed three times with wash buffer. The plates were incubated with 50 μl/well alkaline phosphatase-conjugated streptavidin (Dako) diluted 1:2,000 in diluent containing 1% BSA and Tris buffer (pH 7.4) for 1 h at 37˚C and washed three 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 from 0 to 30 ng/ml were tested in triplicate.

Statistical methods. Differences in the serum Reg IV concentration between two groups were tested using the non-

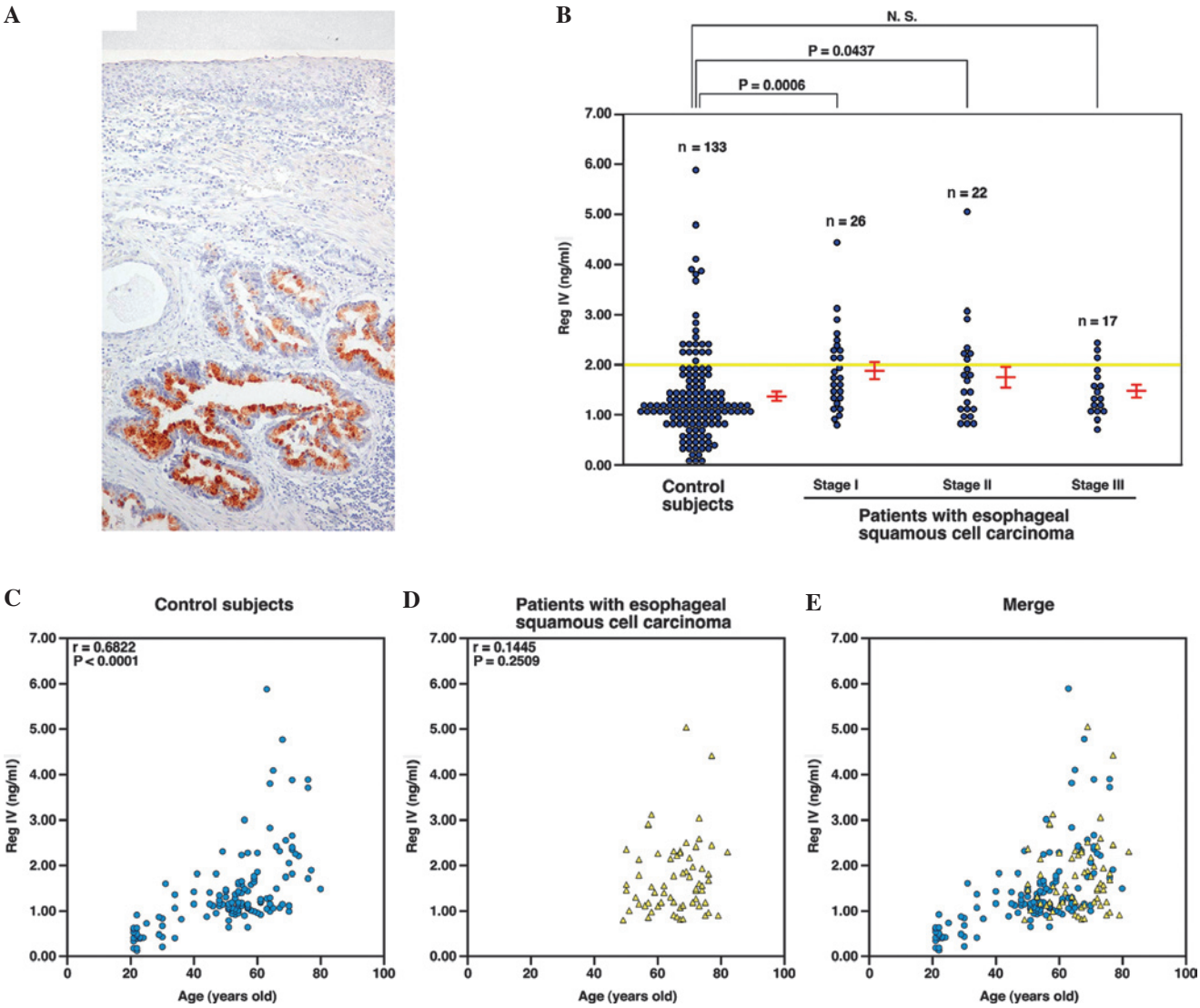

Figure 1. Immunostaining and serum concentration of Reg IV in esophageal cancer. (A) Immunostaining of Reg IV in esophageal adenocarcinoma (original magnification, x100). Reg IV staining is observed in goblet cell-like vesicles of adenocarcinoma cells. (B) Enzyme-linked immunosorbent assay of serum samples from 133 control subjects and 65 patients with esophageal squamous cell carcinoma (SCC). The yellow bar indicates the cut-off levels defined on the basis of a previous study (2 ng/ml) (15). The red bars indicate the mean ± SE. Differences in the serum concentration of Reg IV between two groups were tested using the Mann-Whitney U test. (C) Correlation between the serum concentrations of Reg IV and age in control subjects. The correlation was examined using Spearman's rank correlation test. (D) Correlation between the serum concentrations of Reg IV and age in patients with esophageal SCC. The correlation was examined using Spearman's rank correlation. (E) Panel (C) was merged with panel (D).

parametric Mann-Whitney U test. Correlations between the serum Reg IV concentration and age or gender were assessed using Spearman's rank correlation test. $P<0.05$ was considered to be statistically significant.

Results

Expression of Reg IV in esophageal cancer. An immunohistochemical analysis of Reg IV was performed in 269 human esophageal cancer tissue samples. Although esophageal SCC is the most frequent subtype of esophageal cancer in Asian countries, no Reg IV staining was detected in any of the 255 esophageal SCC samples. Quantitative RT-PCR of *REG4* was performed in 10 esophageal SCC samples; however, no *REG4* expression was found. By contrast, Reg IV was stained in 4 of 10 (40%) adenocarcinoma samples (Fig. 1A). The Reg IV staining was mucin-like; no perinuclear staining was noted. It was confirmed that tumor cells showing mucin-like staining of Reg IV were positive for MUC2 and negative for chromogranin A (data not shown). Although extensive chromogranin A staining was observed in the 4 samples of small cell carcinoma, no Reg IV staining was found (data not shown). Reg IV staining was also not found in corresponding non-neoplastic squamous cells (Fig. 1A).

Serum Reg IV concentration in patients with esophageal squamous cell carcinoma and control subjects. Serum Reg IV levels in 133 control subjects and 65 patients with esophageal SCC measured by ELISA are shown in Fig. 1B. The serum Reg IV concentration of control subjects measured in the

present study (mean ± SE, 1.38±0.08 ng/ml) was higher than that measured in our previous study (0.52±0.05 ng/ml) (15). The correlation between the serum concentration of Reg IV and age or gender was investigated. Although the serum concentration of Reg IV did not correlate with age in our previous study (15), serum concentration was higher in elderly compared to that in young control subjects. Spearman's rank correlation test showed a significant correlation between serum Reg IV and age (r=0.4362, P<0.0001) (Fig. 1C). In our previous study, the cut-off level for Reg IV was set at 2 ng/ml (15). A high level of Reg IV concentration was found in 13 of 23 (57%) control subjects who were more than 65 years old.

Serum Reg IV concentration in esophageal SCC patients at stage I (n=26, 1.88±0.16 ng/ml, P=0.0006, Mann-Whitney U test) and stage II (n=22, 1.76±0.21 ng/ml, P=0.0437, Mann-Whitney U test) was significantly higher than that of the control subjects (Fig. 1B). By contrast, the serum Reg IV concentration in esophageal SCC patients at stage III was not significantly elevated (n=17, 1.48±0.11 ng/ml, P=0.1329, Mann-Whitney U test). In total, serum Reg IV concentration in esophageal SCC patients (n=65, 1.74±0.10 ng/ml) was significantly higher compared to that of the control subjects (P=0.0003, Mann-Whitney U test).

In the present study, no Reg IV expression was detected in esophageal SCC by quantitative RT-PCR and immunohistochemistry. However, serum Reg IV concentration in esophageal SCC patients was significantly higher compared to that of the control subjects. Discrepancy between immunostaining and ELISA results is not likely due only to methodological differences. Spearman's rank correlation test showed that there was no significant correlation between serum Reg IV concentration in esophageal SCC patients and age (r=0.1445, P=0.2509) (Fig. 1D). Additionally, when the distribution of serum Reg IV concentration in esophageal SCC patients was compared to that of the control subjects, it was not significantly different (Fig. 1E).

Discussion

This study aimed to investigate the expression of Reg IV in esophageal cancer. Although Reg IV staining was not detected in esophageal SCC and small cell carcinoma samples, it was present in 40% of adenocarcinoma samples. We confirmed that the adenocarcinoma cells were also positive for MUC2. In our previous study, Reg IV expression was not found in renal cell carcinoma and was noted in only 1% of urothelial carcinoma, neither of which are adenocarcinomas (10). Taken together, these results indicate that the expression of Reg IV is highly specific for adenocarcinoma.

Although Reg IV expression was not found in esophageal SCC at the mRNA and protein levels, serum Reg IV concentrations in esophageal SCC patients at stages I and II were significantly higher compared to those of the control subjects. Spearman's rank correlation test showed a significant correlation between serum Reg IV and age in control subjects. In the ELISA analysis, the mean age of the control subjects (51 years) was younger than that of patients with esophageal SCC (65 years), suggesting that the elevation of the serum concentration of Reg IV in esophageal SCC is age-related and not due to esophageal SCC. Notably, the distribution of serum Reg IV concentration in esophageal SCC patients did not show a significant difference from that noted in the control subjects.

We are unable to thoroughly explain this age-related elevation of serum Reg IV concentration. A number of lines of evidence have suggested that Reg IV protein detected in serum samples is derived from cancer cells. In pancreatic adenocarcinoma patients, postoperative serum Reg IV levels were reduced to within normal range 3 or 4 weeks following tumor resection (5). The Reg IV concentration in serum samples from patients with gastric cancer showing Reg IV-positive immunostaining was significantly higher compared to that with gastric cancer showing Reg IV-negative immunostaining (15). Although the control subjects were confirmed to be free of malignancy by gastrointestinal endoscopy and biopsy, the possibility that the control subjects had cancer cannot be excluded. To address these issues, serum samples from cancer-free subjects confirmed by autopsy should be analyzed.

Another possible explanation for the age-related elevation of serum Reg IV concentration is Reg IV expression in intestinal metaplasia of the stomach. In our previous study, normal stomach cells were not stained by Reg IV, however, extensive staining of Reg IV was observed in intestinal metaplasia of the stomach (4). Since the incidence and prevalence of intestinal metaplasia of the stomach increase with age, age-related elevation of serum Reg IV concentration may be due to Reg IV expression in intestinal metaplasia of the stomach.

In conclusion, we showed that the expression of Reg IV is highly specific for adenocarcinoma, but is not specific for SCC or small cell carcinoma. Although serum Reg IV concentration in esophageal SCC patients was significantly higher than that in control subjects, we hypothesize that serum Reg IV is not suitable as a tumor marker for esophageal SCC detection. The reason for the apparent age-related elevation of serum Reg IV concentration in esophageal SCC patients has yet to be elucidated and further investigation is required to clarify the potential utility of serum Reg IV measurement.

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