Presence of serum tripartite motif-containing 21 antibodies in patients with esophageal squamous cell carcinoma

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SEREX has been applied to esophageal SCC, and the TRIM21 gene was identified as a novel SEREX antigen of esophageal SCC. The presence of s-TRIM21-Abs was confirmed by Western blotting using bacterially expressed TRIM21 gene product and was evaluated for clinicopathological significance in patients with esophageal SCC. s-TRIM21-Abs were detected in 18 (20%) of 91 patients with esophageal SCC but not in 42 healthy donors. The presence of s-TRIM21-Abs was partly associated with tumor size ($P = 0.063$) and poor survival ($P = 0.067$). To measure serum antibody levels, ELISA using purified recombinant TRIM21 protein was developed. The levels of s-TRIM21-Abs were significantly higher in patients with esophageal SCC than in healthy donors ($P = 0.013$). s-TRIM21-Abs may be a useful tumor marker to diagnose and predict disease progression in patients with esophageal SCC. (*Cancer Sci* 2006; 97: 380–386)

Esophageal SCC is one of the most malignant tumors. The 5-year survival rate varies between 20 and 40%.[1–3] Despite improvements in surgical techniques and adjuvant chemoradiotherapy, many patients suffer from rapid recurrence of the disease and have a poor prognosis.[4] Because these tumors are aggressive, patients often have systemic involvement, therefore a delay in diagnosis is a major contributing factor to the poor outlook.[3] Although several tumor markers have been identified in esophageal carcinoma, the percentage of tumors expressing them is not high enough to detect esophageal SCC in the majority of patients.[5–7]

It has been known for several decades that the immune system is able to recognize tumor cells,[8,9] and through the use of the autoimmune system, it is possible to identify tumor markers. This analytical method was developed by Sahin *et al.* and called SEREX.[10] It involved the immunoscreening of cDNA libraries prepared from tumor specimens with autologous or allogeneic sera. The antigens are easily identified by sequencing the isolated cDNA clones, so SEREX is suitable for large-scale screening of tumor antigens. SEREX has been applied to a variety of human tumor types and has already successfully identified over 1000 novel tumor antigens.[11]

The SEREX method has been applied to esophageal SCC and has also identified NY-ESO-1, a gene product of testicular cells. This antigen is overexpressed in various cancer cells compared with normal tissues.[12] SEREX analysis has also led to the isolation of several antigens known to be associated with the malignant state, including a mutated version of p53 tumor suppressor protein in esophageal cancer.[13,14] Serum p53 antibodies have been associated with poor prognosis.[15]

In the previous series of SEREX screening in esophageal SCC, we identified several new SEREX antigens including TROP2, SURF1 and HOOK2.[16,17] In the present series, we have identified TRIM21 as a SEREX antigen, and have evaluated the clinicopathological significance of s-TRIM21-Abs in patients with esophageal SCC.

Materials and Methods

Human esophageal SCC cDNA libraries

Sera were collected from patients after they had given written informed consent. This study was approved by the Local Ethical Review Board of the Graduate School of Medicine, Chiba University. Recombinant DNA work was performed with official permission of the Graduate School of Medicine, Chiba University, and in accordance with the rules of the government of Japan. The human esophageal SCC cell line, T.Tn, was established by the Department of Clinical Molecular Biology, Graduate School of Medicine, Chiba University.[18,19] Total RNA was prepared from T.Tn cells by the acid guanidium thiocyanate-phenol-chloroform method,[20] and purified to poly(A)$^+$RNA using Oligotex-dT_{30} (Super) mRNA Purification Kit (Takara Biochemicals, Kyoto, Japan) according to the manufacturer's instructions. cDNA was ligated into the EcoRI-XhoI site of the λZAP II phage. The original library size was 1.8×10^6.

Patient and healthy donor sera

Sera were obtained from 123 patients with esophageal SCC, 48 with gastric, colon or breast cancer (16 patients each) before treatment and 42 healthy donors. The patients with esophageal

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Abbreviations: CEA, carcino-embryonic antigen; DTT, dithiothreitol; EDTA, ethylenediaminetetra-acetic acid; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; GST, glutathione-S-transferase; IgG, immunoglobulin G; IPTG, isopropyl β-D-thiogalactoside; PBS, phosphate-buffered saline; PBS-T, Tween-20 in phosphate-buffered saline; SCC, squamous cell carcinoma; SCC-Ag, squamous cell carcinoma antigen; SEREX, serological identification of antigens by recombinant cDNA expression cloning; SSA1, Sjogren syndrome antigen A1; s-TRIM21-Abs, serum TRIM21 antibodies; TRIM21, tripartite motif-containing 21.

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SCC consisted of 110 men (89%) and 13 women (11%), with a median age of 65 years (range 39–86 years). Before starting treatment, 123 cancer patients were classified according to the Tumor Node Metastasis/Union Internationale Contre Cancer classification system,[21] as follows: stage I ($n = 27$), stage II ($n = 22$), stage III ($n = 34$), and stage IV ($n = 40$).

Of the SCC patients, 64 underwent R0 resection with extended lymph node dissection and 59 received definitive chemoradiation therapy. After treatment, they were followed-up with clinical examinations and imaging studies on a regular basis, until death or the end of March 2005, whichever was the earliest. The mean follow-up time for survivors was 30 months. Each sample was centrifuged at 3000 *g* for 5 min, then the supernatant was stored at −80°C until use. Repeated thawing and freezing of samples was avoided.

Samples from a total of 91 of 123 patients were analyzed by Western blot analysis and samples from 52 of 123 patients were analyzed by ELISA. Samples from 20 patients were analyzed by both systems.

CEA, CYFRA21-1 and SCC-Ag assays

Serum CEA and CYFRA21-1 concentrations were measured with Enzymun-TEST CEA and Enzymun-TEST CYFRA21-1 (Boehringer Mannheim, Mannheim, Germany), respectively. Levels of SCC-Ag were measured in serum with the SCC Test (Abbott Laboratories, Abbott Park, IL). The cut-off values for serum CEA, CYFRA21-1, and SCC-Ag were 4.6 ng/mL, 2.57 ng/mL and 1.5 ng/mL, respectively, in accordance with the manufacturer's instructions. The specificity at these cut-off values is 95%.[2,22]

Immunological screening of the esophageal carcinoma cell antigens by SEREX

Esophageal carcinoma antigens were screened using the SEREX method previously reported by Sahin *et al*.[10] *Escherichia coli* XL1-Blue MRF′ was infected with λZAP II phages which contained the cDNA library, and the expression of cDNA was induced by blotting on nitrocellulose membranes (NitroBind; Osmonics, Minnetonka, MN), which had been pretreated for 30 min with 10 mM IPTG (Wako Pure Chemicals, Osaka, Japan). The membranes were then washed three times with TBS-T (20 mM Tris-HCl [pH 7.5], 0.15 M NaCl and 0.05% Tween-20), and blocking was performed by treatment with 1% protease-free bovine serum albumin (Wako Pure Chemicals) in TBS-T for 1 h. The membranes were exposed in 1 : 2000-diluted serum with no preabsorption for 1 h. After washing with TBS-T three times, the membranes were treated with 1 : 5000-diluted alkaline phosphatase-conjugated F(ab)′ fragment-specific goat antihuman IgG for 1 h. Positive reactions were detected by incubation in color development solution (100 mM Tris-HCl [pH 9.5], 100 mM NaCl and 5 mM $MgCl_2$) containing 0.3 mg/mL of nitroblue tetrazolium chloride (Wako Pure Chemicals) and 0.15 mg/mL of 5-bromo-4-chloro-3-indolyl-phosphate (Wako Pure Chemicals). Positive clones were recloned twice to obtain monoclonality and retested for serum reactivity.

Sequence analysis of identified antigens

Monoclonalized phage cDNA clones were converted to pBluescript phagemids by *in vivo* excision using ExAssist helper phage (Stratagene, La Jolla, CA). Plasmid DNA was obtained from *E. coli* SOLR strain transformed by the phagemid. The cDNA inserts were sequenced by the dideoxy chain termination method using the DNA sequencing kit BigDye Terminator (Applied Biosystems, Foster City, CA) and ABI PRISM 3700 DNA Analyzer (Applied Biosystems). Sequences were analyzed for homology with public databases of known genes and proteins using BLAST on the National Center for Biotechnology Information's website (http://www.ncbi.nlm.nih.gov/BLAST/).

Western blot analysis

A total of 91 patients were analyzed by Western blot analysis. *E. coli* JM109 cells, which contained cDNA clones recombined in pBluescript II, were cultured with or without 1 mM IPTG for 2.5 h. Cells were then washed with PBS and lyzed by incubation at 100°C for 3 min in sodium dodecyl sulphate sample buffer.[23] *E. coli* lysate was then subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis followed by Western blotting using sera of patients or healthy donors.

Purification of recombinant TRIM21 protein

The cDNA insert of TRIM21 incorporated in pBluescript was cleaved by EcoRI and XhoI, and recombined in pGEX-4T-3. *E. coli* JM109 cells containing pGEX-4T-3-TRIM21 or control pGEX-4T-3 were cultured in 200 mL of Luria broth and treated with 1 mM IPTG for 2.5 h. Cells were harvested, washed with PBS and lyzed by sonication in 10% Trinton X-100, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA and 1 mM DTT. The lysate was then centrifuged at 10 000 *g* for 30 min at 4°C. GST in the supernatant was directly purified by glutathione-Sepharose (Amersham Biosciences, Piscataway, NJ). GST-TRIM21 recovered in the pellet was suspended in 8 M urea, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA and 1 mM DTT, and dialyzed stepwise against 4 M urea, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA and 1 mM DTT for 1 h, 2 M urea, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA and 1 mM DTT for 1 h, then 50 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA and 1 mM DTT for more than 12 h. The samples were centrifuged at 10 000 *g* for 10 min at 4°C, and the supernatant was subjected to affinity purification using glutathione-Sepharose. The purified proteins were concentrated by Apollo centrifugal concentrators (Orbital Biosciences, Topsfield, MA).

ELISA

Samples from a total of 52 patients were analyzed by ELISA. Fifty μL of antigens (GST or GST-TRIM21) diluted at 10 μg protein/mL in PBS was added to the wells of a microtiter plate, and incubated at room temperature overnight. The plate was washed 4 times with 0.1% PBS-T then blocked with 10% fetal calf serum in PBS. The plate was incubated at room temperature for 1 h and washed four times with PBS-T. Fifty μL of sera diluted at 1/100 in 10% fetal calf serum in PBS was added to the wells and incubated for 1 h. The wells were washed with PBS-T four times and the bound IgG antibodies were detected by incubation with horseradish peroxidase-conjugated antihuman IgG antibody (Jackson Immuno Research Laboratories, West Grove, PA) for 1 h,

followed by washing and the addition of 100 μL of a peroxidase substrate (o-phenylenediamine, 0.4 mg/mL) in citrate-phosphate buffer, pH 5.0, containing 0.02% (v/v) H_2O_2. The reaction was stopped with 30 μL of 22% H_2SO_4. Absorbance at 490 nm was determined using a microplate reader (Emax; Molecular Devices, Sunnyvale, CA).

Immunostaining for TRIM21 protein

Paraffin-embedded esophageal SCC tumor tissues were cut and dewaxed through a graded alcohol and xylene. After antigen retrieval with an incubator at 95°C for 40 min in 10 mM citrate buffer (pH 6.0), endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 15 min. After blocking with 5% non-fat dry milk in PBS for 30 min, sections were incubated for 1 h with goat polyclonal antibody against SSA1 (Abcam, Cambridge, UK) diluted at 1 : 200. Parallel sections were incubated with 5% non-fat dry milk as negative controls. All sections were washed three times with wash buffer (DAKO, Carpinteria, CA) for 5 min each. For linking, all sections were incubated with biotinylated antigoat IgG, and reacted with the streptavidin conjugated to horseradish peroxidase reagent (LSAB$^+$HRP system; DAKO). Finally, the reaction was visualized with a chromogen, diaminobenzidine, in 3% hydrogen peroxidase. Sections were then counterstained with hematoxylin, dehydrated and mounted.

Statistical analyses

Fisher's exact (two-sided) probability test and the Mann–Whitney *U*-test were used to determine the significance of the differences between two groups. The survival probabilities were calculated using the product-limit method of Kaplan and Meier, considering all deaths. Survival differences between groups were determined using the log–rank test. All statistical analyses were carried out using the Stat View 5.0 J program for Windows (SAS Institute, Cary, NC). *P*-values lower than 0.05 were considered statistically significant.

Results

Serological screening of cDNA library

A phage expression library was constructed from the mRNA of an esophageal SCC cell line, T.Tn. A total of 1×10^6 clones of cDNA were screened using the serum from a patient with esophageal SCC, and 13 reactive clones were isolated. DNA sequence analysis and homology search, using the National Center for Biotechnology Information databases, revealed that they consisted of four independent clones. Eight out of the 13 clones were PIK3R1, which is p85α regulatory subunit of phosphatidylinositol 3-kinase. The others were Zic family member 2 (ZIC2), mediator of RNA polymerase II transcription (MED9) and TRIM21. Serum ZIC2 antibodies are frequently detected in healthy donors as well as patients.[17] We therefore focused on TRIM21, a member of the tripartite motif family, which includes three zinc-binding domains, a RING, a B-box type 1 and a B-box type 2, and a coiled-coil region.[24] The chromosomal position of TRIM21 is 11p15.5, and the product consists of 475 amino acids.[25] The isolated clone contained the region of amino acid positions between 86 and 475 (approximately 43 kDa). TRIM21 is listed in the cancer immunome database as a SEREX antigen of breast carcinoma.[11]

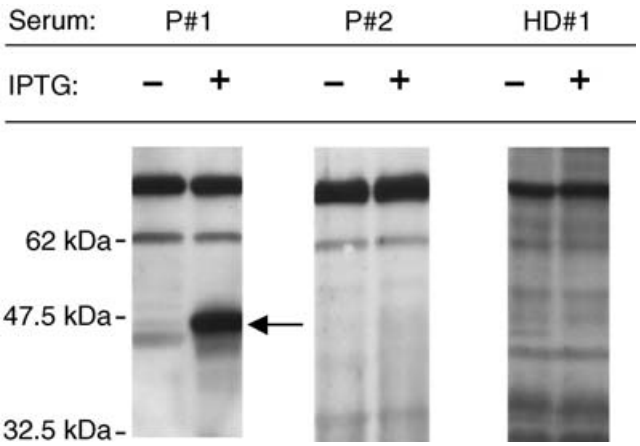

Fig. 1. Recognition of tripartite motif-containing 21 (TRIM21) by serum antibodies in patients with esophageal SCC. *Escherichia coli* containing TRIM21 cDNA expression plasmids was treated with or without isopropyl β-D-thiogalactoside (IPTG) for 2.5 h, with cell lysates being subsequently subjected to western blot analysis using sera from esophageal cancer patient 1 (P#1), patient 2 (P#2), or healthy donor 1 (HD#1). An arrow indicates the IPTG-induced polypeptide that represents TRIM21 cDNA product.

Presence of serum TRIM21 antibodies in patients with esophageal SCC

To confirm the presence of s-TRIM21-Abs in patients with esophageal SCC, Western blot analysis was performed on samples from 91 patients using bacterially expressed TRIM21 protein which was induced by IPTG treatment. Figure 1 shows representative positive and negative results of s-TRIM21-Abs. The signals which appeared only in the samples pretreated with IPTG were judged to be sero-positive. The size of the IPTG-dependent polypeptide was approximately 47 kDa, which was consistent with the estimated size of the fusion protein of pBluescript II-encoded β-Gal (41 amino acids) and amino-terminal truncated TRIM21 protein. The results of Figure 1 indicate that serum of patient 1 (P#1) was positive for s-TRIM21-Abs, whereas the sera of patient 2 (P#2) and healthy donor 1 (HD#1) were negative. We examined the sero-positivity of s-TRIM21-Abs using sera from 91 patients with esophageal SCC and 42 healthy donors. Positive reactions were detected in the sera of 20% (18 out of 91) of patients. Detectable amounts of s-TRIM21-Abs were not found in any of the healthy donors.

Next, we evaluated the relationship between clinico-pathological parameters and the presence of s-TRIM21-Abs (Table 1). Although the differences were not statistically significant, the presence of s-TRIM21-Abs was associated with tumor size ($P = 0.063$) and poor prognosis ($P = 0.067$, Fig. 2), as well as positivity of CYFRA21-1 ($P = 0.043$) and SCC-Ag ($P = 0.056$). There was no correlation between the presence of s-TRIM21-Abs and other clinicopathological variables such as gender, age, tumor location, tumor depth, N factor or M factor. A multivariate analysis was performed to evaluate the prognostic significance of s-TRIM21-Abs (Table 2). Although Tumor Node Metastasis factors were

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Table 1. Relationship between s-TRIM21-Abs and the clinicopathological variables in 91 patients with esophageal SCC

Variables (No. of patients)		No. of patients with s-TRIM21-Abs (positive rate %)	*P*-value
Gender	Male (84)	16 (19)	
	Female (7)	2 (29)	0.621
Age	=65 years (45)	12 (27)	
	>65 years (46)	6 (13)	0.121
Tumor location	Upper (15)	4 (27)	
	Lower (76)	14 (18)	0.486
Tumor size	=50 mm (54)	7 (13)	
	>50 mm (37)	11 (30)	0.063
Tumor depth	T1 T2 (46)	9 (20)	
	T3 T4 (45)	9 (20)	>0.999
N factor	N0 (30)	4 (13)	
	N1 (61)	14 (23)	0.403
M factor	M0 (65)	10 (15)	
	M1 (26)	8 (31)	0.143
Stage	I, II (39)	5 (13)	
	III, IV (52)	13 (25)	0.189
CYFRA21-1	Negative (73)	11 (15)	
	Positive (18)	7 (39)	0.043
SCC-Ag	Negative (59)	8 (14)	
	Positive (32)	10 (31)	0.056
Treatment	Surgery (45)	7 (16)	
	CRT (46)	11 (24)	0.431

P-values were calculated by Fisher's exact probability test. CRT, chemoradiation therapy; SCC, squamous cell carcinoma.

independent risk factors for survival, s-TRM21-Abs was not ($P = 0.437$).

ELISA assay to detect serum antibodies

Although it is possible to evaluate the presence or absence of serum antibodies by Western blotting, the quantitative estimation is limited. ELISA assays were performed on sera from another series of 52 patients, including 20 patients who were analyzed by both systems, using a recombinant antigen protein to quantitatively analyze the levels of s-TRIM21-Abs. cDNA of TRIM21 was recombined into pGEX-4T, which produced the GST-TRIM21 fusion protein after treatment with IPTG. The fusion protein was affinity-purified by glutathione-Sepharose. The levels of serum antibody in patients with esophageal SCC were significantly higher than those in healthy donors (0.505 ± 0.618 *versus* 0.333 ± 0.136, $P = 0.013$) (Fig. 3). Levels of s-TRIM21-Abs were divided into two groups with a border serum level of 0.605 which was the mean + two standard deviations of s-TRIM21-Abs of healthy donors. The positive rate of patients with esophageal SCC was higher than that of healthy donors (15% *versus* 2%, $P = 0.073$).

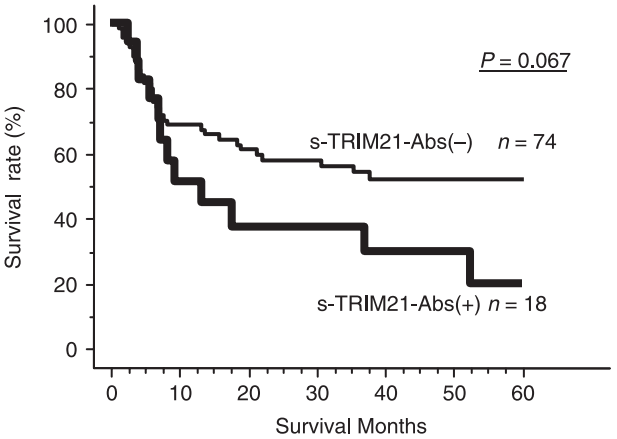

Fig. 2. Kaplan–Meier overall survival curves for the patients with and without serum tripartite motif-containing 21 (TRIM21) antibodies (s-TRIM21-Abs). *P*-value was calculated by log-rank test. The survival of s-TRIM21-Abs-negative patients was superior to that of s-TRIM21-Abs-positive patients.

Table 2. Relationship between s-TRIM21-Abs and survival in patients with esophageal SCC

Variables		Overall 5-year survival rate (%)	Univariate *P*-values†	Multivariate *P*-values†	Adjusted hazard ratio (adjusted 95% CI)
Gender	Male	50			
	Female	30	0.287	0.094	2.71 (0.84–8.72)
Age	=65	50			
	>65	46	0.431	0.315	1.40 (0.73–2.69)
Location	Upper	51			
	Lower	49	0.992	0.698	1.23 (0.44–3.42)
Tumor size	=50 mm	68			
	>50 mm	24	<0.001	0.068	1.94 (0.95–3.94)
Tumor depth	T1T2	69			
	T3T4	25	<0.001	0.031	2.20 (1.07–4.52)
N factor	N0	81			
	N1	32	<0.001	0.022	3.13 (1.18–8.26)
M factor	M0	60			
	M1	25	<0.001	0.017	2.18 (1.15–4.13)
s-TRIM21-Abs	Negative	52			
	Positive	20	0.067	0.437	1.36 (0.63–2.92)

†Log–rank test. CI, confidence interval; s-TRIM21-Abs, serum tripartite motif-containing 21 (TRIM21) antibodies; SCC, squamous cell carcinoma.

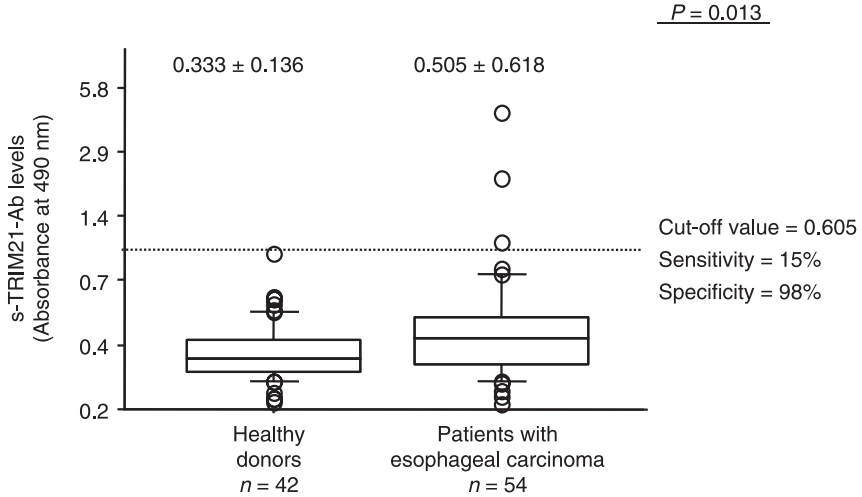

Fig. 3. Serum tripartite motif-containing 21 (TRIM21) antibodies (s-TRIM21-Abs) levels detected by enzyme-linked immunosorbent assay (ELISA). The levels of s-TRIM21-Abs were measured by ELISA using purified glutathione-S-transferase (GST)-TRIM21 protein and control GST protein. The box plots display the 10th, 25th, 50th, 75th, and 90th percentiles. s-TRIM21-Abs levels were significantly higher in patients with esophageal squamous cell carcinoma than those in healthy donors. *P*-values were calculated using the Mann–Whitney *U*-test.

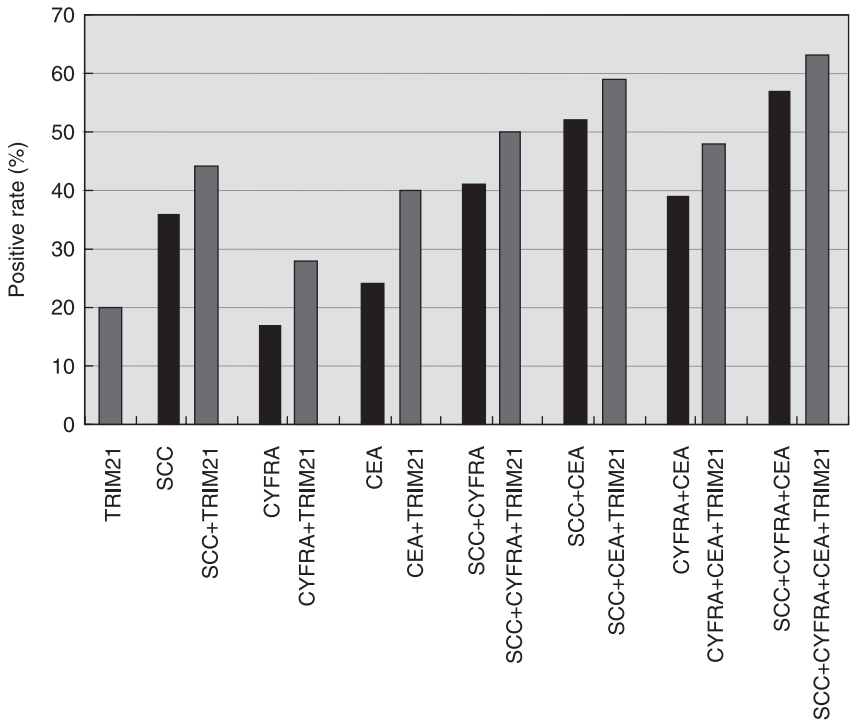

Fig. 4. Comparison of positive rates of serum tripartite motif-containing 21 (TRIM21) antibodies (s-TRIM21-Abs) and conventional tumor markers. Shown are positive rates of s-TRIM21-Abs (TRIM21) and conventional tumor markers, squamous cell carcinoma (SCC)-Ag (SCC), CYFRA21-1 (CYFRA) and carcino-embryonic antigen (CEA), and those obtained by combination assessment with 2, 3 or 4 of either s-TRIM21-Abs, SCC-Ag, CYFRA21-1 or CEA.

Comparison of positive rates between s-TRIM21-Abs and conventional tumor markers

To evaluate the comparative value of s-TRIM21-Abs in detecting esophageal carcinoma, the sensitivity of three conventional tumor markers, SCC-Ag, CYFRA21-1 and CEA, was compared (Fig. 4). Positive rates of s-TRIM21-Abs, SCC-Ag, CYFRA21-1 and CEA were 20%, 36%, 17% and 24%, respectively. The presence of s-TRIM21-Abs associated with the positivity of these conventional serum tumor markers, SCC-Ag ($P = 0.056$) and CYFRA21-1 ($P = 0.043$) (Table 1), but not with positivity of CEA ($P > 0.999$). Thus, the positive rates were improved considerably by combination of s-TRIM21-Abs with CEA (40%, 49 of 123 patients). Combination of s-TRIM21-Abs with other markers also increased the positive rates but less significantly. More than 60% (77 of 123 patients) were sero-positive by combination of s-TRIM21-Abs with all three conventional tumor markers.

Presence of s-TRIM21 Abs in patients with other cancers

s-TRIM21-Abs were further tested with serum samples from patients with other cancers using Western blotting. Patients with colon cancer showed a similar positive rate of s-TRIM21-Abs (19%, 3 of 16 cases) to that of patients with esophageal SCC, whereas the positive rates were lower in patients with gastric (6%, 1 of 16 cases) and breast cancer (6%, 1 of 16 cases).

Immunostaining of TRIM21 in esophageal carcinoma specimens

We investigated the expression and distribution of TRIM21 protein by immunohistochemical analysis. We used formalin-fixed esophageal tissue specimens that contained esophageal

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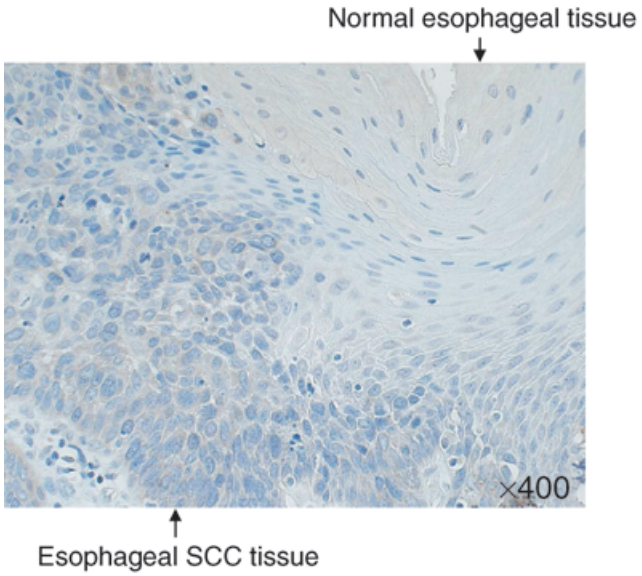

Fig. 5. Immunoperoxidase staining of formalin-fixed paraffin-embedded esophageal carcinoma tissues using anti- tripartite motif-containing 21 (TRIM21) antibody. The areas of normal tissue and carcinoma tissue are shown by arrows. Original magnification × 400.

carcinoma and normal cells of s-TRIM21-Abs-positive patients. Typical results are shown in Figure 5. TRIM21 proteins were relatively abundant in the cytoplasm as compared to the nucleus. Although esophageal SCC tissue was stained more heavily than normal esophageal tissue, the staining intensity was very low in both tissues. Thus, the development of autoantibodies against TRIM21 cannot be attributed simply to the overexpression of TRIM21 protein.

Discussion

SEREX screening has been performed in esophageal carcinomas by Chen *et al.*[12] and Tureci *et al.*[26] who succeeded in identifying NY-ESO-I and NY-ESO-II. In the present study, we identified TRIM21 as a new SEREX antigen of esophageal SCC; s-TRIM21-Abs were present in 20% of patients with esophageal SCC. Although this rate was not higher than the positive rates of CYFRA 21–1, CEA or SCC-Ag (24–39%),[14] s-TRIM21-Abs were completely undetectable in all the healthy donors so far examined. Such specificity suggests that s-TRIM21-Abs might be useful in the detection of esophageal SCC.

The TRIM family consists of 37 members, which possess the large tripartite motif.[24] The function of the motif has been speculated to include multimerization, scaffolding or ubiquitination.[27,28] TRIM21 is also known as SSA1 (Ro/SSA, RO52).[29] The autoantibodies against TRIM21/SSA1 have been found in 35–50% of patients with systemic lupus erythematosus,[30] and in up to 97% of patients with Sjogren syndrome.[31] Thus, this protein appears to be highly immunoreactive in autoimmune disease. The presence of s-TRIM21-Abs in patients with esophageal SCC and colon cancer, but their absence in healthy donors, suggests that these carcinogenesis might be affected by the regulatory mechanisms of the autoimmune system.

The expression levels of TRIM21 in tumor tissues are not necessarily higher than those in normal tissues (http://harvester.embl.de/harvester/P194/P19474.htm). Our results of immunohistochemical analysis showed that the expression levels of TRIM21 were low in both esophageal SCC and normal tissues with slightly higher levels in SCC (Fig. 5). Therefore, overexpression of TRIM21 may not be a cause of the development of the autoantibodies. It was reported that TRIM21 is most abundant in the cytoplasm and present to a lesser extent in the nucleus.[32] Upon induction of apoptosis by ultraviolet radiation,[33] or tumor necrosis factor-α,[34] TRIM21 was relocalized and exposed on the cell surface whereas other proteins were sequestered in apoptotic bodies. It is thus possible that the exposed TRIM21 protein induces serum autoantibodies. As tumor cells are frequently apt to die by apoptosis by means of activated *ras* and c-*myc* oncogenes,[35,36] this mechanism might account for the development of autoantibodies.

However, Poumand and Pettersson have demonstrated that the Zn^{2+}-binding domain is a target for conformation-dependent anti-TRIM21 autoantibodies in patients with Sjogren syndrome and systemic lupus erythematosus.[37] The antigenicity is dramatically increased by the same reducing conditions that promote Zn^{2+} binding. If the oxidoreduction status is altered during tumorigenesis, TRIM21 protein with different conformation could evoke the autoimmune system.

In terms of clinical application, ELISA can be useful because of its quantitativity and high sensitivity in the detection of serum antibodies. In contrast, specific recognition of antigens by serum antibodies can be confirmed by expected molecular weights of reactive bands in Western blotting (Fig. 1). We first examined the presence of s-TRIM21-Abs by Western blotting, then undertook quantitative analysis by an ELISA system. Although serum samples were limited and only a small number of patients were assessed by both Western and ELISA systems, nevertheless the presence of serum antibodies was correlated in both systems (data not shown).

We evaluated the clinical significance of s-TRIM21-Abs in the patients with esophageal SCC. Presence of s-TRIM21-Abs was partly associated with tumor size and poor prognosis. The serum levels of other conventional tumor markers, such as CYFRA21-1 and SCC-Ag, reflect disease progression.[2,22] Interestingly, the positivity of s-TRIM21-Abs was significantly correlated with the presence of CYFRA21-1 ($P = 0.043$) and partly related with SCC-Ag ($P = 0.056$) (Table 1). As the positivity of s-TRIM21-Abs was unrelated to that of CEA ($P > 0.999$), combination of these two markers improved the positive rate considerably (Fig. 4). Thus, in addition to the conventional tumor marker, s-TRIM21-Abs might be useful as a new diagnostic marker for monitoring tumor progression and also for predicting the survival of patients with esophageal SCC.

Acknowledgments

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