Identification of a novel SEREX antigen, SLC2A1/GLUT1, in esophageal squamous cell carcinoma

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Abstract. We have carried out SEREX (serological identification of antigens by recombinant cDNA expression cloning), and identified SLC2A1 (solute carrier family 2/facilitated glucose transporter, member 1) as an antigen recognized by serum IgG antibodies in patients with esophageal squamous cell carcinoma (SCC). The levels of serum anti-SLC2A1 antibodies (s-SLC2A1-Abs), examined by enzyme-linked immunosorbent assay using bacterially expressed glutathione-S-transferase-SLC2A1 fusion protein, were significantly higher in patients with esophageal SCC than in healthy donors. When using a cut-off level as the mean + 2x standard deviations of healthy donors, a total of 12 (21%) out of 57 SCC patients were revealed as positive for s-SLC2A1-Abs. The presence of s-SLC2A1-Abs was not associated with either clinicopathological factors or survival. Because s-SLC2A1-Abs were not associated with the positivity of other conventional serum markers, a combination assay of s-SLC2A1-Abs with these conventional serum markers may be useful for the diagnosis and monitoring of esophageal SCC.

Introduction

It has been well documented that tumor markers are indispensable for the early detection of cancer. Although several serum tumor markers have been linked with esophageal carcinoma, only limited positivity has been reported for esophageal squamous cell carcinoma (SCC) (1-5). SEREX (serological identification of antigens by recombinant cDNA expression cloning) is an effective and convenient method for identifying tumor markers (6). This involves the immuno-screening of cDNA libraries, prepared from tumor specimens, with autologous or allogeneic sera. Since the antigens are easily identified by sequencing the isolated cDNA clones, SEREX is suitable for the large-scale screening of tumor antigens. SEREX has been applied to a variety of tumor types, and more than 1000 SEREX antigens have already been identified (7).

A previous application of the SEREX method to esophageal cancer resulted in the identification of NY-ESO-1, which is a cancer-testis antigen that is expressed in various cancer cells (8). In addition, SEREX analysis has led to the isolation of several antigens known to be related to cancer, including the p53 tumor suppressor protein. Serum antibodies to p53 have been used for the detection of superficial cancers and in the prediction of a poor prognosis for patients with esophageal SCC (9-11).

We have applied the SEREX methodology to esophageal SCC with the aim of adding to the catalogue of defined immunogenic proteins in human cancer, and have previously reported several new SEREX antigens of esophageal SCC such as TROP2, SURF1 and HOOK2 (12,13). In the present study, we have identified SLC2A1/GLUT1 as a new SEREX antigen. This provides a potentially new diagnostic method for esophageal SCC, i.e. the examination of SLC2A1 antibody levels in sera instead of SLC2A1 antigen expression levels in tumor tissue.

Materials and methods

Human esophageal squamous cell carcinoma cDNA libraries. This study was approved by the local ethics review board of Chiba University Graduate School of Medicine. Recombinant DNA work was conducted with official permission and in accordance with the rules of the government of Japan. All patients gave their written informed consent before participating in the study. A human esophageal SCC cell line, T.Tn, was established by the Department of Clinical Molecular Biology, Chiba University Graduate School of Medicine (14,15). Total RNA was prepared from T.Tn cells by the acid guanidium thiocyanate-phenol-chloroform method and purified to poly(A)$^+$ RNA using an Oligotex-dT$_{30}$ (Super) mRNA purification kit (Takara Biochemicals, Kyoto, Japan) according to the manufacturer's instructions (16). cDNA was ligated into the

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EcoRI-XhoI site of the λZAP II phage. The original library size was 1.8×10^6.

Patients and healthy donor sera. Sera were obtained from 57 patients with esophageal SCC before treatment was started, and from 31 healthy donors. The SCC patients consisted of 51 men (89%) and 6 women (11%), with a median age of 65 years (range 44-82 years). They were pathologically classified according to the Tumor Node Metastasis/Union Internationale Contre Cancer (pTNM/UICC) classification (17) as follows: stage I cancer (n=19), stage II (n=6), stage III (n=13) and stage IV (n=19). After the operation, patients were followed-up with clinical examinations and imaging studies on a regular basis until death or the end of March 2005. The mean follow-up time for survivors was 26 months. Each sample was centrifuged at 3,000 x g for 5 min and then frozen at -80˚C until use. Repeated thawing and freezing of samples was avoided.

CEA, CYFRA-Ag and SCC-Ag assays. Serum carcino-embryonic antigen (CEA) and CYFRA-Ag concentrations were measured with Enzymun-Test CEA and Enzymun-Test CYFRA21-1 (Boehringer Mannheim, Mannheim, Germany), respectively. Serum SCC-Ag levels were measured using the SCC Test (Abbott Laboratories, Abbott Park, IL). The cut-off values for serum CEA, CYFRA-Ag and SCC-Ag were 4.6, 2.57 and 1.5 ng/ml, respectively, in accordance with the manufacturer's instructions. The specificity at these cut-off values is 95% (4,5).

Immunological screening of esophageal carcinoma cell antigens by SEREX. Esophageal carcinoma antigens were screened using the SEREX method previously published by Sahin *et al* (6). *E. coli* XL1-Blue MRF' was infected with λZAP II phage which contained cDNA library, and the expression of cDNA was induced by blotting on nitrocellulose membranes (NitroBind, Osmonics Inc., Minnetonka, MN), which had been pretreated for 30 min with 10 mM isopropyl ß-D-thiogalactoside (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 1:2,000-diluted serum for 1 h. After washing with TBS-T three times, the membranes were treated with 1:5,000-diluted alkaline phosphatase-conjugated F(ab)' fragment-specific goat anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) 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 NCBI-BLAST.

Purification of recombinant SLC2A1 protein. cDNA insert of SLC2A1 incorporated in pBluescript was cleaved by EcoRI and XhoI, and recombined in pGEX-4T-3. *E. coli* JM109 cells containing pGEX-4T-3-SLC2A1 or control pGEX-4T-3 were cultured in 200 ml of Luria broth (LB) and treated with 1 mM IPTG for 2.5 h. Cells were harvested, washed with phosphate-buffered saline (PBS) and lysed by sonication in 10% Triton X-100, 50 mM Tris-HCl (pH 8.0), 1 mM ethylene-diaminetetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT). The lysate was then centrifuged at 10,000 x g for 30 min at 4˚C. Glutathione-S-transferase (GST) was harvested in the supernatant, and directly purified by glutathione-Sepharose. GST-SLC2A1 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, and then 50 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA and 1 mM DTT for >12 h. The samples were centri-fuged at 10,000 x g for 10 min at 4˚C and affinity-purified by glutathione-Sepharose (Amersham Biosciences, Piscataway, NJ). The purified proteins were concentrated using Apollo centrifugal concentrators (Orbital Biosciences, Topsfield, MA).

Western blot analysis. *E. coli* JM109 cells which contained cDNA clones recombined in pGEX-4T-3 or pGEX-4T-2 were cultured with or without 1 mM IPTG for 2.5 h. Cells were then washed with PBS and lysed by incubation at 100˚C for 3 min in SDS sample buffer (18). *E. coli* lysate was then subjected to SDS-PAGE followed by Western blotting using sera of patients or healthy donors.

Enzyme-linked immunosorbent assay (ELISA) using bacterially expressed glutathione-S-transferase-SLC2A1 fusion protein. Antigens (50 µl) (GST or GST-SLC2A1) diluted at 10 µg protein/ml in PBS were added to the wells of a microtiter plate, and incubated at room temperature overnight. The plate was washed 4 times with 0.1% Tween-20 in PBS (PBS-T) and then blocked with 10% fetal calf serum in PBS (PBS-FCS). The plate was incubated at room temperature for 1 h and washed 4 times with PBS-T. Sera (50 µl) diluted at 1/100 in PBS-FCS were added to the wells and incubated for 1 h. The wells were washed with PBS-T 4 times and the bound IgG antibodies were detected by incubation with horseradish peroxidase-conjugated anti-human IgG antibody (Jackson ImmunoResearch 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 by using a microplate reader (Emax, Molecular Devices, Sunnyvale, CA).
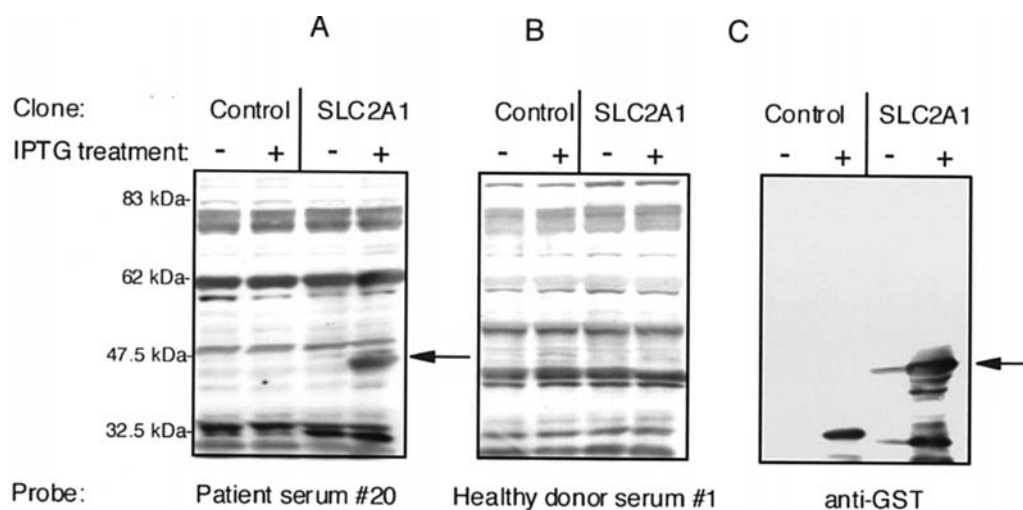
Figure 1. Recognition of SLC2A1 by serum antibodies in patients with esophageal SCC. *E. coli* containing pGEX-4T-3-SLC2A1 (SLC2A1) or control pGEX-4T-2-SLC2A1 (control, SLC2A1 frameshift product) was treated with or without IPTG for 2.5 h. Cell lysates were subsequently subjected to Western blot analysis using esophageal cancer patient serum #20 (A), or healthy donor serum #1 (B). The same samples were also probed with anti-GST-antibody (C). An arrow indicates the IPTG-induced polypeptide that represents SLC2A1 cDNA product.

Statistical analyses. 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. Fisher's exact probability test and the Mann-Whitney U test were used to determine the significance of the differences between two groups. All statistical analyses were carried out using the StatView 5.0J program for Windows (SAS Institute Inc., Cary, NC). P-values <0.05 were considered statistically significant.

Results

Serological screening of cDNA library. Phage expression library was constructed from the mRNA of an esophageal cancer cell line, T.Tn. A total of $1x10^6$ clones of cDNA were screened using serum from a patient with esophageal SCC, and 13 reactive clones were isolated. DNA sequence analysis and an homology search using the National Center for Bio-technology Information (NCBI) databases revealed SLC2A1 [Solute carrier family 2 (facilitated glucose transporter), member 1] (Accession number: NM_006516), which was further investigated in this study. SLC2A1 is a member of the membrane transporter (SLC) family, and is an integral membrane glycoprotein involved in transporting glucose into cells (19). The chromosomal position of SLC2A1 is 1p35-p31.3, and the product consists of 492 amino acids (54,117 Da). The isolated clone contained the region between amino-acid positions 292 and 492 (approximately 22 kDa). SLC2A1 is not listed as a SEREX antigen in the cancer immunome database (7). However, its family member, SLC2A11 (NM_030807), which is also a glucose transporter but located at a different locus (22q11.2), is listed as a SEREX antigen of pancreatic adenocarcinoma, melanoma and fibrosarcoma.

Presence of serum SLC2A1 antibodies in patients with esophageal squamous cell carcinoma. To confirm the presence of serum SLC2A1 antibodies (s-SLC2A1-Abs) in patients with esophageal SCC, Western blotting was performed using
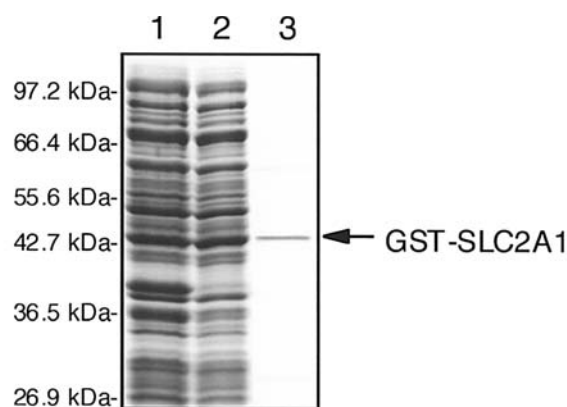

Figure 2. SDS-PAGE after purification of recombinant SLC2A1 protein. Lane 1, whole bacterial lysates; lane 2, supernanant of Triton X-100 lysate; lane 3, purified fraction after glutathione-Sepharose affinity-column chromatography.

bacterially-expressed SLC2A1 protein. The expression vector was constructed by ligation of EcoRI/XhoI insert of SLC2A1 in pBluescript in pGEX-4T-3, which produces GST-SLC2A1 fusion protein after treating *E. coli* with IPTG. As a control, the SLC2A1 insert was inserted in the EcoRI/XhoI site of pGEX-4T-2, which produced a frameshift mutation and an early translational termination. Fig. 1 shows representative positive and negative results of s-SLC2A1-Abs. When the *E. coli* extracts were probed with anti-GST antibody, the reaction of polypeptides of approximately 33 and 47 kDa was markedly enhanced by treatment with IPTG (Fig. 1C), suggesting that these bands were IPTG-induced GST-fusion proteins. The size of 47 kDa was almost equal to the estimated size of the fusion protein of pGEX-4T-encoded GST (25 kDa) and amino-terminal truncated SLC2A1 protein (22 kDa). This polypeptide was also observed in the serum from patient #20 (P#20) but not in the serum from healthy donor #1 (HD#1) (Fig. 1A and B). Control polypeptide did not react with the serum from patient #20 or healthy donor #1. These results
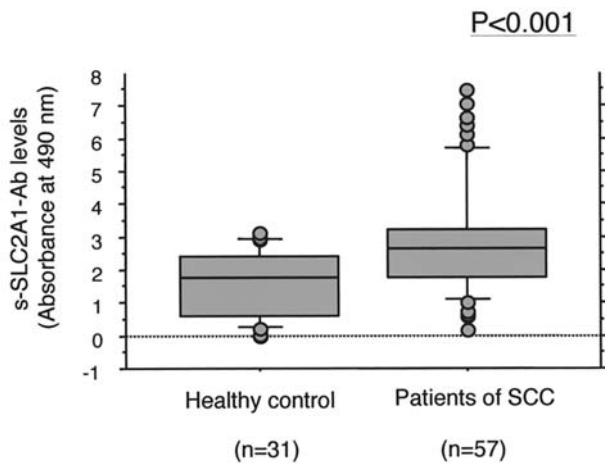
Figure 3. Results of ELISA. The levels of s-SLC2A1-Abs were measured by ELISA using purified GST-SLC2A1 protein and control GST protein. The boxplots display the 10th, 25th, 50th (bold horizontal line is the median), 75th and 90th percentiles. P-values calculated by the Mann-Whitney test were found to be <0.001 between levels of s-SLC2A1-Abs in patients versus healthy donors.

Table I. Relationship between s-SLC2A1-Abs and the clinico-pathological variables in 57 patients with esophageal SCC.

Variables (No. of patients)	No. of patients with s-SLC2A1-Abs (positive rate, %)	P-value[a]
Gender		
Male (50)	12 (24)	
Female (7)	0 (0)	0.325
Age		
≤65 (30)	6 (20)	
>65 (27)	6 (22)	>0.999
Location		
Upper (15)	3 (20)	
Lower (42)	9 (21)	>0.999
Tumor size		
≤40 mm (33)	5 (15)	
>40 mm (24)	7 (29)	0.324
Tumor depth		
T1,T2 (26)	5 (19)	
T3,T4 (31)	7 (23)	>0.999
N factor		
N0 (22)	4 (18)	
N1 (35)	8 (23)	>0.999
M factor		
M0 (51)	10 (20)	
M1 (6)	2 (33)	0.596
Stage		
I (18)	4 (22)	
II, III, IV (39)	8 (21)	>0.999
CYFRA-Ag		
Positive (8)	2 (29)	
Negative (49)	10 (20)	0.670
SCC-Ag		
Positive (13)	4 (31)	
Negative (44)	8 (18)	0.440
CEA-Ag		
Positive (20)	5 (25)	
Negative (37)	7 (19)	0.736

[a]P-values were calculated by Fisher's exact probability test.

indicated that the serum of P#20 was positive for s-SLC2A1-Abs while the serum of HD#1 was negative.

To analyze the levels of s-SLC2A1-Abs quantitatively, we performed ELISA by using recombinant antigen protein. GST-SLC2A1 fusion protein was induced by treatment with IPTG, and affinity-purified by glutathione-Sepharose. The reactive levels against control GST were subtracted from those against GST-SLC2A1 of each serum sample. The mean (± SD) serum antibody levels of patients with esophageal SCC were 2.892 (±1.662), which were significantly higher than those of healthy donors (1.588±0.982) (P<0.001, Fig. 3).

The levels of s-SLC2A1-Abs were divided into two groups with a border value of 3.552 which was the mean + two standard deviations of s-SLC2A1-Abs of healthy donors. The positive rate of patients with esophageal SCC was 21% (12 out of 57) while no healthy donors were positive for s-SLC2A1-Abs.

We then examined the relationship between the presence of s-SLC2A1-Abs and clinicopathological features of the patients (Table I). There was no correlation between the presence of s-SLC2A1-Abs and clinicopathological variables such as location, tumor depth and TNM factors. Because there was no association between the presence of s-SLC2A1-Abs and positivity of other serum markers, a combination assay using conventional serum markers and s-SLC2A1-Abs revealed high positive rates (32, 37 and 47%, respectively; Fig. 4). Although sero-positive patients showed better survival rates than sero-negative patients, the difference was not statistically significant (Fig. 5).

Discussion

SEREX screening for esophageal carcinomas has previously been performed by Chen *et al* (8) and Tureci *et al* (20), and NY-ESO-1 and NY-ESO-2 have been identified. In the present study, we identified SLC2A1 as a new SEREX antigen of esophageal SCC. The levels of s-SLC2A1-Abs were significantly higher in patients with esophageal SCC than in healthy donors.

Such a clear difference between patients and healthy donors suggests that s-SLC2A1-Abs may be a useful diagnostic marker for esophageal SCC.

SLC2A1/GLUT1 has been reported to be overexpressed in tumor tissue from lung (21), gastric (22), head and neck (23) and pancreatic (24) cancers. Expression of SLC2A1 has been observed in all of 72 esophageal cancer tissues examined
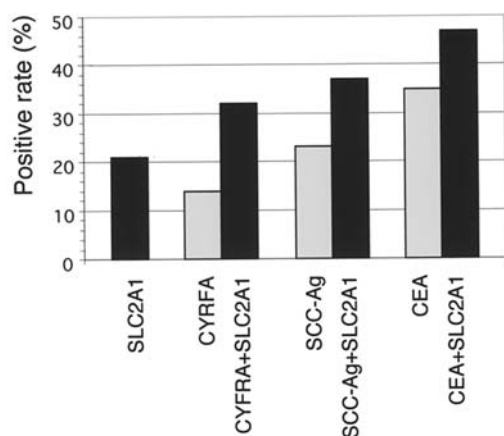
Figure 4. Positive rate in combination assay of serum markers with s-SLC2A1-Abs. Gray box indicated positive rates of conventional serum markers. Black box indicated positive rates of conventional serum markers when used in combination with s-SLC2A1-Abs (32, 37 and 47%, respectively).
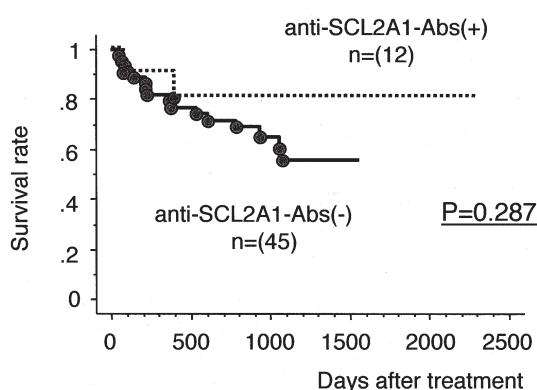

Figure 5. Kaplan-Meier survival curves for positive and negative cases of esophageal SCC with respect to detection of s-SLC2A1-Abs. No significant difference was seen between the two curves by the log-rank test.

(25). There are five members of the glucose transporter family. Matsuzu *et al* have reported that SLC2A1/GLUT1 but not SLC2A2/GLUT2, SLC2A3/GLUT3, SLC2A4/GLUT4 nor SLC2A11 (GLUT10/11) was overexpressed in thyroid carcinoma compared to normal tissue (26). The expression of SLC2A1 is transcriptionally regulated by the oncogene product, c-Myc (27). The suppression of SLC2A1 expression by anti-sense mRNA inhibited tumor cell growth (28). Among glucose transporter family members, only SLC2A1 may be a necessary factor for the rapid growth of tumor cells.

Solute carrier proteins are engaged in the transport of small molecules across membranes. Although SLC2A1/GLUT1 has not yet been reported as a SEREX antigen, other SLC family members, such as SLC22A17, SLC25A2, SLC25A20, SLC25A6, SLC4A1AP, SLC4A5, SLC9A3R2 and SLC06A1, have been identified as SEREX antigens in the cancer immunome database (7). For example, SLC2A11 is a SEREX antigen of pancreatic adenocarcinoma, melanoma and fibro-sarcoma. It should be noted that SEREX screening identifies not only the original antigen but also other proteins which have the same epitope structure. This raises the possibility that some, if not all, solute carrier proteins might have been isolated by SEREX as cross-reactive proteins recognized by anti-SLC2A1 antibodies.

Because SLC2A1 is highly expressed in many cancer tissues, SLC2A1 is an appropriate molecular target for the diagnosis of cancer. The present study showed that the serum levels of SLC2A1 antibodies were significantly higher in patients with esophageal SCC than in healthy donors. We had previously reported that serum p53 antibodies are a useful marker for esophageal SCC (9-11). Thus, s-SLC2A1-Abs may also become available as a rapid and convenient diagnostic method for esophageal SCC and various other types of cancer. Because s-SLC2A1-Abs were not associated with the positivity of other conventional serum markers (Table I), a combination assay of s-SLC2A1-Abs with these conventional serum markers may further improve the diagnostic efficiency of esophageal SCC.

Acknowledgements

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