EXPRESSION OF GAGE, NY-ESO-1, MAGE-A, AND SSX PROTEINS IN ESOPHAGEAL CANCER: IMPLICATONS FOR IMMUNOTHERAPY

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CTA: cancer/testis antigen

TAA: tumor-associated antigen

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Although there was no correlation between cancer/testis antigen expression and prognosis, we showed the importance of examining different sections of the same tumor to overcome the heterogeneous expression problem. In the cases of coexpression of more than one cancer/testis antigen, the protein expressing parts of the tumors usually overlapped, which means polyvalent vaccines targeting cancer/testis antigens do not have to cover a larger tumor area and therefore the success rate of the immunotherapy may be lower than expected.

ABSTRACT

Cancer/testis antigens (CTAs) elicit immune response in cancer patients and are therefore targets of immunotherapy. Current information on CTA expression is primarily based on mRNA assays and little is known about their expression at the protein level. The objectives of this study are to analyze GAGE, NY-ESO-1, MAGE-A and SSX protein expression in esophageal cancer and to correlate their expression patterns with clinicopathologic parameters and survival. We examined CTA protein expression in 213 patients with esophageal cancer by immunohistochemistry. Antigen-positive tumors were evaluated once and antigen-negative tumors were evaluated three times by examining different parts of the cancer specimen. GAGE, NY-ESO-1 and MAGE-A were heterogeneously expressed in 42 (20%), 44 (21%) and 111 (52%) tumors, respectively, whereas SSX expression was not detected. Of the 126 (59%) patients expressing CTAs, 70 (33%) expressed one, 41 (19%) expressed two and 15 (7%) expressed three antigens. The expression of MAGE-A was correlated with those of GAGE (P=0.001) and NY-ESO-1 (P=0.002), and the expression of GAGE was correlated with that of NY-ESO-1 (P=0.002). One hundred and fifty-six (79%) sections were positively stained in the first evaluation, whereas 37 (19%) and four (2%) positive sections were identified in the second and third evaluations, respectively. Particularly, MAGE and GAGE expression showed overlaps. GAGE, NY-ESO-1 and MAGE-A protein expression was not correlated with the disease progression, TNM factors or survival. The detection of immunonegative cells in every specimen suggests addition of other drugs to increase the therapeutic effect of CTA-specific cancer vaccines.

INTRODUCTION

Esophageal cancer is a relatively common and highly malignant neoplasm. After curative surgical resection, the three-year survival rate remains low at 36 to 40% [1, 2]. The combination of surgery with chemotherapy and radiotherapy has no benefit on the overall survival and therefore, the development of new adjuvant therapies is needed. Immunization against defined tumor-associated antigens (TAAs) is considered to be an attractive modality in addition to existing treatments. However, the main obstacle is the identification of relevant target TAAs. Several categories of TAAs have been identified, of which cancer/testis antigens are of particular interest due to their restricted expression in normal testis and cancer cells. CTAs are expressed in tumors of different histological origins and elicit humoral and/or cellular immune responses [3], including esophageal cancer [4-6]. Therefore, CTA-specific immunotherapy presents a new hope for esophageal cancer patients.

Recent studies of MAGE, BAGE, GAGE [7], NY-ESO-1 [6], LAGE-1, SCP-1 and SSX [8] expression in esophageal cancer at the mRNA level have provided important information of CTA expression profiles and shown the potential of CTAs for use in the development of antigen-specific vaccines. However, CTA expression studies suffer from a number of limitations, the first of which is mRNA-protein expression discrepancy [9, 10], which has been explained by the low sensitivity of immunohistochemical analysis, tissue sampling variations or the heterogeneous expression patterns of the CTAs [3]. Nevertheless, this discrepancy has also been shown at the protein level when the same monoclonal antibody was used [10, 11]. It is likely that examination of

only one part of the tumor did not reveal the complete CTA expression pattern and resulted in the discrepancy at both mRNA and protein levels. Second, most of the previous studies evaluated a small number of fresh tumor samples by reverse transcriptase polymerase chain reaction (RT-PCR) and statistical analysis of the correlation between CTA expression and tumor progression or prognosis did not reach a significant level or could not be performed. However, analysis of the correlation between CTA expression pattern and survival may lead to the identification of subgroups of patients who may potentially benefit from CTA immunotherapy and help physicians set improved guidelines for the administration of vaccine preparations. Third, the administration of poly-CTA vaccines is thought to cover a large portion of the tumor area, thereby solving the problem of heterogeneous CTA expression. However, if the expression of multiple CTAs overlaps with each other instead of covering different parts of the tumor, the targeted areas may be limited, resulting in failure of the immunotherapy.

Therefore, the expression patterns and the prognostic significance of CTAs in esophageal cancer are currently unknown. In order to resolve the incomplete typing data that may have contributed to earlier discrepant findings, where CTA expression was studied in relation to clinicopathologic data and survival, we analyzed GAGE, NY-ESO-1, MAGE-A and SSX protein expression in a large number of patients with esophageal cancer and evaluated the correlation between CTA expression and clinicopathologic factors or survival.

PATIENTS AND METHODS

Patients

Three hundred and fourteen patients with esophageal cancer, who were treated at the Department of Surgery, Niigata University Medical Hospital, were enrolled from January 1991 through December 2000. Fifty-eight patients with extraesophageal second primary cancers and 43 patients who had undergone some form of surgical palliative procedure or had received neoadjuvant therapy were found to be ineligible. The remaining 213 patients who had undergone esophagectomy were qualified and included in the present study. All patients provided written informed consent prior to the study.

Data including age, sex, tumor location and size, treatment protocol, curability, histology, tumor node metastasis (TNM) stage and outcome were obtained from clinical and pathologic records. Definitions of surgical resections, clinical staging and histopathologic classification were performed according to the UICC-TNM classification [12]. Final pathologic staging was determined for all the patients. The clinicopathologic characteristics of the patients are summarized in Table 1. The duration of the follow-up was from the time of surgery to death, dropout, or 31 March 2004.

Immunohistochemistry

Formalin-fixed, paraffin-embedded esophageal tumor samples were obtained from the archives of the Department of Pathology, Niigata University Medical Hospital. Three observers (AA, SK and TT) evaluated

the slides without knowledge of the clinical data. Antigen-positive tumors were evaluated once and antigen-negative tumors were evaluated three times by examining different parts of the tumor specimen.

Immunohistochemistry was performed with a Histofine SAB-PO kit (Nichirei Corporation, Tokyo, Japan). All incubations were conducted at room temperature unless stated otherwise. After deparaffinization, microwave treatment was performed in citrate buffer (10 mM, pH 6) and EDTA buffer (1 mM, pH 8) for 15 minutes each for MAGE and NY-ESO-1 antigen retrieval, respectively. For SSX antigen retrieval, slides were incubated in EDTA buffer at 90°C for 30 minutes. For GAGE antigen retrieval, slides were treated with 0.1% trypsin in PBS (pH 7.8) at 37°C for 20 minutes. Endogenous peroxidase activity was quenched by treatment with 0.3% H_2O_2 in methanol. After blocking, the sections were incubated overnight at 4°C with mouse monoclonal anti-MAGE antibody (clone 6C1; reacts with MAGE-1, -2, -3, -4, -6 -10, and -12 proteins)(1:200 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse monoclonal anti-GAGE antibody (clone 26; reacts with GAGE-3, -4, -5, -6, and 7B proteins)(1:8000 dilution; BD Biosciences, San Jose, CA), mouse monoclonal anti-NY-ESO-1 antibody (clone E978)(1:100 dilution; Zymed Laboratories Inc., South San Francisco, CA) and goat polyclonal anti-SSX antibody (reacts with SSX2, SSX3, and SSX5 proteins)(1:400 dilution; a kind gift from Santa Cruz Biotechnology, Inc.). A biotin-labeled secondary antibody was used for detecting the primary antibody, followed by peroxidase-labeled streptavidin. The reaction was developed by adding 3,3-diaminobenzidine tetrachloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and the slides were counterstained with hematoxylin. The number of stained tumor cells was

graded as follows: focal or <5%, -; 5 to 50%, +; and 50%<, ++. The antibody concentrations were determined by titration of testis tissue. Positive and negative control slides consisted of testis tissue, and negative control slides were incubated with buffer instead of the primary antibody.

Statistical Analysis

Patients were censored if lost to follow-up, alive at the end of the study period or five years (1825 days) after enrollment. Statistical analysis was performed with Fisher’s exact test and Spearman’s rank correlation test. Survival curves were plotted by means of the Kaplan-Meier method and compared by using the log-rank test. A *P* value (two-tailed) less than 0.05 was considered to be significant.

RESULTS

GAGE, NY-ESO-1, MAGE-A and SSX Expression

Staining of testis tissue revealed that GAGE (Fig. 1A), NY-ESO-1, MAGE-A and SSX were expressed mainly in spermatogonia and rarely in primary spermatocytes close to the basement membrane. The staining pattern was nuclear for all the four antigens. Only a fraction of spermatogonia were reactive for SSX (Fig. 1B).

In esophageal cancer, there was no staining with anti-SSX antibody. One hundred and twenty-six (59%) patients expressed CTAs. Immunopositivity on staining with anti-MAGE, anti-NY-ESO-1 and anti-GAGE antibodies was observed in 111 (52%), 44 (21%) and 42 (20%) cases, respectively. Immunoreactivity to anti-MAGE antibody was strong and 73 (66%) of the MAGE-positive sections showed >50% staining of the tumor cells. On the other hand, 33 (75%) of the NY-ESO-1-positive and 23 (55%) of the GAGE-positive cases showed <50% staining. Anti-GAGE antibody usually showed focal (<5%) reactivity. The staining pattern was cytoplasmic and nuclear, and heterogeneous for all the three antibodies. In most cases of co-expression, anti-MAGE and anti-GAGE antibodies were found to react with the same parts of the tumor (Figs. 1C-F). Fifty-six patients (25%) expressed at least two and 15 (7%) patients expressed three CTAs (Fig. 2). The immunoreactivity of MAGE-A was correlated with those of GAGE ($r=0.381$, $P=0.001$) and NY-ESO-1 ($r=0.211$, $P=0.002$), and the immunoreactivity of GAGE was correlated with that of NY-ESO-1 ($r=0.213$, $P=0.002$).

One hundred and ninety-seven cancer sections were immunoreactive with anti-GAGE, anti-NY-ESO-1 and anti-MAGE antibodies. One hundred and fifty-six (79%) cancer sections were positively stained in the first evaluation, whereas 37 (19%) and four (2%) positive sections were identified in the second and third evaluations, respectively.

Clinicopathologic Parameters and GAGE, NY-ESO-1 and MAGE-A Expression

The relationship between CTA expression and clinicopathologic parameters is summarized in Table 2. GAGE expression was significantly higher in patients 65 years old and over, who had tumors greater than 60 mm in size, and who had tumors located in the lower/abdominal esophagus, whereas MAGE-A expression was significantly higher only in patients with lower/abdominal esophagus located tumors. CTA expression was not correlated with disease progression or TNM factors (Table 3).

Survival and GAGE, NY-ESO-1 and MAGE Expression

Survival rates were indistinguishable between patients with GAGE- and NY-ESO-1-positive tumors and those with GAGE- and NY-ESO-1-negative tumors, respectively (P=0.7212 and P=0.8177, respectively). Patients with MAGE-A-negative tumors showed slightly better prognosis; however, the difference between patients with MAGE-A-positive tumors and those with MAGE-A-negative tumors was not significant (P=0.1756) (Figs. 3A-C). The three-year survival rates of antigen–positive and antigen-negative patients were, respectively, 49.3% and 52.3% for GAGE, 52.1% and 51.6% for NY-ESO-1, and 46.1% and 57.6% for MAGE-A.

The patients were grouped according to the number of CTAs expressed: Group A (87 patients, 41%) did not express any antigen, Group B (70 patients, 33%) expressed one antigen, Group C (41 patients, 19%) expressed two antigens, and Group D (15 patients, 7%) expressed three antigens. No significant association was found between the number of CTAs expressed and the survival rate (P=0.3376) (Fig. 3D). The patients were also divided into any-CTA-positive (126 patients, 59%) and CTA-negative (87 patients, 41%) groups, and no significant difference in the survival curves was found (P=0.1481).

DISCUSSION

In our series, MAGE-A protein was dominant and detected in 52% of the esophageal cancer specimens. This high expression frequency and the associated strong immununoreactivity can be explained by the use of a poly-MAGE antibody detecting seven members of the MAGE-A family. Previous studies have shown that at least one member of the MAGE-A gene family is expressed in 34 to 84% [7, 13-16] of the examined esophageal tumors. In most of the studies, including ours, MAGE-A protein expression rate is lower than MAGE-A mRNA expression rate [9, 10]. This may be an indication of the low sensitivity of immunohistochemistry compared to that of RT-PCR. However, the more likely explanation for these discrepancies is the heterogeneous expression of CTAs, as some of the melanoma tumors that stained positive for NY-ESO-1 by immunohistochemistry were negative by RT-PCR and quantitative RT-PCR [17], respectively.

In esophageal cancer, NY-ESO-1 mRNA expression was observed in 33% of tumors and protein expression in 41% of those [6]. However, in our study NY-ESO-1 protein expression rate was 21%. The reported high protein expression rate could be due to the difference in grading of number of stained tumor cells. If “focal” or “<5% of cells stained” were graded as negative, the protein expression rate would decrease to 19% [6], which is consistent with our result.

The reported mRNA expression rate of GAGE in esophageal cancer is 24% [7], and is very similar to our result of 20% for GAGE antigen. We used a polyclonal anti-SSX antibody that reacted with SSX2, SSX3 and SSX5. Esophageal cancer expresses SSX4 but not SSX1 and SSX2 [8]; our

findings suggest that SSX3 and SSX5 are not expressed as well.

The present analysis showed that 38 of 42 (90%) GAGE-positive cases and 33 of 44 (75%) NY-ESO-1-positive cases exhibited co-expression with MAGE-A, and there was only one case of GAGE and NY-ESO-1 co-expression without MAGE-A expression. Esophageal cancer [7] and malignant gammopathies [18] showed this high co-expression pattern of CTAs with MAGE family members at both mRNA and protein levels. Genomewide demethylation may explain the activation of GAGE, NY-ESO-1, MAGE-A, and SSX gene expression in cancer cells and show that they may be co-regulated [19]. However, demethylation alone may not explain the selective activation of different cancer/testis genes in different cancers.

Although we found no correlation between CTA expression and disease progression or survival in this study, there is increasing evidence that they are correlated. For instance, CTA expression is correlated with increasing tumor stage and/or grade in ovarian neoplasms [20], bladder cancer [21] and malignant gammopathies [18]. The analysis of CTA expression in different cancers has shown variations in the number of CTAs expressed and their expression frequency. Esophageal cancer, along with head and neck cancer, ovarian cancer and sarcoma, is included in the group of moderate CTA expressers [3]. Thus, the generalized conclusion of a correlation between CTA expression and survival for every cancer may be incomplete.

As CTA-expressing esophageal cancer patients will be eligible for specific immunotherapy against CTAs, it is important to evaluate the quantity and pattern of expression of each antigen prior to the

immunotherapy. We have observed that in the cases of co-expression, NY-ESO-1-positive areas and particularly GAGE-positive areas are mostly overlapped with MAGE-positive areas and rarely stained different parts of the tumor tissue. Therefore, a combination of GAGE, NY-ESO-1 and MAGE antigens may enlarge the immunopositive area, although the targeted area will still be limited. The detection of immunonegative cancer cells in every specimen regardless of the size of the immunopositive area covered by multiple antigens may indicate the addition of other drugs to increase the therapeutic effect of poly-CTA-specific vaccines. For instance, 5-aza-2'-deoxycytidine is an effective agent as it induces CTA expression preferentially in tumor cells [22] and at both mRNA and protein levels even in CTA-negative cells [23, 24].

To our knowledge, this is the first study that dealt with the analysis of the expression of multiple CTA proteins in esophageal cancer on a large scale and showed the staining of GAGE protein. By evaluating three sections of the antigen-negative tumors, we were able to overcome the false negative results produced by the heterogeneous expression patterns. After staining the second series of slides, 98% of the antigen-expressing tumors were identified. For future studies, we suggest evaluation of two different sections of the antigen-negative tumors as the evaluation of only one section led to our overlooking 21% of the antigen-positive cases, and the evaluation of three sections did not provide important data.

In conclusion, we demonstrated that GAGE, NY-ESO-1 and MAGE-A proteins were heterogeneously expressed in esophageal cancer and their expression was not correlated with disease progression or survival.

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Table 1. Patients’ characteristics.

Characteristic	No.
Age: years, mean (range)	64 (40-85)
Gender	
Female	25
Male	188
Histopathology	
Squamous cell carcinoma	202
Adenocarcinoma	6
Adenosquamous carcinoma	4
Undifferentiated carcinoma	1
Tumor differentiation	
Well	56
Moderate	121
Poor	35
Lymphatic permeation	
Absent	87
Present	126
Vascular permeation	
Absent	118
Present	95
Tumor location	
Cervical esophagus	11
Upper esophagus	11
Middle esophagus	108
Lower/abdominal esophagus	83
Tumor size: mm, mean (range)	60 (7-247)
Type of resection	
R0	183
R1	4
R2	26
UICC Classification	
Tumor	
in situ	3
1	66
2	21
3	98
4	25
Node	
0	86
1	127
Metastasis	
0	175
1	38
Stage grouping	
0	3
I	46
II	51
III	75
IV	38

Table 2. Clinicopathologic parameters and GAGE, NY-ESO-1 and MAGE-A expression.

	GAGE			NY-ESO-1			MAGE-A		
Parameter	**-**	**+**	***P***	**-**	**+**	***P***	**-**	**+**	***P***
Age									
<65 years	94	15	0.038*	78	26	0.132	47	57	0.493
≥65 years	77	27		91	18		55	54	
Gender									
Female	21	4	0.791	20	5	1.000	15	10	0.209
Male	150	38		149	39		87	101	
Histopathology									
SCC**	162	40	1.000	162	40	0.244	95	107	0.359
Others	9	2		7	4		7	4	
Tumor differentiation									
Well-moderate	144	33	0.356	140	37	1.000	87	90	0.358
Poor	26	9		28	7		14	21	
Lymphatic permeation									
Absent	74	13	0.164	73	14	0.228	47	40	0.163
Present	97	29		96	30		55	71	
Vascular permeation									
Absent	98	20	0.300	97	21	0.307	59	59	0.581
Present	73	22		72	23		43	52	
Tumor location									
Cervical/Upper/Mid	114	16	0.001*	106	24	0.386	71	59	0.017*
Lower/Abdominal	57	26		63	20		31	52	
Tumor size									
<60 mm	104	17	0.023*	93	28	0.393	64	57	0.099
≥60 mm	67	25		76	16		38	54	
Type of resection									
R0	149	34	0.324	144	39	0.636	88	95	1.000
R1-2	22	8		25	5		14	16	
Tumor									
T0-2	74	16	0.603	69	21	0.494	50	40	0.071
T3-4	92	26		100	23		52	71	
Node									
N0	72	14	0.381	67	19	0.731	46	40	0.209
N1	99	28		102	25		56	71	
Metastasis									
M0	143	32	0.266	139	36	1.000	85	90	0.702
M1	28	10		30	8		17	21	
Stage grouping									
0-II	82	18	0.607	78	22	0.735	50	50	0.585
III-IV	89	24		91	22		52	61	

*Statistically significant.
**Squamous cell carcinoma.

Table 3. Major prognostic factors and GAGE, NY-ESO-1 and MAGE-A expression.

	GAGE Positive*	NY-ESO-1 Positive*	MAGE-A Positive*	Total no. tested
Tumor				
in situ	0	0	0	3
1	9 (14)	13 (20)	28 (42)	66
2	7 (33)	8 (38)	12 (57)	21
3	19 (19)	20 (20)	58 (59)	98
4	7 (28)	3 (12)	13 (52)	25
Node				
0	14 (16)	19 (22)	40 (47)	86
1	28 (22)	25 (20)	71 (56)	127
Metastasis				
0	33 (18)	36 (21)	90 (51)	175
1	10 (26)	8 (21)	21 (55)	38
Stage grouping				
0	0	0	0	3
I	6 (13)	9 (19)	18 (39)	47
II	12 (24)	13 (26)	32 (63)	50
III	14 (19)	14 (19)	40 (53)	75
IV	10 (26)	8 (21)	21 (55)	38

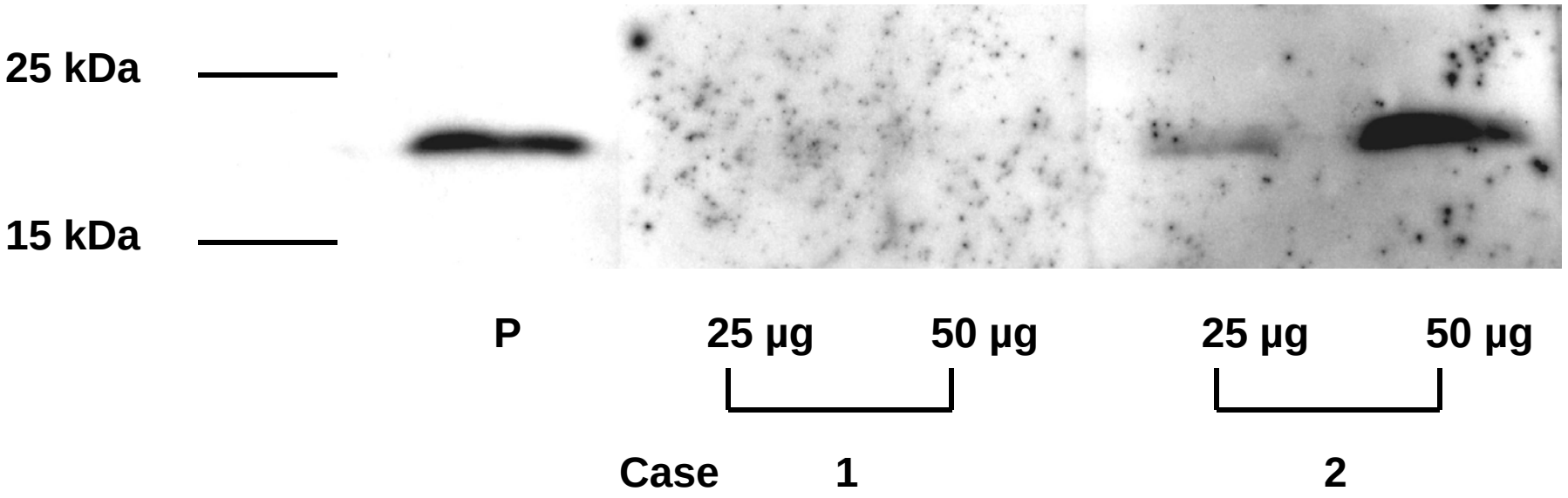
* The percentage of positive cases with respect to the total number of cases is shown in parentheses.

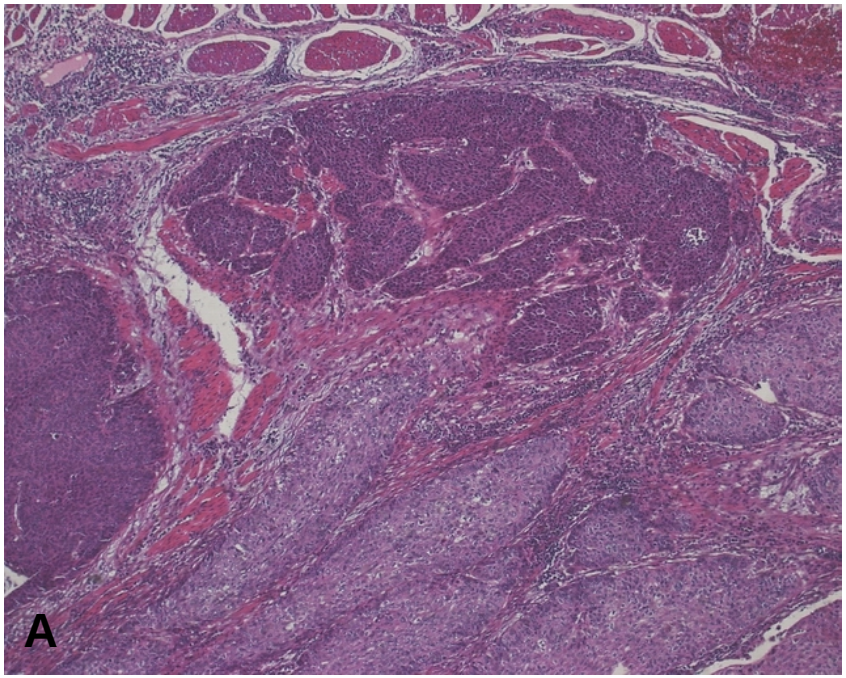
FIGURE LEGENDS

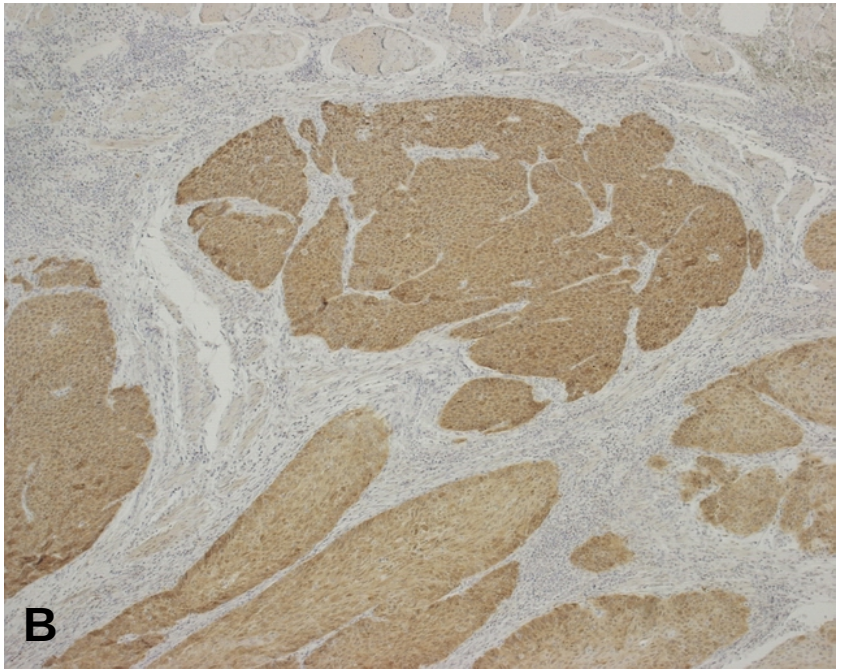
Figure 1. Immunohistochemical staining. Normal testis tissue, (A) GAGE antigen staining of spermatogonia, homogeneous expression (original magnification x300), (B) Immunoreactive with SSX antigen, showing strong nuclear staining of spermatogonia and heterogeneous expression (original magnification x250). Esophageal squamous cell cancer, (C) H&E, (D) GAGE, (E) NY-ESO-1 and (F) MAGE-A stainings, cytoplasmic and occasionally nuclear staining of tumor cells, heterogeneous expression pattern showing mixture of immunopositive and immunonegative cells surrounded by negative intervening connective tissue (original magnification x100).

Figure 2. GAGE, NY-ESO-1 and MAGE-A expression frequencies.

Figure 3. Survival curves of (A) GAGE-, (B) NY-ESO-1- and (C) MAGE-A-positive and -negative patients. (D) Survival curves of patients grouped according to the number of CTAs expressed. Each dot represents the point at which patients' data were censored.


25 kDa
15 kDa
P
25 µg
50 µg
25 µg
50 µg
Case
1
2


A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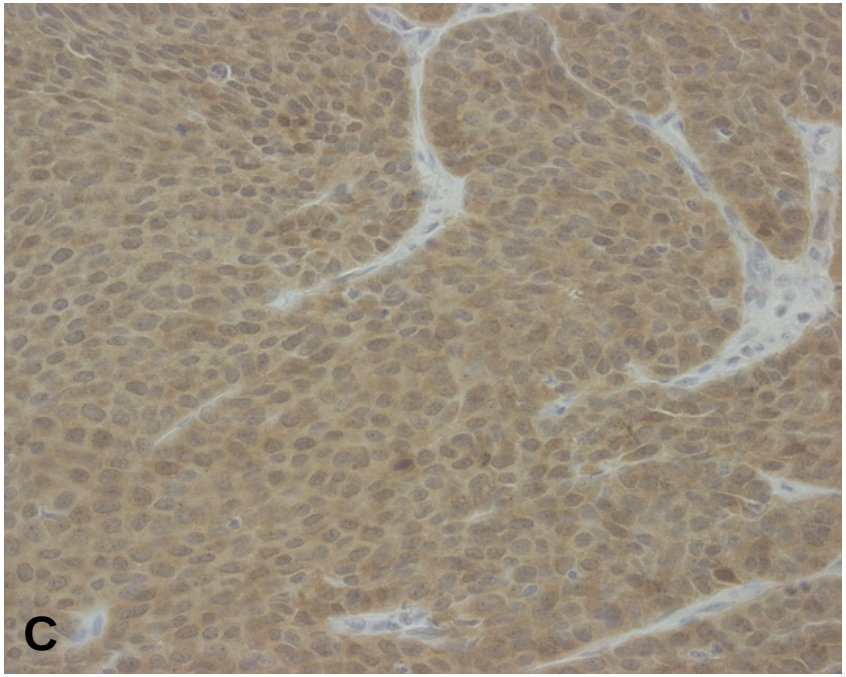
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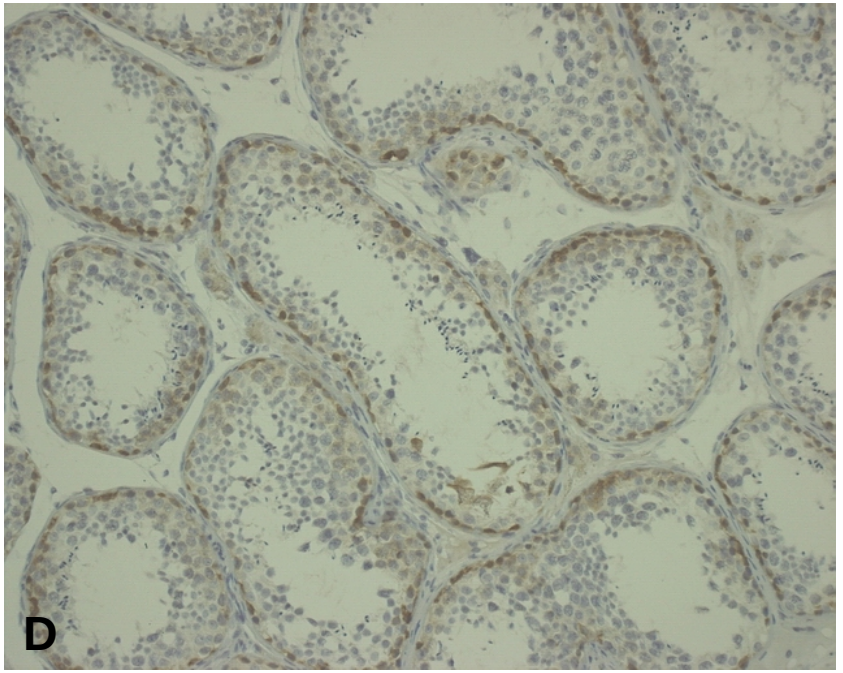
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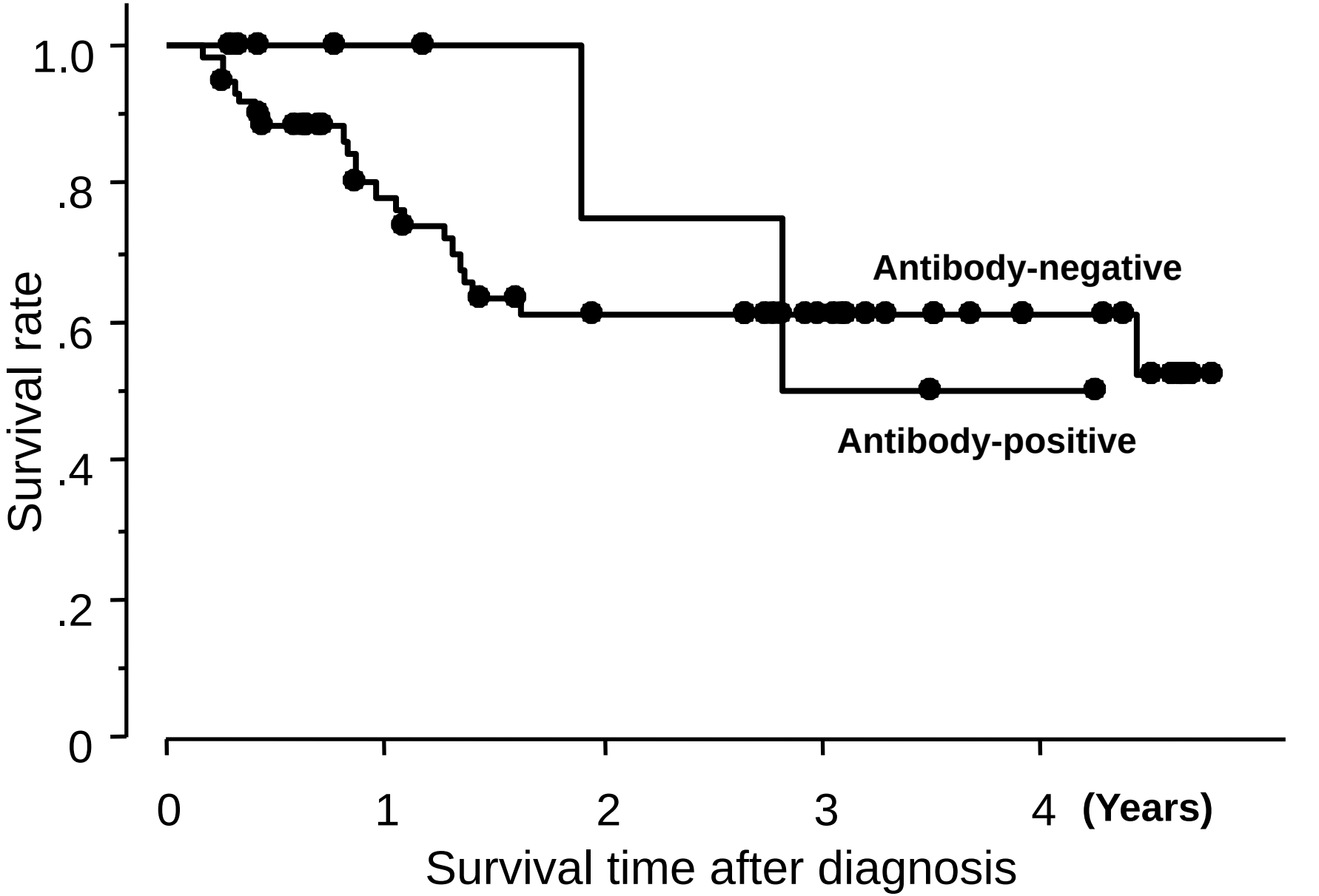
1.0
.8
.6
.4
.2
0
Survival rate
Antibody-negative
Antibody-positive
0
1
2
3
4
(Years)
Survival time after diagnosis