NY-ESO-1 EXPRESSION AND ITS SERUM IMMUNOREACTIVITY IN ESOPHAGEAL CANCER

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ABSTRACT

PURPOSE. NY-ESO-1, a member of the cancer/testis antigen (CTA) family, elicits humoral and cellular immune response in patients with advanced cancer. Unresectable or metastatic esophageal carcinoma patients do not benefit from the present multimodality treatment regimens for survival. The objectives of this study are to analyze the antibody response to NY-ESO-1 antigen in patients with esophageal cancer and to determine the potential of NY-ESO-1 for use in tumor-specific immunotherapy.

METHODS. Sera from 69 patients with esophageal cancer were investigated for antibody production against NY-ESO-1 by Western blot analysis. Fifty-six tissue samples from those patients were also analyzed for NY-ESO-1 protein expression by immunohistochemistry.

RESULTS. NY-ESO-1 protein expression was found in 18 of 56 (32%) esophageal carcinomas. Serum immunoreactivity specific for NY-ESO-1 was found in nine patients (13%). Eight of the nine patients were in the advanced stage (Stages III and IV). There was no relationship between clinicopathologic features and serum immunoreactivity for NY-ESO-1. NY-ESO-1 protein expression was detected in three of five antibody-positive patients whose tissues were available for analysis. Survival analysis showed no significant difference between antibody-positive and antibody-negative patient groups.

CONCLUSIONS. Humoral immune response to NY-ESO-1 antigen was established in patients with esophageal cancer in the advanced stage. NY-ESO-1 is a good candidate for vaccine-based immunotherapy for advanced esophageal carcinoma.

KEYWORDS

Esophageal cancer; tumor antigens; NY-ESO-1; humoral immunity

INTRODUCTION

Esophageal cancer is one of the most common malignancies in the world. At the time of detection, most patients already present with the advanced disease. Tumor depth, lymphatic spread and distant metastases are strong independent prognostic variables [1]. Although currently, the curative resection rate is higher than 90%, the three-year survival rate after curative resection remains at 36-40% [7,23]. Postoperative chemotherapy slightly improves disease-free survival but provides no benefit to overall survival [2,6]. Moreover, the treatment is rather toxic [16]. Thus, postoperative chemotherapy is not regarded as a standard therapy and the development of novel therapeutic approaches is awaited. Recently, reports of the clinical efficacy of immunotherapy have generated new hope. Activation of the immune system against tumor cells has led to distinct tumor regression, particularly in patients with melanoma [17] and renal cell carcinoma [27]. There are only a few clinical trials of immunotherapy for advanced esophageal carcinoma [18,24,25]. However, the results are promising and suggest that cancer vaccines may represent an effective therapeutic strategy for esophageal cancer patients.

Tumor-associated antigens (TAAs) induce spontaneous immune responses in different types of human cancers [20]. Their growing list presents several target antigens for the construction of cancer vaccines. Among the TAAs, cancer/testis antigens (CTAs) are of particular interest because of their restricted expression patterns in different cancers and in normal tissues, particularly in the testis [4]. NY-ESO-1, which is one of the CTAs, was originally identified by serological expression techniques using serum from an

esophageal cancer patient [3]. NY-ESO-1 is one of the most potent immunogenic CTAs, eliciting both humoral and cellular immune responses in patients with NY-ESO-1 expressing tumors [8]. Because of its cancer/testis restricted expression pattern and immunogenicity, cancer patients expressing NY-ESO-1 protein with associated immune response are likely to be candidates for anti-NY-ESO-1 immunotherapy. However, the expression rate and the rate of antibody response differ with the malignancy type; for example, 80% of sarcoma patients express NY-ESO-1 protein [10] but only 4% exhibit antibody response [13]. Therefore, NY-ESO-1 expressing esophageal carcinoma may be an optimal target for anti-NY-ESO-1 immunotherapy. Before evaluating the potential of antigen-specific therapy against NY-ESO-1, however, both expression rate and humoral immunogenicity have to be determined. In this regard, we examined the presence of anti-NY-ESO-1 antibodies in sera of esophageal cancer patients by Western blotting and analyzed NY-ESO-1 protein expression in formalin-fixed paraffin-embedded tumor sections.

MATERIALS AND METHODS

Patients and sera

One hundred esophageal cancer patients were treated at the Department of Surgery, Niigata University Medical Hospital from June 1998 to March 2003 were enrolled in the study. Seventeen patients who were diagnosed with double cancers were excluded from the study. Fourteen patients who had undergone some kind of surgical procedure other than biopsy or had received neoadjuvant therapy before serum collection were also excluded. The remaining 69 patients were considered qualified and included in the present study. Blood was collected before the start of treatment. Sera were immediately separated and stored at -80^{o}C until use. Informed consent was obtained from all the patients.

Data including age, sex, treatment protocol, tumor node metastasis (TNM) stage and outcome were obtained from clinical and pathologic records and our esophageal cancer database. Tumor stage was determined according to the TNM classification (5th ed.) of the International Union Against Cancer (UICC). Final pathologic staging was determined for patients undergoing surgical excision of the tumor without prior treatment. Clinical staging was determined for the remaining patients. The clinical characteristics of the patients are summarized in Table 1. Patients were followed up until their demise, dropout, or June 30, 2003.

Western Blot Analysis and Cell Line

We evaluated patient's serum immunoreactivity specific for NY-ESO-1 by means of Western blot analysis using the melanoma cell line SK-MEL-37

as a positive control antigen. SK-MEL-37 cells were kindly provided by the Ludwig Institute for Cancer Research (New York, NY).

Eight microliters of lysate per lane (containing 50 µg of protein) was mixed with 2x SDS-sample buffer and electrophoresis was conducted on 15% SDS-polyacrylamide gel. After blotting on a cellulose nitrate filter (0.20 µm, Advantec MFS, Inc., Dublin, CA) and blocking with 10% low-fat milk in Tris-buffered saline/0.1% Tween-20 for 6 h at 37^{o}C, blots were incubated overnight at 4^{o}C with patient's serum at 1:50 dilution or with a mouse monoclonal antibody against NY-ESO-1 (Zymed Laboratories Inc., South San Francisco, CA) at 2 µg/ml as a positive control. The membranes were then incubated with goat anti-human IgG (Fc specific; Sigma-Aldrich Inc., St. Louis, MO) at 1:5000 dilution or goat anti-mouse IgG (Zymed Laboratories Inc.) at 1:2000 dilution for 3 h. Serum antibodies binding to NY-ESO-1 were visualized by a chemiluminescence system (ECL; Amersham Pharmacia Biotech Inc., Piscataway, NJ). Sera were considered positive for NY-ESO-1 antibody if a 22 kDa band was detectable. Positive sera were analyzed three times and negative sera were tested two times.

Immunohistochemistry

We used archival samples of the Department of Pathology, Niigata University Hospital for the immunohistochemical analysis of NY-ESO-1 protein. Of the 69 cases where serum immunoreactivity for NY-ESO-1 was analyzed, 60 were available for the immunohistochemical analysis of tumor for NY-ESO-1 expression. We excluded four cases because they received chemotherapy, radiotherapy or chemoradiotherapy before resection, and analyzed the

remaining 56 cases. Three observers (TK, AA and KS) evaluated the slides independently with masking of clinical data.

Immunohistochemistry was performed using a Histofine SAB-PO(M) kit (Nichirei Corporation, Tokyo, Japan). All incubations were conducted at room temperature unless stated otherwise. Formalin-fixed sections of 4 μm thickness were placed on coated glass slides and deparaffinized with xylene. After rehydration, microwave treatment was performed in EDTA buffer (1 mM, pH 8) for 15 min at 500 W. The slides were treated with 0.3% (v/v) H_2O_2 in methanol and incubated with 10% rabbit serum for 30 min. Mouse monoclonal NY-ESO-1 antibody was then added at a concentration of 2.5 μg/ml and incubation was carried out for 1 h at room temperature, then overnight at 4^{o}C. A biotin-labeled anti-mouse antibody was used to detect primary antibody for 30 min, followed by peroxidase-labeled streptavidin for another 30 min. The reaction was developed by diaminobenzidine tetrahydrochloride and the slides were counterstained with hematoxylin. The number of stained tumor cells was graded as follows: <5% -, 5-50% +, and >50% ++. The concentration of NY-ESO-1 antibody was determined by titration in testis tissue and testis was also used as a control.

Statistical analysis

Statistical analysis was performed using the Fisher's exact test, the Mann-Whitney *U* test and the log-rank test. A *P* value (two-tailed) less than 0.05 was considered to be significant.

RESULTS

Antibody response to NY-ESO-1

A specific 22 kDa band corresponding to NY-ESO-1 was unequivocally detected by Western blot analysis using SK-MEL-37 lysates (Fig. 1). Two more bands were detected at high molecular weight range (>45 kDa) because of non-specific interactions. Antibody specific for NY-ESO-1 was found in sera collected from nine of 69 (13%) patients with esophageal carcinoma. One patient showing serum immunoreactivity was in Stage I, whereas the other eight patients were in Stages III and IV (Stages 0/I/II vs. Stages III/IV; P=0.07). Age (P=0.70), sex (P=0.22), treatment protocol (resection vs. others; P=0.25), tumor (*in situ*/T1/T2 vs. T3/T4; P>0.99), node (negative vs. positive; P=0.50), and metastasis (negative vs. positive; P=0.20) were not related to the serological status of the patients. The clinical characteristics of the antibody-positive patients are listed in Table 2.

NY-ESO-1 protein expression in esophageal carcinoma

Eighteen of the 56 (32%) esophageal carcinomas analyzed showed expression of the NY-ESO-1 antigen. The extents of the antigen expression were 50% or less in eight carcinomas and more than 50% in 10 carcinomas. The staining pattern was cytoplasmic, and half of the carcinomas showed intratumoral heterogeneity (Fig. 2).

Relationship between NY-ESO-1 protein expression and serum immunoreactivity

Of the nine antibody-positive patients, five were analyzed for NY-ESO-

1 expression in the tumors. One tumor showed strong and homogeneous expression and two tumors showed heterogeneous expression in less than 50% of the tumor cells. The remaining two showed no expression of NY-ESO-1 protein. Serum immunoreactivity for NY-ESO-1 was higher in patients with tumors expressing NY-ESO-1 protein than in those with non-NY-ESO-1 expressing tumors (3/18 vs. 2/38), although there was no statistically significant difference (*P*=0.31).

NY-ESO-1 antibody response and survival

The three-year survival rates of antibody-positive and antibody-negative patient groups were 50% and 61%, respectively. Survival analysis showed no significant difference between the two groups (*P*=0.71). Figure 3 shows survival curves for groups of patients separated according to antibody response.

DISCUSSION

Locally advanced esophageal cancer results in obstruction and malnourishment. Starvation suppresses cellular immune response and increases serum IgG and IgA levels [5,21]. The deficiency of cell-mediated immunity indicates a poor prognosis [26]. Although serum immunoglobulin levels were reported to be altered, we were not able to find any reports showing the presence of a functional impairment of humoral immune response in esophageal cancer patients. In 1997, the detection of anti-NY-ESO-1 antibodies in a patient with esophageal squamous cell carcinoma revealed the presence of an activated immune system interacting with the NY-ESO-1 antigen presented by the tumor [3]. Interestingly, a subsequent study that analyzed 12 esophageal cancer cell lines by reversed transcription-polymerase chain reaction (RT-PCR) did not reveal any NY-ESO-1 expression [12], whereas other studies that employed esophageal tumor samples revealed frequent expression [15,19], making NY-ESO-1 a promising candidate for antigen-specific immunotherapy.

In the present study, we addressed the incidence of humoral immunoreactivity for NY-ESO-1 in patients with esophageal carcinoma. Our consecutive and middle-sized sample analysis revealed that nine of 69 (13%) patients had anti-NY-ESO-1 antibodies and that a majority of the NY-ESO-1 antibody-positive patients (89%) were in Stages III and IV. The incidence of specific antibody response is comparable to those in a previous study of ovarian cancer and melanoma [22]. In that survey of the sera of patients with melanoma, ovarian, lung, breast and colon cancers, no correlation between serum antibody positivity and disease stage could be shown. It has been

surmised that a large tumor mass and long exposure to the tumor antigen may facilitate the increase of the anti-NY-ESO-1 antibody titer [9]. However, similar to the previous study [22], the relationship between serum antibody positivity and disease stage did not reach the level that is statistically significant in our study of esophageal cancer patients. A large series is needed to confirm whether there is a relationship between serum immunoreactivity and disease progression.

To date, there has been no study that analyzed NY-ESO-1 protein in a consecutive series of esophageal cancer patients. Only two studies in which NY-ESO-1 expression was analyzed by RT-PCR have revealed the mRNA expression in 24% [15] and 50% of esophageal carcinoma patients [19]. The expression rate in our study was somewhat between those values. Our study and the previous study [19] that showed a considerably high rate of NY-ESO-1 gene expression (50%) using RT-PCR holds true also in the case of NY-ESO-1 protein expression.

We had initially expected that the NY-ESO-1 protein should be expressed by the tumor in all the antibody-positive patients. However, of the nine antibody-positive patients, two had tumors that were negative for NY-ESO-1 expression. It is unlikely that autoimmunity against NY-ESO-1 occurred without exposure to the antigen originating from cancer, because no immunoreactivity was found in serum collected from healthy volunteers (data not shown). NY-ESO-1 is known to show heterogeneous expression in different cancers [11] except synovial sarcomas [10]. In our analysis, NY-ESO-1 expression was also heterogeneous. We speculate that the false negativity result caused by the intratumoral heterogeneity is the main

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contributor to the seemingly unlikely phenomenon. The results may likewise indicate that we should sample from different areas of the tumor in order to evaluate NY-ESO-1 protein expression.

As regards cancer immunity, it is interesting to know whether the prognosis of patients possessing specific antibodies against CTAs is improved or not, because specific cancer immunity may be established in such patients. In an analysis of antibody response to NY-ESO-1 in sporadic medullary thyroid carcinoma patients, the possible correlation between disease recurrence and presence of serum anti-NY-ESO-1 antibodies was investigated but no significant correlation was found [14]. In the present study, there was no significant difference in the survival rate between antibody-positive and antibody-negative esophageal cancer patients. As shown in Figure 3, the two survival curves intersect each other and seem to be similar, and the expected survival rates are higher than those of previous studies [7,23]. This may be a consequence of the small number of antibody-positive patients and the recent enrollment of some patients with a rather short follow-up. Together with results from the above-mentioned study of thyroid cancers, we conclude that the presence of anti-NY-ESO-1 antibodies has no significant effect on a patient's prognosis. However, it has been suggested that NY-ESO-1 gene expressing tumors may induce development of antibodies over time and recurrence or metastases may result in an increase in the anti-NY-ESO-1 antibody titer [22]. Therefore, a period of observation may reveal changes in the expression patterns of NY-ESO-1 and repeated analysis may be necessary to understand the correlation between the presence of specific antibodies and the clinical course or outcome of the disease. In order to

determine if NY-ESO-1 has malignant potential, more extensive studies are needed.

The clinical experience of immunotherapy for advanced esophageal carcinoma is limited. The administration of IL-2 in conjunction with preoperative chemotherapy [18] and the regional injection of autologous tumor-activated lymphocytes [24,25] were reported to produce a clinical response with tolerable toxicities. Those reports are small-scale clinical trials and their results encourage further investigation. Not only NY-ESO-1 but also other members of the CTA family have been analyzed to date. Esophageal carcinoma has been shown to express MAGE, BAGE, GAGE [28], LAGE-1, SCP-1 and SSX-4 genes [15], which leads to the possibility of developing polyvalent cancer vaccines. Those studies suggest that esophageal cancer is a good candidate for immunotherapy.

The current study demonstrated that antibody response to NY-ESO-1 antigen occurs in esophageal cancer patients. Further studies are necessary to understand the antibody response and its influence on the course of the malignant disease. This data suggests that NY-ESO-1 could be a novel target for tumor-specific antigen-based immunotherapy for the treatment of patients with esophageal carcinoma, alone or in combination with other tumor-specific antigens [15,28].

書式変更: 最初の行 ： 0 字，1 行の文字数を指定時に右のインデント幅を自動調整する，改ページ時 1 行残して段落を区切らない，日本語と英字の間隔を自動調整する，日本語と数字の間隔を自動調整する

削除: The induction of an immune response may suppress tumor growth and yield a favorable prognosis even in advanced cases. Esophageal cancer patients may benefit from specific immunotherapy for NY-ESO-1 antigen, alone or in combination with other tumor-specific antigens.

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Table 1. Patients' characteristics.

Characteristic	No.
Number of patients	69
Age: years, mean (range)	66 (41-82)
Gender	
Male	62
Female	7
Histology	
Squamous cell carcinoma	67
Adenocarcinoma	1
Small cell carcinoma	1
Initial Treatment	
Esophagectomy	50
Others*	19
UICC Classification	
Tumor	
in situ	2
1	23
2	1
3	24
4	19
Node	
0	31
1	38
Metastasis	
0	53
1	16
Stage Grouping	
0	2
I	20
II	6
III	25
IV	16

*Others include radiotherapy, chemotherapy, chemoradiotherapy, esophageal stenting, and endoscopic mucosal resection.

Table 2. Clinical characteristics of NY-ESO-1 antibody-positive patients.

Patient no.	Age, sex	TNM	Initial treatment	IHC
14	59, M	T4N1M1	Exploration only	N. A.
18	58, M	T2N1M1	Resection	+
22	71, M	T4N1M0	Resection	+
24	66, F	T3N0M1	Resection	++
30	80, M	T4N1M0	Radiotherapy	N. A.
42	73, M	T4N0M0	Chemotherapy	N. A.
57	61, F	T1N0M0	Resection	-
60	69, M	T1N1M1	Resection	-
65	73, M	T4N1M0	Chemoradiotherapy	N. A.

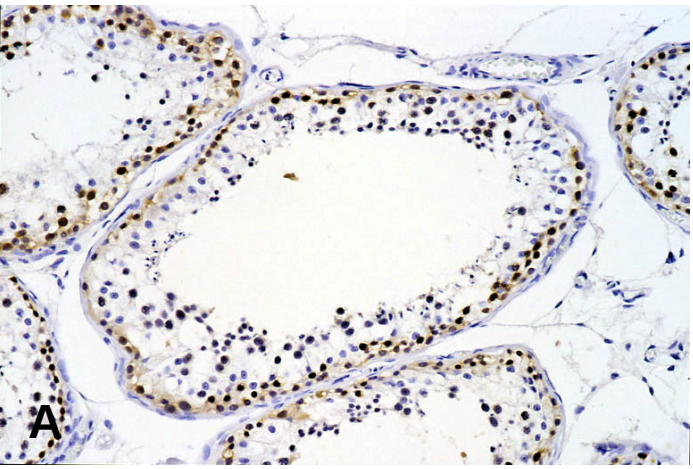
IHC: Immunohistochemistry; N. A.: not analyzed.

FIGURE LEGENDS

Fig. 1. Western blot analysis. (P) Positive control, mouse monoclonal anti-NY-ESO-1 antibody (2 µg/ml). Cases 1 and 2, patients' sera (1:50) tested against 25 and 50 µg protein extracted from melanoma cell line, negative and positive samples, respectively.

Fig. 2A-D. NY-ESO-1 protein expression. Esophageal squamous cell cancer: (A) hematoxylin and eosin staining, (B) immunostaining, diffuse and strong expression of NY-ESO-1 (original magnification, x60), and (C) cytoplasmic and nuclear staining pattern (original magnification, x300). Testis: (D) strong staining within seminiferous tubules, spermatogonia and primary spermatocytes (autopsy specimen from a patient with no testicular disease) (original magnification, x150).

Fig. 3. Survival curves of antibody-positive and antibody-negative patients. Each dot represents the point at which patients' data were censored.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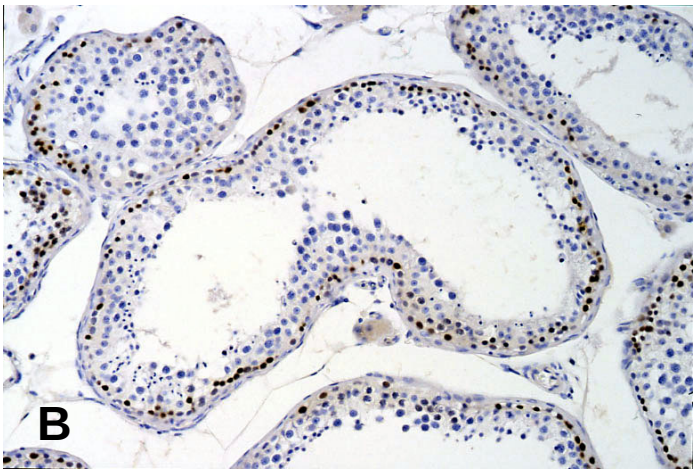
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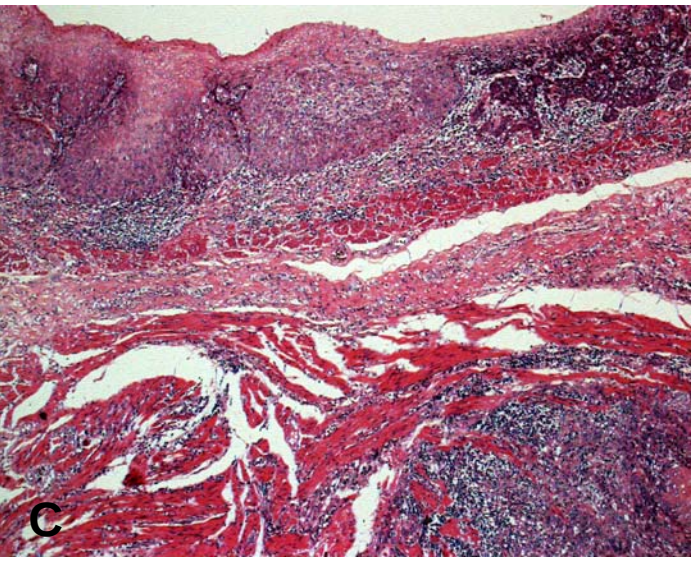
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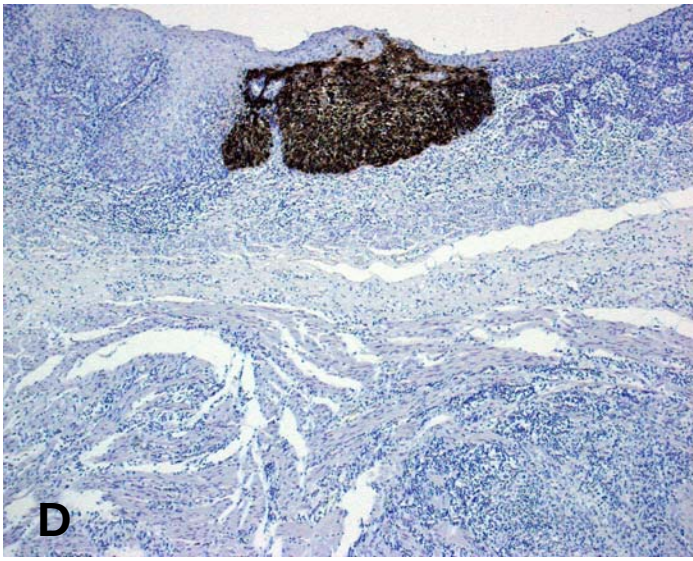
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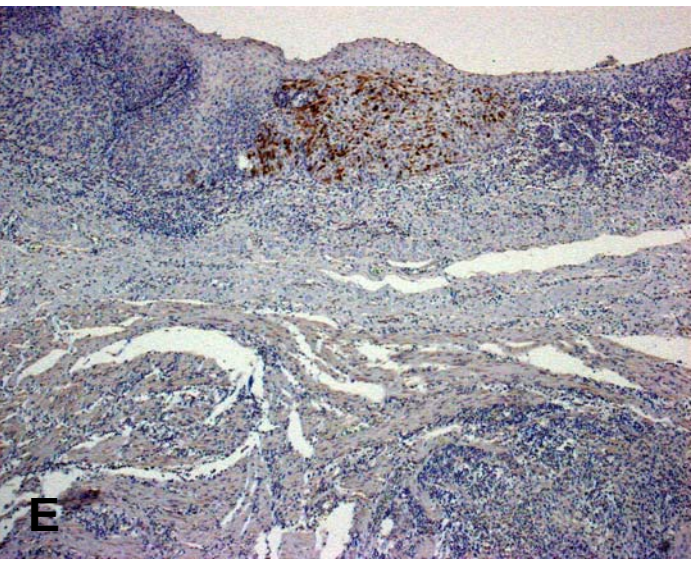
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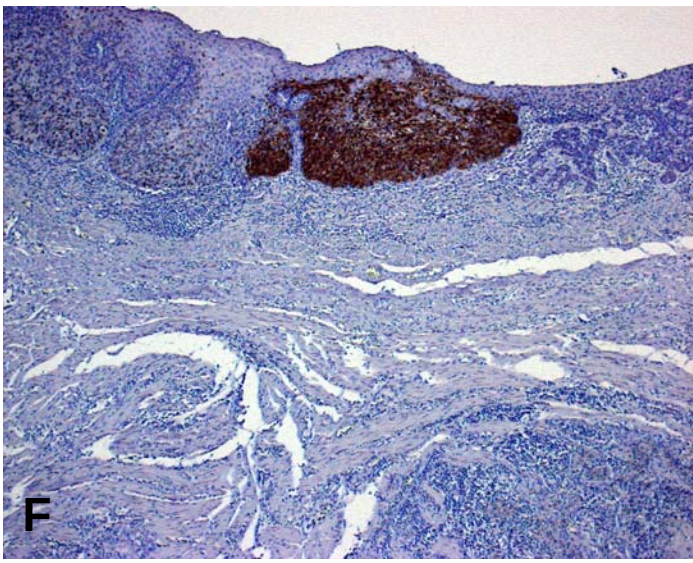
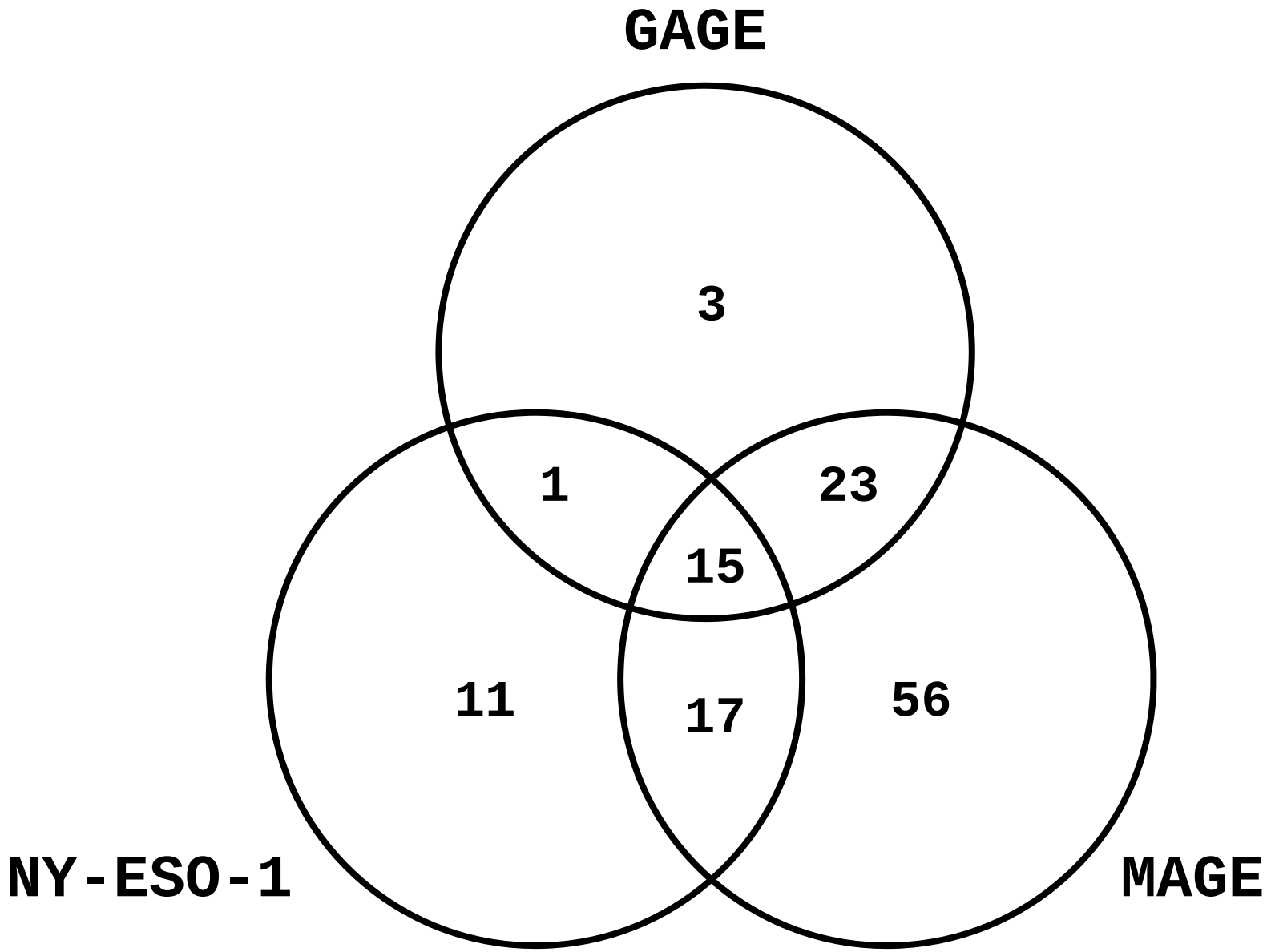
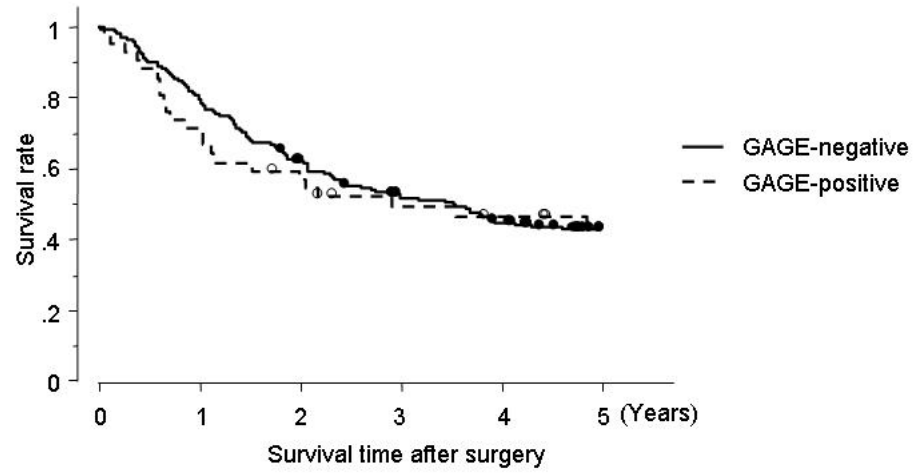
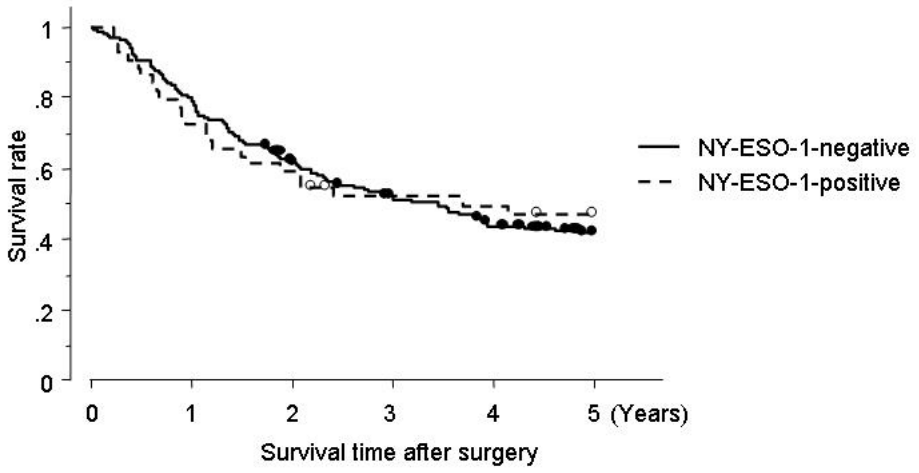

F

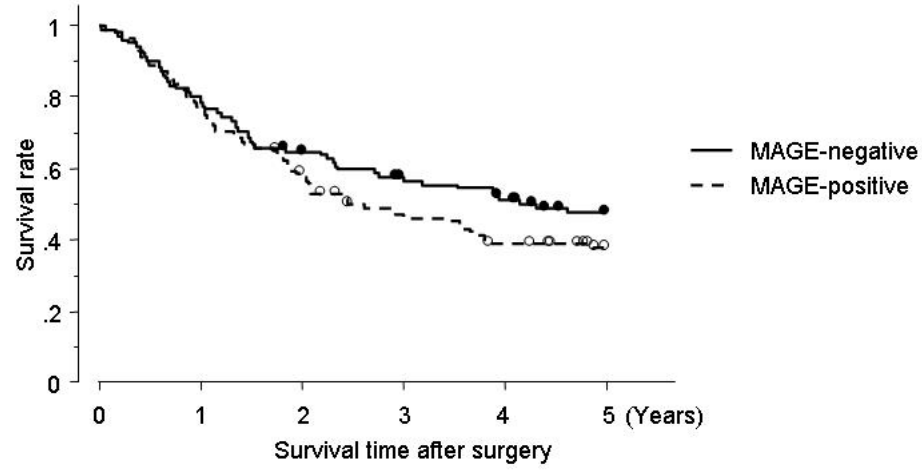

GAGE
3
1
23
15
11
17
56
NY-ESO-1
MAGE


Survival rate
0
.2
.4
.6
.8
1
0
1
2
3
4
5 (Years)
Survival time after surgery
GAGE-negative
GAGE-positive

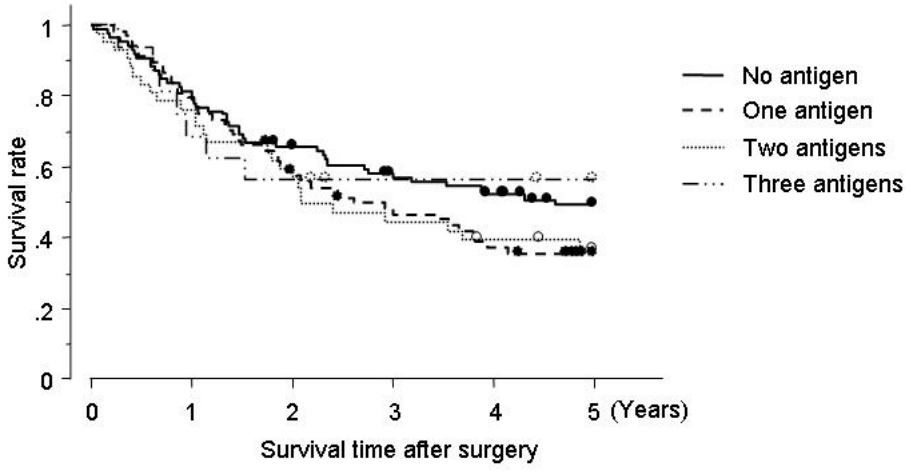
A


Survival rate
0
.2
.4
.6
.8
1
0
1
2
3
4
5 (Years)
Survival time after surgery
NY-ESO-1-negative
NY-ESO-1-positive

B


Survival rate
0
.2
.4
.6
.8
1
0
1
2
3
4
5 (Years)
Survival time after surgery
MAGE-negative
MAGE-positive

C


Survival rate
0
.2
.4
.6
.8
1
0
1
2
3
4
5 (Years)
Survival time after surgery
No antigen
One antigen
Two antigens
Three antigens

D