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ORIGINAL ARTICLE

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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 responses in patients with advanced cancer. Unresectable or metastatic esophageal carcinoma patients do not benefit from the present multimodality treatment regimens in terms of survival. The objectives of this study were 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*: Serum from 69 patients with esophageal cancer was investigated for antibody production against NY-ESO-1 by Western blot analysis. Also analyzed by immunohistochemistry were 56 tissue samples from these patients for NY-ESO-1 protein expression. *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 9 patients (13%) of whom 8 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 tissue was available for analysis. Survival analysis showed no significant difference between antibody-positive and antibody-negative patient groups. *Conclusions*: A humoral immune response to NY-ESO-1 antigen was established in patients with advanced esophageal cancer. 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

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Introduction

Esophageal cancer is one of the most common malignancies in the world. At the time of detection, most patients already present with 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 3-year survival rate after curative resection remains at 36–40% [7, 10]. Postoperative chemotherapy slightly improves disease-free survival but provides no benefit in terms of overall survival [2, 6]. Moreover, the treatment is rather toxic [17]. 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 [18] and renal cell carcinoma [27]. There are only a few clinical trials of immunotherapy for advanced esophageal carcinoma [19, 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 [21]. The growing list of TAAs provides 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 an 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 [11] but only 4% exhibit an antibody response [14]. 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 the serum from 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 serum

Enrolled in the study were 100 esophageal cancer patients treated at the Department of Surgery, Niigata University Medical Hospital from June 1998 to March 2003. Of these patients, 17 diagnosed with double cancers were excluded from the study, and also excluded were 14 patients who had undergone some kind of surgical procedure other than biopsy or had received neoadjuvant therapy before serum collection. The remaining 69 patients were considered to qualify and were included in the present study. Blood was collected before the start of treatment. The serum were immediately separated and stored at −80°C until use. Informed consent was obtained from all 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 edition) 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 30 June 2003.

Western blot analysis and cell line

We evaluated the patients' serum immunoreactivity specific for NY-ESO-1 by 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). Lysate (8 μl per lane containing 50 μg of protein) was mixed with 2× SDS sample buffer and electrophoresis was conducted on 15% SDS-polyacrylamide gel. After blotting on a cellulose nitrate filter (0.20 μm; Advantec MFS, Dublin, Calif.) and blocking with 10% low-fat milk in Tris-buffered saline/0.1% Tween-20 for 6 h at 37°C, blots were incubated overnight at 4°C with patient's serum at a dilution of 1:50 or with a mouse monoclonal antibody against NY-ESO-1 (Zymed Laboratories, South

Table 1 Patient characteristics. Values are number of patients, except age in years

Number of patients	69
Age (years)	
Mean	66
Range	41–82
Gender	
Male	62
Female	7
Histology	
Squamous cell carcinoma	67
Adenocarcinoma	1
Small cell carcinoma	1
Initial treatment	
Esophagectomy	50
Other[a]	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

[a]"Other" includes radiotherapy, chemotherapy, chemoradiotherapy, esophageal stenting, and endoscopic mucosal resection

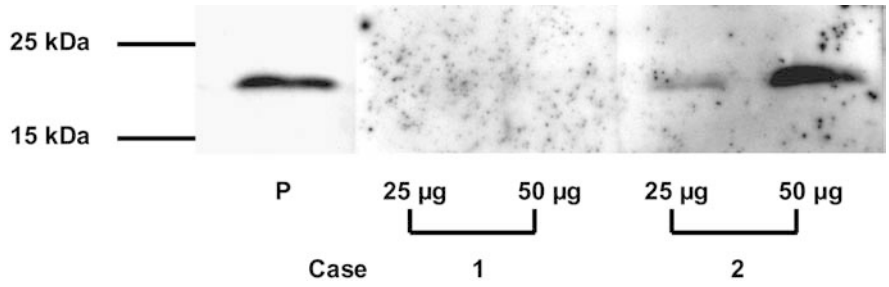

Fig. 1 Western blot analysis (*P* positive control, mouse monoclonal anti-NY-ESO-1 antibody, 2 µg/ml). *Cases 1 and 2* patient serum (1:50) tested against 25 and 50 µg protein extracted from melanoma cell line, negative and positive samples, respectively

San Francisco, Calif.) at 2 µg/ml as a positive control. The membranes were then incubated with goat anti-human IgG (Fc specific; Sigma-Aldrich, St. Louis, Mo.) at a dilution of 1:5000 or goat anti-mouse IgG (Zymed Laboratories) at a dilution of 1:2000 for 3 h. Serum antibodies binding to NY-ESO-1 were visualized using a chemiluminescence system (ECL; Amersham Pharmacia Biotech, Piscataway, N.J.). Serum was considered positive for NY-ESO-1 antibody if a 22 kDa band was detectable. Positive serum samples were analyzed three times and negative samples were tested two times.

Immunohistochemistry

We used archival samples from the Department of Pathology, Niigata University Hospital, for the immunohistochemical analysis of NY-ESO-1 protein. Of the 69 patients in whom 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 patients because they had received chemotherapy, radiotherapy or chemoradiotherapy before resection, and analyzed samples from the remaining 56 patients. Three observers (T.K., A.A. and K.S.) 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 m*M*, 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°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.

Table 2 Clinical characteristics of NY-ESO-1 antibody-positive patients (*NA* not analyzed)

Patient no.	Age (years)	Sex	TNM	Initial treatment	Immuno histochemistry
14	59	M	T4N1M1	Exploration only	NA
18	58	M	T2N1M1	Resection	+
22	71	M	T4N1M0	Resection	+
24	66	F	T3N0M1	Resection	+ +
30	80	M	T4N1M0	Radiotherapy	NA
42	73	M	T4N0M0	Chemotherapy	NA
57	61	F	T1N0M0	Resection	−
60	69	M	T1N1M1	Resection	−
65	73	M	T4N1M0	Chemoradiotherapy	NA

Statistical analysis

Statistical analysis was performed using Fisher's exact test, the Mann-Whitney *U*-test and the log-rank test. *P* values (two-tailed) less than 0.05 were 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). Antibody specific for NY-ESO-1 was found in the serum from 9 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

Of the 56 esophageal carcinomas analyzed, 18 (32%) showed expression of the NY-ESO-1 antigen. The extent of the antigen expression was 50% or less in eight

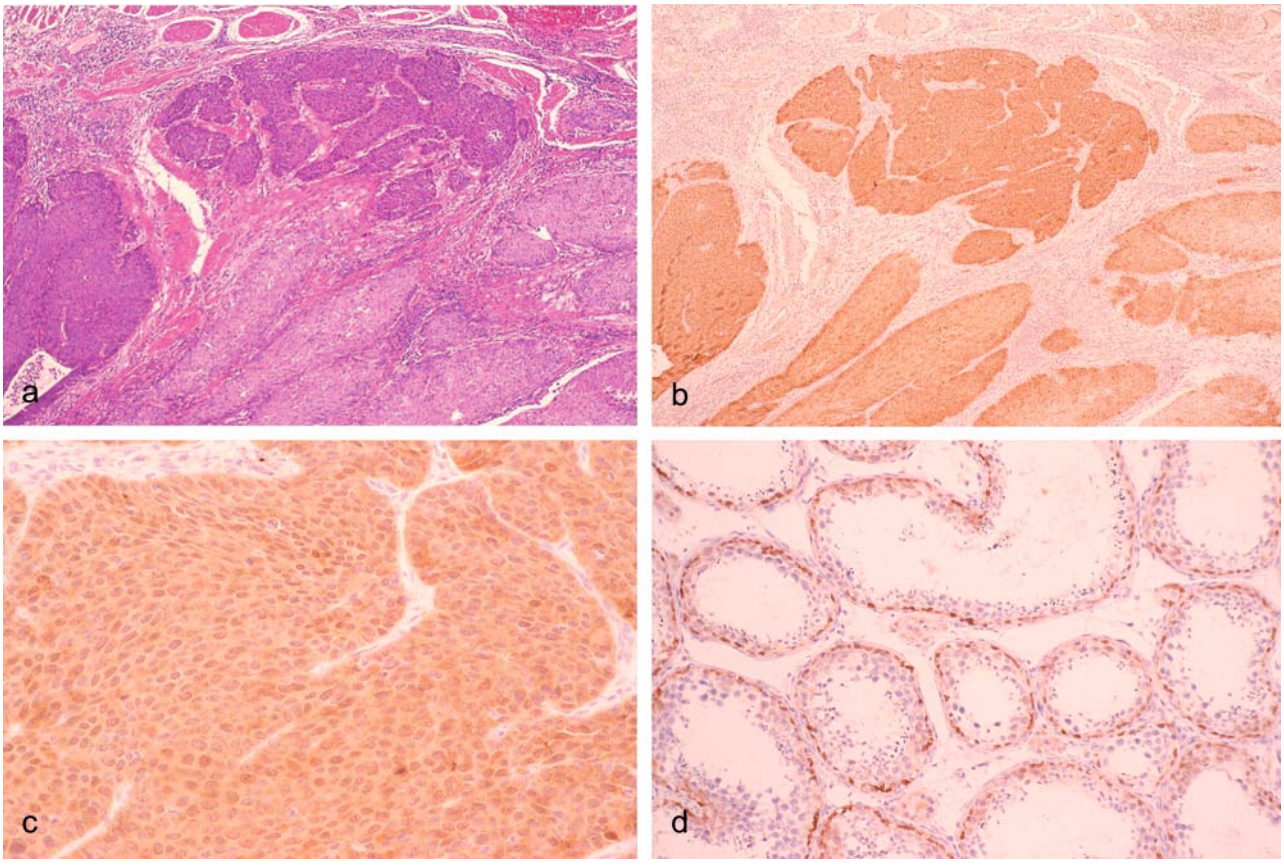

Fig. 2a–d NY-ESO-1 protein expression. Esophageal squamous cell cancer: **a** hematoxylin and eosin staining; **b** immunostaining, diffuse and strong expression of NY-ESO-1 (original magnification, ×60); **c** cytoplasmic and nuclear staining pattern (original magnification, ×300). **d** Testis: strong staining within seminiferous tubules, spermatogonia and primary spermatocytes (autopsy specimen from a patient with no testicular disease) (original magnification, ×150)

carcinomas and more than 50% in ten 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 the difference was not statistically significant ($P = 0.31$).

NY-ESO-1 antibody response and survival

The 3-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.

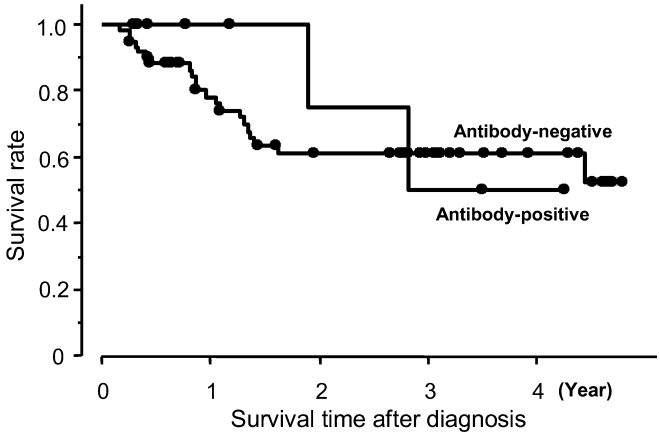

Fig. 3 Survival curves of antibody-positive and antibody-negative patients. Each dot represents the point at which patients' data were censored

Discussion

Locally advanced esophageal cancer results in obstruction and malnourishment. Starvation suppresses the cellular immune response and increases serum IgG and

IgA levels [5, 22]. Deficiency of cell-mediated immunity indicates a poor prognosis [26]. Although serum immunoglobulin levels have been reported to be altered, we were not able to find any reports of functional impairment of the humoral immune response in esophageal cancer patients. Anti-NY-ESO-1 antibodies have been detected in a patient with esophageal squamous cell carcinoma revealing the presence of an activated immune system interacting with the NY-ESO-1 antigen presented by the tumor [3]. Interestingly, a subsequent study in which 12 esophageal cancer cell lines were analyzed by reversed transcription-polymerase chain reaction (RT-PCR) did not reveal any NY-ESO-1 expression [13], whereas other studies in which esophageal tumor samples were investigated revealed frequent expression [16, 20], 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 medium-sized sample analysis revealed that 9 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 a specific antibody response is comparable to those found in a previous study of ovarian cancer and melanoma [23]. In that survey of the serum 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 in the anti-NY-ESO-1 antibody titer [9]. However, similar to the finding of the previous study [23], the relationship between serum antibody positivity and disease stage did not reach the level of statistical significance 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 in which NY-ESO-1 protein has been analyzed in a consecutive series of esophageal cancer patients. Only two studies in which NY-ESO-1 expression was analyzed by RT-PCR revealed the mRNA expression in 24% [16] and 50% of esophageal carcinoma patients [20]. The expression rate in our study was between these values. Our study and the previous study [20] in which a moderately high rate of NY-ESO-1 gene expression (50%) was shown 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 would 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 [12] except synovial sarcomas [11]. 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 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 would be 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 [15]. 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 Fig. 3, the two survival curves intersect and seem to be similar, and the expected survival rates are higher than those of previous studies [7, 10]. 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. Taking our results together with those of 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 [23]. Therefore, a period of observation may reveal changes in the expression patterns of NY-ESO-1 and repeated analysis may be necessary to elucidate 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 [19] and the regional injection of autologous tumor-activated lymphocytes [24, 25] have been reported to produce a clinical response with tolerable toxicities. Those reports are of small-scale clinical trials and their results encourage further investigation. Other members of the CTA family as well as NY-ESO-1 have been analyzed. Esophageal carcinoma has been shown to express MAGE, BAGE, GAGE [28], LAGE-1, SCP-1 and SSX-4 genes [16], 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 elucidate the antibody response and its influence on the course of the malignant disease. Our findings suggest 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 [16, 28].

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