Expression of the MAGE-A4 and NY-ESO-1 cancer-testis antigens and T cell infiltration in non-small cell lung carcinoma and their prognostic significance

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Abstract. Cancer-testis (CT) antigens were identified as a group of highly attractive targets for cancer immunotherapy because of their expression in a variety of malignant tumors but solely in the testis among the normal adult tissues. To evaluate the potential of two members of this family, MAGE-A4 and NY-ESO-1 antigens, for cancer vaccine in non-small cell lung carcinoma (NSCLC), we examined the expression of these antigens and T cell infiltration in tumor tissue, and evaluated their prognostic significance. One hundred fifty-seven patients with NSCLC were studied. Reverse transcription-PCR was performed to evaluate MAGE-A4 and NY-ESO-1 expression. Immunohistochemistry was performed for NY-ESO-1 expression and T cell infiltration into the tumor site. Survival analysis was also performed. MAGE-A4 and NY-ESO-1 were expressed in 40 of 141 (28.4%) and 13 of 157 (8.3%) NSCLC respectively. Both CT antigens were more frequently expressed in squamous cell carcinoma (SCC) than in adenocarcinoma. An inverse correlation was found between MAGE-A4 expression and patient survival in advanced stage cancers. Combined infiltration of both $CD4^+$ and $CD8^+$ T cells into tumor nest predicted better survival. There was no correlation, however, between lymphocyte infiltration and antigen expression in the tumor. MAGE-A4 expression in advanced group and T cell infiltration may provide prognostic information. Lastly, these CT antigens, especially MAGE-A4, may represent potential targets for cancer immunotherapy in patients with NSCLC.

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Introduction

Non-small cell lung carcinoma (NSCLC) is one of the most common fatal malignancies because of challenges for early detection and increasing incidence. Surgical resection, platinum-based chemotherapy, and irradiation are regularly attempted for the therapy of NSCLC; however, they have low effectiveness due to frequent recurrence and metastasis. The overall 5-year survival rate after diagnosis remains at 10-15% (1). Even for operable cases, the 5-year survival rate was reported as 51% (2). To improve the poor prognosis of this type of tumor, there is a need to establish new therapeutic methods including immunotherapy.

As one line of evidence for the presence of cancer immunosurveillance in humans, cancer patients were found to mount a spontaneous immune response to autologous tumors (3). Analysis of humoral (4) and cellular (5) immune responses to autologous cancer led to the identification of many tumor antigens (6). Among them, CT antigens are recognized as particularly attractive targets for immunotherapy because of their unique expression profile. The characteristics of these antigens are high expression in adult male germ cells but complete absence in other normal adult tissue, along with high and aberrant expression in a variety of malignant neoplasms (6,7). Moreover, tumor expression of certain CT antigens has been reported to elicit specific humoral and cellular immune responses to these antigens (7-9), and cancer vaccine trials to target CT antigens resulted in successful induction of immune response or tumor regression (7,10).

CT antigens can be classified into several super families. MAGE genes are one of the well-characterized members of the CT antigen family that includes six subfamilies, MAGE-A, MAGE-B, MAGE-C, MAGE-D, MAGE-E, and MAGE-F. All of their genes are located on chromosome X. Among these, MAGE-A, MAGE-B, and MAGE-C show expression patterns as CT antigens (11-13). Tumor expression of MAGE-A4 was reported in human malignancies including uterine malignancies (14), ovarian neoplasms (15), hepatocellular carcinomas (16),

mucosal melanomas of the head and neck (17), esophageal adenocarcinomas (18), and colorectal cancer (19). A limited study has identified specific MAGE-A4 expression in lung cancer (20), although its clinicopathological effects, such as prognostic significance, are unclear.

NY-ESO-1 is another well-characterized CT antigen that appears to be the most immunogenic CT antigen known to date (7). Integrated humoral and cellular immune response against NY-ESO-1 are frequently observed in patients with tumors with NY-ESO-1 expression (8,21). As a member of CT antigens, NY-ESO-1 is expressed in a variety of malignant neoplasms, including melanomas, and esophageal, breast, prostate, urinary tract, ovarian, and lung cancers (22-24). Recently, immunohistochemical and molecular analytical approaches for the detection of NY-ESO-1 expression were compared (25,26), defining the reliability of each method. However, its clinical significance, e.g. in prognosis, in lung cancer is unclear.

Another line of evidence for the existence of cancer immunosurveillance in humans is the correlation between the presence of lymphocytes infiltrating the tumor site and a favorable prognosis for patients. Abundant infiltration of $CD3^+$, $CD8^+$, and natural killer cells has been frequently reported to predict an improved clinical outcome (3,27-29). Therefore, the analysis of T cell infiltration into tumor sites is a useful approach to evaluate the degree of immune reaction against tumors in individual cancer patients.

Although the expression of tumor-associated antigens and the presence of tumor infiltrating lymphocytes support the idea of cancer immunosurveillance, the clinicopathological significance of tumor expression of CT antigens and its relationship to T cell infiltration remains unclear. In this study, we examined the expression of the MAGE-A4 and NY-ESO-1 antigens in NSCLC in a Japanese population and analyzed the infiltration of $CD4^+$ and $CD8^+$ T cells into tumor sites to evaluate the clinicopathological impact of these factors.

Patients and methods

Patients and specimens. We studied 157 consecutive patients with primary NSCLC who underwent curative surgery at Hokkaido University Hospital or its affiliated hospitals between 1997 and 2003. All of the tissue samples were collected after obtaining informed consent from the patients. Distant metastasis was not detected in any patient upon preoperative examination. No patient had received prior anticancer treatment. Cases of in-hospital death were excluded from the study. The clinical typing of tumors was determined according to the tumor node metastasis (TNM) classification system of the International Union Against Cancer (30). The histopathological subtype, stage and grade of the tumors were determined by three pathologists according to the World Health Organization (WHO) Classification of Tumors of Lung (31). For RNA isolation, freshly collected tumor specimens of the size of 5 mm^3 were frozen in liquid nitrogen-chilled isopentane and stored at -80˚C until used. For immunohistochemistry, archived formalin-fixed paraffin-embedded specimens of 70 cases were obtained. As shown in Table I, 87 patients were determined only by reverse transcription-polymerase chain reaction (RT-PCR). Fifty-four patients were determined by both RT-PCR and immunohistochemistry. Sixteen patients were determined

Table I. The clinicopathological characteristics of patients with primary NSCLC.

Characteristics	No.
Total	157
RT-PCR + IHC	54
Only RT-PCR	87
Only IHC	16[a]
Obtained clinical data	125
Age	
>60 years	93
≤ 60 years	32
Median	67 (range 31-84)
Gender	
Male	86
Female	39
Histology	
Squamous cell carcinoma	46
Adenocarcinoma	64
Large cell carcinoma	9
Other	6
pT status	
pT0	1
pT1	28
pT2	72
pT3	20
pT4	4
pN status	
pN0	87
pN1	17
pN2	19
Unknown	2
Systemic metastasis (post-operation)	
Metastasis (+)	27
Metastasis (-)	60
Unknown	38
Stage	
I	71
II	26
III	25
IV	2
Unknown	1
Grade	
Well-differentiated	23
Moderately differentiated	41
Poorly differentiated	43
Unknown	18
Median follow-up (days)	652

[a]Investigated for NY-ESO-1, CD4, and CD8 expression by immunohistochemistry only.

only by immunohistochemistry with NY-ESO-1 mAb. Clinical data was obtained from 125 to 157 patients.

RT-PCR analysis of NY-ESO-1 and MAGE-A4 expression. Total tissue RNA was isolated from frozen tumor tissue using Isogen (Nippon Gene Ltd., Tokyo, Japan) according to the manufacturer's protocol. Total RNA (5 μg) was primed with an oligo$(dT)_{18}$ oligonucleotide and reverse-transcribed with Superscript II (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. Obtained cDNA was tested for integrity by amplification of ß-actin and p53 transcripts in PCR reaction as described elsewhere (32). PCR was subsequently performed to analyze expression of MAGE-A4 and NY-ESO-1. The primers for MAGE-A4 were MAGE-A4 F (5'-ATGTCTTCTGAGCAGAAGAGT-3') and MAGE-A4 R (5'-TCAGACTCCCTCTTCCTCCTC-3'), and the primers for NY-ESO-1 were NY-ESO-1A (5'-CA GGGCTGAATGGATGCTGCAGA-3') and NY-ESO-1B (5'-GCGCCTCTGCCCTGAGGGAGG-3'). Amplification for MAGE-A4 gene products was 1 min at 94˚C, 1 min at 68˚C, and 1 min at 72˚C for 35 cycles. Amplification for NY-ESO-1 gene products was 1 min at 94˚C, 1 min at 60˚C, and 1 min at 72˚C for 35 cycles. These cycles were followed by a 6-min elongation step at 72˚C. Testicular tissue was used as a positive control. The PCR products were visualized by ethidium bromide staining after separation over a 1% agarose gel.

Antibodies. Mouse monoclonal antibody (mAb) against NY-ESO-1 protein (clone E978) was obtained from Ludwig Institute for Cancer Research. Clone E978 (IgG1) was generated against a 23-kDa NY-ESO-1 recombinant protein and specifically detects NY-ESO-1 as described previously (8,24). Anti-human CD4 and anti-human CD8 were the mouse monoclonal primary antibodies (Histofine CD4 mouse IgG1 monoclonal antibody and Histofine CD8 mouse IgG1κ monoclonal antibody; Nichirei Corp., Tokyo, Japan).

Immunohistochemical staining. Specimens were fixed in 10% formalin and embedded in paraffin. Serial 4-μm-thick sections were examined by immunohistochemistry. Deparaffinized specimens were rinsed in phosphate-buffered saline (PBS, pH 7.4), followed by endogenous peroxidase blockade by a 10-min incubation with 0.3% hydrogen peroxide in methanol. After a rinse in PBS, specimens were saturated with 10% normal goat serum (Histofine SAB-PO kit; Nichirei Corp.) for 5 min. Specimens were then incubated at room temperature for 30 min with primary antibody that was diluted at an appropriate concentration (2.5 μg/ml for E978, 1:500 dilution for anti-CD4 and anti-CD8). Detection was carried out using the streptavidin-biotin-peroxidase method by Histofines SAB-PO kit (Nichirei Corp.) according to the manufacturer's instructions. 3,3-Diaminobenzidine tetrahydrochloride (Histofine SAB-PO kit; Nichirei Corp.) was employed as a chromogen. Slides were counterstained with hematoxylin and mounted. As positive control, normal adenoid tissue was used for CD4 and CD8. As negative control, primary antibodies were replaced with PBS.

Evaluation and classification of NY-ESO-1 expression and T cell infiltration. The NY-ESO-1 expression level detected by immunohistochemistry was classified into 4 groups by the percentage of positive tumor cells: group A, >50% tumor cells; group B, 11-50% tumor cells; group C, 1-10% tumor cells; and group D, no positive signal. $CD4^+$ and $CD8^+$ cell infiltration was evaluated according to the previous reports of Naito *et al* (27) and Schumacher *et al* (28), with some modifications. The degree of immune cell infiltration was observed in more than 10 independent high-power (x200) microscope fields for each tissue sample. The average value of the five areas with the most abundant distribution was determined as the number for that slide. The number of $CD4^+$ T cells and $CD8^+$ T cells was counted both in the mesenchymal stroma and within the cancer cell nest. We classified patients into high infiltration group [average count ≥ median value: CD4 (+) and CD8 (+)], and low infiltration group [average count < median value: CD4 (-) and CD8 (-)]. Then, we further classified patients into the following four groups for analysis: patients classified as both CD4 (+) and CD8 (+) were described as CD4/8 (+/+). By the same manner, other three groups were designated as CD4/8 (+/-), CD4/8 (-/+), and CD4/8 (-/-). Two investigators blinded to the patients' clinical information evaluated all specimens. Cases with variability between the observers were classified by the average of cell count of both observers.

Statistical analysis. χ^2 test, Spearman's rank correlation coefficient and Student's paired t-test were used to confirm the association between variables. The Kaplan-Meier method was used to estimate overall survival, and survival differences were analyzed by the log-rank test. P-values of <0.05 were regarded as significant. All analyses were performed with statistical software (StatView J Version 5.0; SAS Institute Inc., Cary, NC).

Results

Study population. The clinicopathological characteristics of patients with primary NSCLC in this study are summarized in Table I. A total of 141 flash-frozen NSCLC tissues were analyzed by RT-PCR, and paraffin-embedded sections of 70 archival NSCLC specimens were investigated by IHC. For 54 cases, frozen and archival specimens were available. The total number of tissue specimens examined by RT-PCR and/or IHC was 157. The median age of the study population was 67 years (range 31-84 years), and the median duration of follow-up was 652 days (range 24-1335 days).

The expression of MAGE-A4 and NY-ESO-1 mRNA in primary lung cancer tissue. Expression of MAGE-A4 and NY-ESO-1 mRNA in primary NSCLC specimens was analyzed by RT-PCR. The size of the PCR product in the tumor was identical to that in the testis, and representative PCR products were confirmed as MAGE-A4 and NY-ESO-1 by DNA sequencing. Cases with only weak amplicon band yields were regarded positive only if the result could be reproduced by a repeated RNA extraction and specific RT-PCR from the same tumor specimen. Cases with faint transcript levels that were not reproducibly positive were not scored as positive. As shown in Table IIa, MAGE-A4 was expressed in 28.4% of all NSCLC samples examined by RT-PCR (40/141 patients). NY-ESO-1

Table II. Expression of CT antigens in 141 primary NSCLC.

a, Expression of CT antigen mRNA by RT-PCR.

	Samples	MAGE-A4	NY-ESO-1	Expression (any, both, none)
Total	141	40 (28.4%)	11 (7.8%)	(42, 9, 99)
Squamous cell carcinoma	41	22	8	(24, 2, 17)
Adenocarcinoma	53	7	2	(7, 6, 46)
Large cell carcinoma	9	4	0	(4, 0, 5)
Other	6	2	0	(2, 0, 4)
Unknown	32	5	1	(5, 1, 27)
Reported frequency in Caucasian		51%	16-21%	

b, Expression of NY-ESO-1 by IHC, and comparison to RT-PCR.

	RT-PCR positive	RT-PCR negative	Total	Only IHC
Group A >50%	1	1	2	1
Group B 11-50%	1	0	1	0
Group C 1-10%	1	0	1	0
Group D 0%	1	49	50	15

c, Correlation between CT antigen expression and clinicopathological factors. P-value (log-rank).

	MAGE-A4 (+)	NY-ESO-1 (+)	MAGE-A4 (+) and/or NY-ESO-1 (+)
Gender (male/female)	0.0525	0.1605	0.0315
Age (>60/≤ 60)	0.0055	0.4541	0.028
p-stage (I/II/III/IV)	0.6911	0.3438	0.6514
Grade (well/moderate/poor)	0.6432	0.5783	0.7359
Histology (SCC/adenocarcinoma)	0.0004	0.0347	<0.0001
pT status (I/II/III/IV)	0.1619	0.9632	0.2665
L/N metastasis	0.1692	>0.9999	0.1145
Systemic metastasis (+/-)	>0.9999	>0.9999	>0.9999

was expressed in 7.8% (11/141 patients) of all NSCLC. The expression frequencies of both CT antigens were less than those reported in a Caucasian population [51% for MAGE-A4 (22), 16-21% for NY-ESO-1 (22,24)]. Co-expression of MAGE-A4 and NY-ESO-1 mRNA was demonstrated in 6.4% (9/141) of specimens. MAGE-A4 and NY-ESO-1 was expressed in 53.7% (22/41) and 19.5% (8/41) of SCC respectively. In contrast, MAGE-A4 and NY-ESO-1 was expressed only in 13.2% (7/53) and 3.8% (2/53) of adenocarcinoma respectively.

Comparison of the RT-PCR and immunohistochemistry of NY-ESO-1 expression in tumor tissue. By E978 anti-NY-ESO-1 mAb, NY-ESO-1 expression signals were restrictively distributed in spermatogonia in the testis (Fig. 1A) as reported (24). In lung cancer specimens, NY-ESO-1 expression was heterogeneous or focal and localized predominantly in the cytoplasm as shown in Fig. 1B. Four of 54 specimens (7.4%) were classified as positive for NY-ESO-1 by immunohistochemistry (Table IIb). We also examined 16 cases where only paraffin sections were available. Including these cases, NY-ESO-1 expression frequency detected by RT-PCR and/or IHC was 8.3% (13/157). NY-ESO-1 expression level detected by immunohistochemistry was classified as described in Patients and methods. Comparison of immunohistochemical detection and mRNA expression of NY-ESO-1 is shown in Table IIb. Of the 54 tumors, 4 cases showed immunohistochemical expression of NY-ESO-1, including one case that was negative by conventional RT-PCR; while, among the 50 cases that were negative by immunohistochemistry, 1 was positive for NY-ESO-1 by conventional RT-PCR. Although the expression frequency was lower, our data here is consistent with the previous report in other tumor types that there is a good correlation between the two methods with some discrepancies (25).

Relationship between CT antigen expression and clinicopathological factors. The correlation between CT antigen expression and various clinicopathological features was analyzed by χ^2 test (Table IIc). MAGE-A4 expression significantly correlated with age (frequently expressed in the group with patients

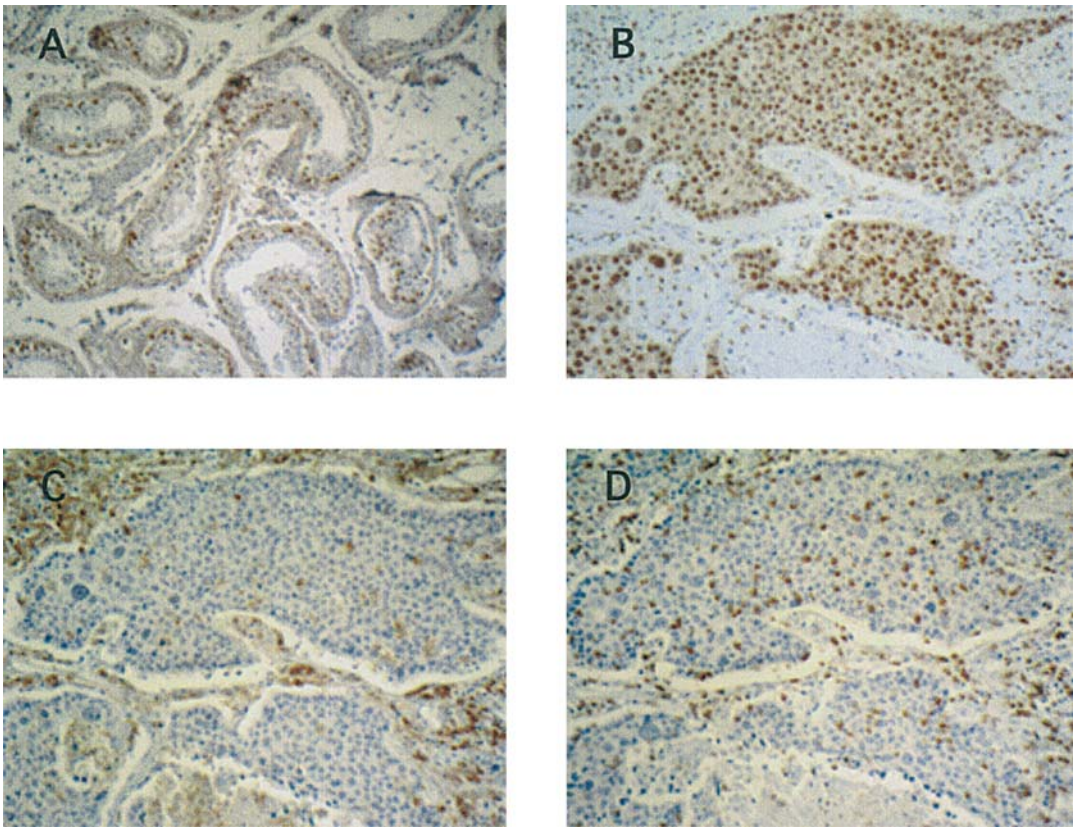

Figure 1. Immunohistochemical staining with anti-NY-ESO-1 mAb E978, anti-CD4 mAb and anti-CD8 mAb. Magnification, x200 for normal adult testis (A) and tumor tissue (B)-(D). (A) Seminiferous tubules with strong intratubular staining of mostly early spermatogenic cells, no reactivity with spermatids or sertoli cell, negative interstitial tissue. (B) Heterogeneous E978 staining of primary NSCLC tissue. (C) Staining of $CD4^+$ T cells within NSCLC tissue. (D) Staining of $CD8^+$ T cells within NSCLC tissue.

over 60 years old, P=0.0055) and histology (frequently expressed in SCC group compared to adenocarcinoma group, P=0.0004). However, MAGE-A4 expression did not correlate with p-stage, differentiation grade, pT status, lymph node metastasis, or systemic metastasis. Although MAGE-A4 was expressed more frequently in male patients than female patients, it was not statistically significant (P=0.0525). NY-ESO-1 expression did not correlate to most clinicopathological factors except histology (frequently expressed in SCC group compared to adenocarcinoma group, P=0.0347).

Prognostic value of MAGE-A4 and NY-ESO-1 expression. By log-rank test, the stage of disease, existence of systemic metastasis and lymph node metastasis were significantly prognostic, as shown in Table III (P<0.0001, P=0.0002 and P=0.0090 respectively). Neither MAGE-A4 expression nor NY-ESO-1 expression were prognostic for overall survival (Fig. 2A, B and Table III). We next applied classification based on p-stage, early group (stage I+II) and advanced group (stage III+IV), to examine the prognostic value of CT antigen expression (Fig. 2C and Table III). We found a significantly poor prognosis in the advanced stage cases expressing MAGE-A4 compared to any other combinations (P=0.0065). NY-ESO-1 expression was not prognostic in either group (data not shown).

Prognostic significance of $CD4^+$ and $CD8^+$ T cells in the cancer cell nest. To evaluate the spontaneous immune reaction *in situ* of NSCLC, $CD4^+$ and $CD8^+$ cell infiltration into tumor sites was detected by immunohistochemistry. As shown in Fig. 1C and D, $CD4^+$ and $CD8^+$ T cell infiltration was detected in the cancer cell nest as well as mesenchymal stroma. Association between $CD4^+$ and $CD8^+$ T cells in the tumor nest was analyzed by Spearman's rho test. There was a significant correlation (Fig. 3, r=0.586; P<0.0001) between the number of $CD4^+$ and $CD8^+$ T cells. Classification based on median value was adopted (Table IVa) and we found a favorable prognostic value with abundant infiltration of $CD4^+$ T cells in the cancer cell nest (Fig. 2D). In contrast, $CD8^+$ T cell infiltration in the cancer cell nest was not prognostic (Fig. 2E). T cell infiltration was further classified into four groups as CD4/8 (+/+), CD4/8 (+/-), CD4/8 (-/+), and CD4/8 (-/-). The number of cases classified in the CD4/8 (+/+), CD4/8 (+/-), CD4/8 (-/+), and CD4/8 (-/-) groups was 25, 10, 10, and 25, respectively (Table IVb). The CD4/8 (+/+) group showed a significantly better overall survival compared with the group with all other groups combined, including the CD4/8 (+/-), (-/+), and (-/-) groups (Fig. 2F, P=0.0370). No correlation was found between stromal infiltrating cells and patient prognosis (data not shown).

CT antigen expression and T cell infiltration into tumor sites. Finally, we addressed the correlation between CT antigen expression and the infiltration of $CD4^+$ or $CD8^+$ cells into the tumor site. Fifty-four patients were available for both T cell infiltration analysis and expression analysis of CT antigens

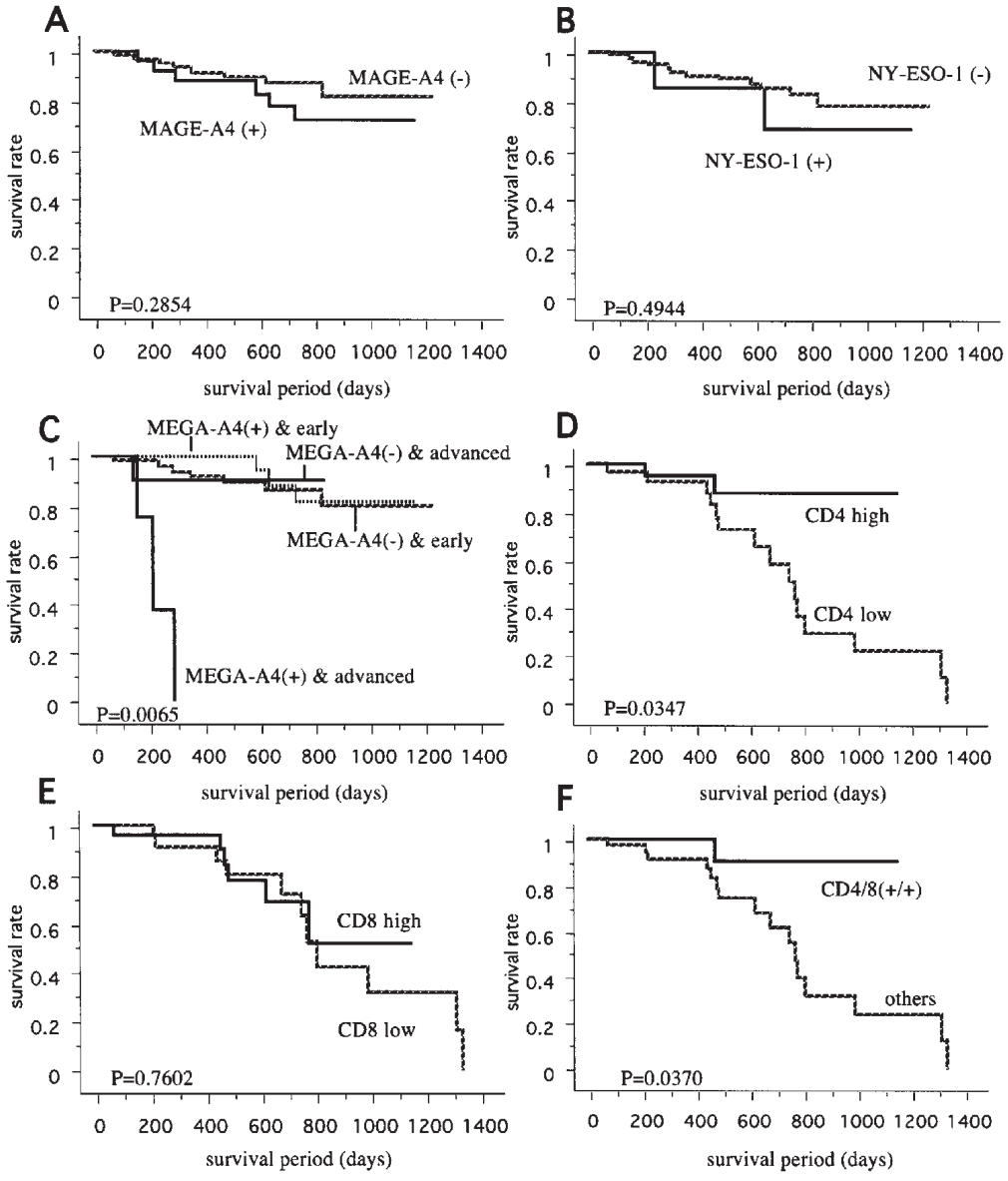

Figure 2. Univariate analysis of survival in NSCLC (P-value: log-rank). Patients shown in Tables III and IV were analyzed by Kaplan-Meier method. Pair-wise differences were analyzed using the log-rank test. (A) No correlation between MAGE-A4 expression and survival rate. (B) No correlation between NY-ESO-1 expression and survival rate. (C) Correlation between MAGE-A4 expression and survival rate in early group and advanced group. (D) Correlation between the number of CD4 and survival rate. (E) No correlation between the number of CD8 and survival rate. (F) Correlation between CD4/8 status and survival rate.

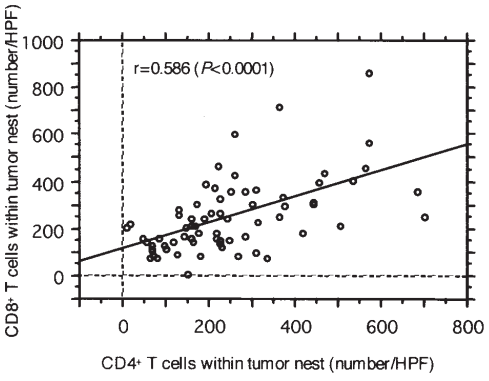

Figure 3. Association between the number of $CD4^+$ and $CD8^+$ T cells in the tumor nest was analyzed by Spearman's rho test. Statistically, there was a significant correlation (r=0.586; P<0.0001) between the number of $CD4^+$ and $CD8^+$ T cells.

using Student's paired t-test. No significant correlation was established between $CD4^+$ or $CD8^+$ T cells in the tumor site and the expression of CT antigens (Table IVc).

Discussion

This report addressed the integrated relationship among expression level of MAGE-A4 and NY-ESO-1, clinicopathological characteristics including overall survival, and infiltration of T cells into tumor site in patients with NSCLC. We chose these antigens because of their potential to mount strong immune reactions, and also because of the lack of detailed analyses in NSCLC. MAGE-A4 expression was an unfavorable prognostic factor for survival in patients with advanced stages. Combined infiltration of $CD4^+$ and $CD8^+$ T cells into tumor nest was positively associated with patient survival.

Table III. Correlation of clinicopathological factors and CT antigen expression with patient survival.

	No. of cases	3-year survival rate (%)	P-value
Stage of disease			<0.0001
Early (stage I+II)	91	67.9	
Advanced (stage III+IV)	24	0	
Systemic metastasis			0.0002
(+)	27	53.2	
(-)	60	87.0	
Lymph node metastasis			0.0090
(+)	32	28.2	
(-)	83	65.6	
Grade			0.8047
Well-differentiated	22	59.6	
Moderately differentiated	36	55.5	
Poorly differentiated	41	57.0	
Histological type			0.3046
SCC	44	54.0	
Adenocarcinoma	60	56.5	
MAGE-A4 expression			0.2859
(+)	32	71.4	
(-)	70	81.1	
NY-ESO-1 expression			0.4944
(+)	9	68.6	
(-)	93	78.6	
MAGE-A4 (+) and/ or NY-ESO-1 (+)			0.1288
(+)	34	69.3	
(-)	68	82.4	
Combination of MAGE-A4 expression and stage[a]			0.006
Early and MAGE-A4 (+)	25	81.4	
Early and MAGE-A4 (-)	57	86.7	
Advanced and MAGE-A4 (+)	7	0	
Advanced and MAGE-A4 (-)	12	90	

P-value (log-rank). [a]2-year survival rate (%).

Table IV. Evaluation of the number of $CD4^+$ and $CD8^+$ T cells in the cancer cell nest.

a, Classification by the number of $CD4^+$ and $CD8^+$ T cells in the cancer cell nest.

	No. of T cells/microscopic field, mean ± SD (range, no. of patients)	
	High	Low
$CD4^+$ T cells	323±177 (221-701.35)	127±57.3 (9-215.35)
$CD8^+$ T cells	363±143 (216-865.35)	137±47.4 (10-215.35)

b, Classified number by the groups of $CD4^+$ and $CD8^+$ T cells in the cancer cell nest.

	CD8 high group	CD8 low group
CD4 high group	CD4/8 (+/+) = 25	CD4/8 (+/-) = 10
CD4 low group	CD4/8 (-/+) = 10	CD4/8 (-/-) = 25

CD4 (+), CD4 high; CD4 (-), CD4 low; CD8 (+), CD8 high; CD8 (-), CD8 low. We obtained 70 formalin-fixed tissues for the evaluation of infiltrating cells.

c, Correlation between CT antigen expression and the number of T cells. P-value (log-rank).

	P-value
No. of CD4 in stroma	
MAGE-A4 (+) < MAGE-A4 (-)	0.1046
NY-ESO-1 (+) > NY-ESO-1 (-)	0.8819
No. of CD4 in nest	
MAGE-A4 (+) > MAGE-A4 (-)	0.7859
NY-ESO-1 (+) < NY-ESO-1 (-)	0.2581
No. of CD8 in stroma	
MAGE-A4 (+) < MAGE-A4 (-)	0.9709
NY-ESO-1 (+) < NY-ESO-1 (-)	0.7810
No. of CD8 in nest	
MAGE-A4 (+) > MAGE-A4 (-)	0.0945
NY-ESO-1 (+) < NY-ESO-1 (-)	0.2962

The frequency of MAGE-A4 and NY-ESO-1 mRNA expression in NSCLC in a Japanese population in this study (28.4% for MAGE-A4 and 7.8% for NY-ESO-1) was lower than that in previous studies in a Caucasian population (33). The reason for this difference in CT antigen expression is unclear, although the genetic differences between Asian and Caucasian populations or technical differences are speculated as a possible reason. In fact, a lower frequency of MAGE-A4 and NY-ESO-1 expression in lung cancer in an Asian population compared to Caucasian populations has been suggested (20). Genetic control of CT antigen expression or genetic difference in the restriction elements in immunological reaction against CT antigen might underlie the observed expression difference in CT antigens.

Of the 54 tumors available for both immunohistochemical and RT-PCR analysis of NY-ESO-1 expression, 4 were positive by RT-PCR and 1 of them was negative by staining with E978. Among 50 samples that were negative by RT-PCR,

1 was positive by immunohistochemical analysis. One likely explanation for this discrepancy is heterogeneity in the distribution and expression level of NY-ESO-1 as suggested previously in other tumor types (25,26). The RT-PCR method cannot provide information about the expression level of tumor antigens in individual cells or the heterogeneous distribution of antigen expression in tissue. Therefore, it is desirable to combine immunohistochemistry and RT-PCR to obtain information about both expression level and antigen distribution. Important information that has not yet been provided is the required expression level and distribution of antigens in tumor tissue to achieve effective immunotherapy. This should be addressed by clinical trials in the future designed to provide comprehensive information on antigen expression.

In this study, MAGE-A4 expression was associated with a poor patient survival. Bolli *et al* reported an association between MAGE tumor-associated antigen expression detected by immunohistochemistry using mAb 57B and poor prognosis in lung squamous cell carcinomas (34). Since 57B preferentially binds to MAGE-A4 in paraffin-embedded section among other MAGE-A family members that can be detected by this mAb (35,36), it is likely that their report reflected our present findings. We also found a significantly poor prognosis with MAGE-A4 expression in advanced stage tumors in patient subgroups restricted to squamous cell carcinoma (data not shown). When we combined early and advanced stages, however, we did not find a relationship between MAGE-A4 expression and patient prognosis, in contrast to their report. The reason for this discrepancy is unclear, although the difference in the specificity between staining with 57B and RT-PCR might be responsible. A similar inverse correlation between MAGE-A4 expression and patient survival was reported in serous ovarian carcinoma (15). Moreover, they found the poorest prognosis in advanced stage cases of serous ovarian carcinoma expressing MAGE-A4. These results suggest a possible role for the MAGE-A4 gene product in determining greater malignant characteristics of cancer cells such as severe cellular atypia or high proliferation rates, although the function of the MAGE-A4 gene product has not been revealed. It is also possible that MAGE-A4 may act as an antigen for regulatory T cells in non-manipulated immune response as reported for another CT antigen, LAGE-1 (37). However, the role of MAGE-A4 antigen in spontaneous immune reaction may differ from its role in immunotherapy. In fact, tumor specific cytotoxic T lymphocytes (CTL) recognizing MAGE gene products were reported, and a large number of CTL epitopes within MAGE proteins were identified (7,38-41). Also, CTL epitopes of MAGE-A4 presented by HLA-A1 (42), HLA-A2 (43), and HLA-B37 (44) were reported. Moreover, it was shown that serologically defined tumor antigens could act as targets for both suppressive and protective immunity depending on the immunization conditions in a murine model (45,46). Persistent induction of toll-like receptor signals was shown to reverse regulatory T cell-mediated tumor-specific T cell tolerance (47).

Intratumoral infiltrating CTL have been considered as one of the most important effector cells in anti-tumor immune response because of their cytotoxic function and specificity on antigen recognition. Intensive infiltration of $CD8^+$ T cells into the tumor nest has been reported to correlate with good patient prognosis in many tumor types including colorectal, esophageal, and renal cell carcinomas (28,33,34). However, we did not find a positive correlation between $CD8^+$ T cell number in tumor nests and patient prognosis in NSCLC. This result is consistent with a previous report by Wakabayashi *et al* (48) which showed that more $CD8^+$ T cells and a higher labeling index of Ki-67/$CD8^+$ T cells within cancer nests was not positively correlated with a favorable prognosis in NSCLC. The reasons why higher numbers of $CD8^+$ T cells did not show better prognosis in NSCLC remain unclear. The functional analysis of $CD8^+$ T cells within tumor nests might help to address the question. In contrast, a higher number of $CD4^+$ T cells infiltrating tumor nests was found to be a predictive factor of better survival with NSCLC in this study. Wakabayashi *et al* also reported the importance of $CD4^+$ T cells in NSCLC. In their study, a higher $CD4^+$ T cell number in cancer stroma was found to associate with a favorable prognosis in patients with NSCLC. Importantly, when we divided a patient group with abundant $CD4^+$ T cells into CD4/8 (+/+) and CD4/8 (+/-), only the CD4/8 (+/+) group showed a good prognosis. This result suggests that $CD8^+$ T cells with appropriate help from $CD4^+$ T cells within a tumor nest *in situ* environment is necessary for effective anti-tumor immune responses, and that simple evaluation of the number of $CD8^+$ T cells does not easily show the prognostic significance. The collaborative effect of $CD4^+$ and $CD8^+$ T cells in improvement of prognosis was also reported in patients with esophageal squamous cell carcinoma (49). Although $CD4^+$ helper T cells may contribute to effective anti-tumor immune responses, $CD4^+$ $CD25^+$ regulatory T cells might be able to function negatively in the eradication of tumor cells. Therefore, it will be important to distinguish helper T cells and regulatory T cells in the future analysis of immune cells in cancer patients.

The poor prognosis of MAGE-A4-expressing tumors suggests that the development of an aggressive therapy is required for patients with tumors expressing MAGE-A4. On the other hand, the insignificant correlation between MAGE-A4/NY-ESO-1 expression and T cell infiltration suggests that the spontaneous immune response is not sufficient against these antigens. We have shown a critical role for induction of type-1 immunity in tumor immunotherapy in murine models (50). We have demonstrated that therapy to actively induce type-1 reaction accelerated APC/Th1 cell-cell interaction at the draining lymph node and induced tumor-specific tetramer$^+$-CTL at the tumor site. These mice were able to overcome their immunosuppressive state and eradicate the established tumor. Immunotherapy strategies against MAGE-A4/NY-ESO-1 to induce not only CTL but also appropriate helper T cell immunity might be an effective approach for cancer immunotherapy in NSCLC.

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