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Article

Prolonged survival of patients with lung adenocarcinoma expressing XAGE-1b and HLA class I antigens

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XAGE-1b is a cancer/testis antigen that has been shown to be expressed at a significant frequency and to be immunogenic in non-small cell lung cancer (NSCLC). In the present study, we investigated correlations between XAGE-1b expression and NSCLC patient survival. XAGE-1b expression was examined immunohistochemically using USO9-13, an anti-XAGE-1b monoclonal antibody, in 121 NSCLCs (83 adenocarcinomas and 38 other histological types). XAGE-1b expression was observed in 27 (32.5%) adenocarcinoma specimens. In the other histological types, positive staining was observed in only 1 specimen. HLA class I expression in these samples was assessed previously. XAGE-1b expression had no correlation with overall survival. However, both XAGE-1b and HLA class I expression correlated with prolonged survival ($P = 0.019$). Moreover, expression of XAGE-1b combined with down-regulated HLA class I expression correlated with poor survival ($P = 0.01$). The density of cancer nest-infiltrating CD8+ T-cells in tumors expressing both XAGE-1b and HLA class I was higher than that in other groups. The findings suggest that XAGE-1b and HLA class I expression elicited a CD8+ T-cell response against minimal residual disease after surgery and resulted in prolonged survival of NSCLC patients.

Keywords: human, non-small cell lung cancer, XAGE-1, HLA class I antigen, immunohistochemistry, prognosis

Introduction

Cancer/testis (CT) antigens are expressed in normal adult tissues only in the testis, as well as in a variety of tumors of different histological origins (1). Based on their characteristic expression profile, CT antigens are thought to be promising targets for tumor immunotherapy (1-3). Several CT antigens have been shown to elicit a spontaneous immune response in cancer patients (4), and both HLA class I and class II restricted epitopes have been identified in those antigens (4-8). Various CT antigens have been shown to be expressed in non-small cell lung cancer (NSCLC) (9, 10). Nevertheless, the expression of CT antigens generally correlated with poor prognosis in lung cancer (10, 11).

The *XAGE-1* gene was originally identified as a *PAGE*/*GAGE*-related gene on the X chromosome using an expression sequence tag database and has CT-like characteristics (12, 13). There are 4 alternative splice variants, *XAGE-1a*, *b*, *c* and *d* (13, 14). Among these, *XAGE-1b* has been shown to be dominantly expressed in lung adenocarcinoma. *XAGE-1b* mRNA expression was observed in 14 of 31 (45%) lung adenocarcinomas and 1 of 18 (6%) lung cancers of other histological types (15). Immunohistochemical analysis showed that XAGE-1b protein expression was observed in most of the NSCLCs that expressed *XAGE-1b* mRNA (15). Expression has also been observed in hepatocellular and gastric carcinomas (16). Moreover, an immune response to XAGE-1b protein (81 amino acids) was shown in lung cancer patients (15-17). In the present study, we analyzed the relevance of XAGE-1b expression on survival in lung cancer patients. We showed that prolonged survival was observed in patients with tumors expressing both XAGE-1b and HLA class I. On the other hand, even shorter survival was observed in patients with tumors expressing XAGE-1b and with down-regulated HLA class I expression.

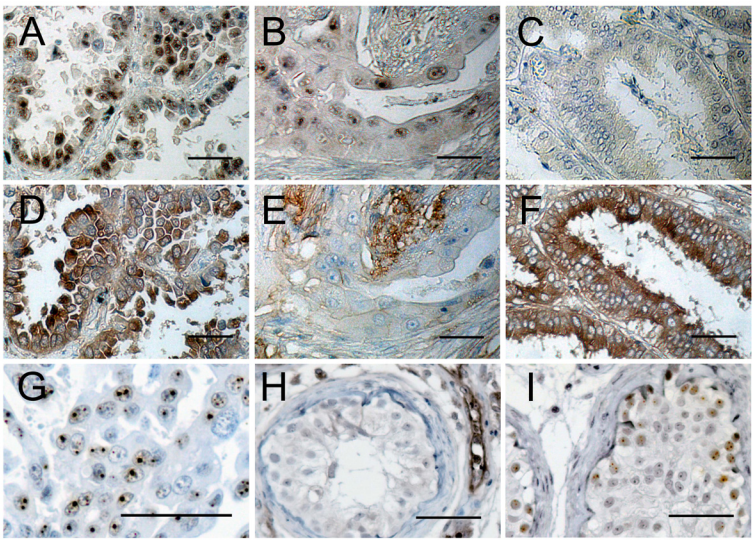
Results

XAGE-1b and HLA class I expression in non-small cell lung cancer

XAGE-1b expression in non-small cell lung cancer was examined by immunohistochemistry using USO9-13 mAb. Expression was observed in 27 of 83 (32.5%) adenocarcinoma specimens. The staining pattern was diffuse in 14, intermediate in 10, and focal in 3 specimens. No positive staining was observed in 33 squamous cell carcinomas. Among the 5 other non-small cell carcinomas, expression was observed in one undifferentiated tumor. The results showed highly restricted expression of XAGE-1b in lung adenocarcinoma, which was consistent with previous observations (15, 17).

HLA class I expression was examined previously in these adenocarcinoma specimens by immunohistochemistry using EMR8-5 mAb (18). Expression was observed in 43 of 83 (51.8%) specimens. Representative immunostainings for XAGE-1b and HLA class I are shown in Figure 1.

Figure 1



XAGE-1b and HLA class I expression in NSCLC samples. Representative immunostaining with USO9-13 anti-XAGE-1b monoclonal antibody (A-C, G, I) and EMR8-5 anti-HLA class I monoclonal antibody (D-F, H) is shown. (A, D) Sequential sections of a specimen expressing both XAGE-1b and HLA class I. (B, E) Sequential sections of a specimen expressing XAGE-1b and with down-regulated HLA class I expression. (C, F) Sequential sections of a specimen expressing no XAGE-1b but HLA class I. (G) XAGE-1b positive adenocarcinoma. (H) Normal testis (control). Spermatogonia and spermatocytes were not stained by anti-HLA class I antibody, while vascular endothelial cells were stained. (I) Normal testis (positive control of XAGE-1b expression). The nuclei of spermatogonia and spermatocytes were stained. Scale bar, 50 μm.

Table 1

XAGE-1b expression and clinicopathological features of patients with lung adenocarcinoma.

Feature		XAGE-1b		*P*
		Positive	Negative	
Age (years)	<65	19	28	0.13
	≥65	8	28	
Sex	Male	13	30	0.82
	Female	14	26	
Smoking	Never smoked	10	21	0.88
	Current or past smoker	12	31	
	Unknown	5	4	
Pathological stage	I	15	35	0.71
	II/III/IV	12	21	
Grade	Well-differentiated	10	28	0.38
	Moderate/poor	17	25	
	Unclassified	0	3	
HLA class I	Positive	13	30	0.32
	Down-regulated	14	26	

Correlation between XAGE-1b expression and clinicopathological features

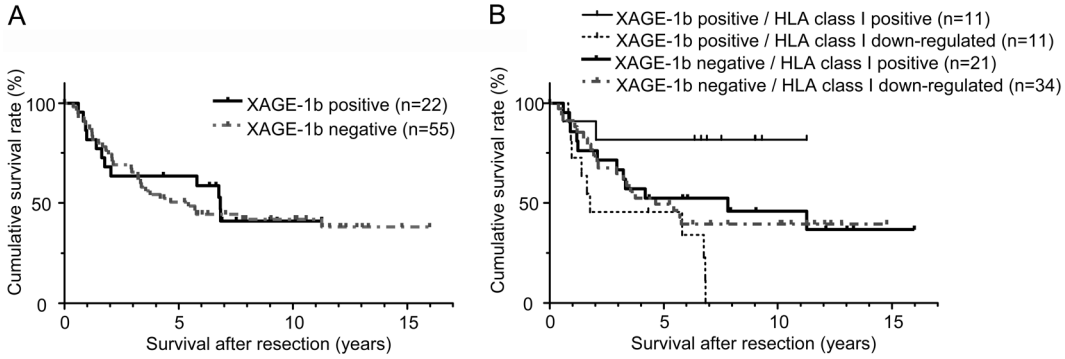
As shown in Table 1, no significant correlation was found between XAGE-1b expression and clinicopathological features including age, sex, smoking status, tumor stage, tumor differentiation, and HLA class I expression in adenocarcinoma patients according to chi-square test results.

Prolonged survival of patients with lung adenocarcinoma expressing XAGE-1b and HLA class I antigens

In 77 adenocarcinoma patients, the relationship between XAGE-1b and HLA class I expression and survival was investigated. Kaplan-Meier analysis using the log-rank test revealed no correlation between XAGE-1b expression and overall postoperative survival (Figure 2A). However, patients with adenocarcinoma expressing both XAGE-1b and HLA class I showed prolonged postoperative survival compared to the other groups of patients combined ($P = 0.019$; Figure 2B). Patients with adenocarcinoma expressing XAGE-1b and down-regulated HLA class I expression showed even shorter survival compared to the other groups of patients combined ($P = 0.010$). Furthermore, no difference in survival was observed between patients with HLA class I positive tumors and those with HLA class I down-regulated tumors when the tumors did not express XAGE-1b.

Univariate analysis showed that advanced pathological stage and tumor grade were correlated with poor prognosis (Table 2). Down-regulated HLA class I expression showed a tendency toward shorter survival time ($P = 0.056$). No correlation was found between the expression pattern (diffuse, intermediate, or focal) and overall survival. Multivariate analysis using a Cox proportional hazards regression model showed down-regulated HLA class I expression and advanced pathological stage to be poor prognostic factors (Table 2). In addition, a tendency toward a synergistic effect was found between HLA class I and XAGE-1b expression ($P = 0.061$), suggesting that XAGE-1b has a different effect on overall survival depending on HLA class I expression.

Figure 2



Kaplan-Meier survival analysis. Curves showing overall survival of patients with lung adenocarcinoma ($n = 77$) in terms of expression of XAGE-1b (A), and XAGE-1b and HLA class I (B), after resection.

Table 2

Uni- and multivariate analyses for clinicopathological factors affecting overall survival in lung adenocarcinoma patients after resection.

Variable		Hazard Ratio (95% CI)	*P*
Univariate analysis			
Pathological stage	I vs. II-IV	5.48 (2.89-10.4)	<0.001
Grade	Non-well vs. well	2.66 (1.42-4.99)	0.0014
HLA class I	Down-regulation vs. strongly positive	1.85 (0.98-3.49)	0.056
Sex	Male vs. female	1.67 (0.92-3.03)	0.088
Smoking	Ever smoking vs. never smoking	1.68 (0.87-3.24)	0.12
Age	1 year increment	1.02 (0.98-1.05)	0.31
XAGE-1b	Negative vs. positive	1.08 (0.56-2.11)	0.82
Multivariate analysis			
Pathological stage	I vs. II-IV	4.42 (2.26-8.66)	<0.001
Grade	Non-well vs. well	1.82 (0.92-3.62)	0.087
HLA class I	Down-regulation vs. strongly positive	4.88 (1.02-23.4)	0.048
Sex	Male vs. female	1.28 (0.66-2.47)	0.46
XAGE-1b	Negative vs. positive	3.45 (0.76-15.6)	0.11
Interaction between XAGE-1b and HLA class I		0.19 (0.034-1.08)	0.061

Abbreviation: CI, confidence interval.

Infiltration of CD8+ T-cells in lung adenocarcinoma expressing XAGE-1b and HLA class I antigens

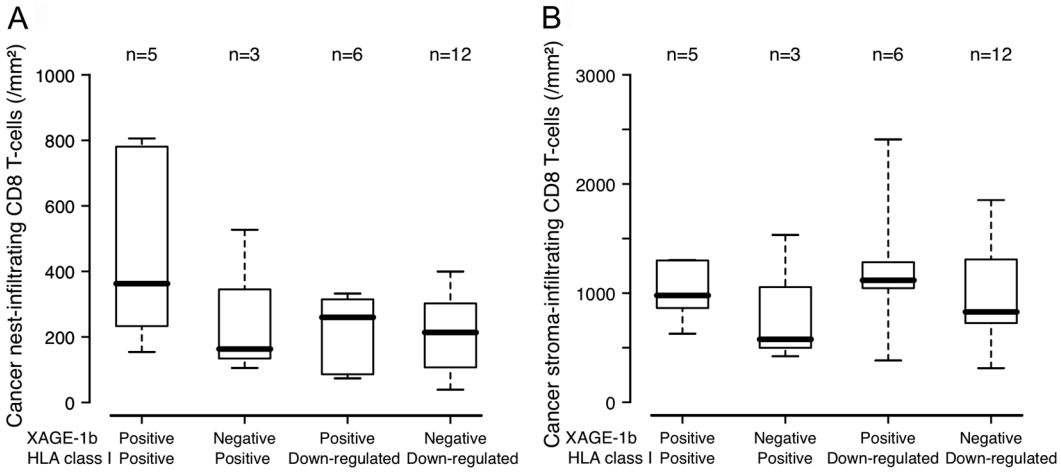
Infiltration of CD8+ T-cells was investigated by immunohistochemistry in 30 adenocarcinoma specimens with tumor cell nests and no pulmonary alveolar structure replacing the tumor. Median counts of cancer nest-infiltrating CD8+ T-cells in adenocarcinomas expressing XAGE-1b and those not expressing XAGE-1b were 289.3 cells/mm^2 (range 73.7-806.0 cells/mm^2) and 183.4 cells/mm^2 (range 38.9-527.2 cells/mm^2), respectively ($P = 0.28$). Median counts of these cells in tumors expressing HLA class I and those with down-regulated HLA class I expression were 297.8 cells/mm^2 (range 105.4-806.0 cells/mm^2) and 237.2 cells/mm^2 (range 38.9-399.5 cells/mm^2), respectively ($P = 0.16$). The differences were not statistically significant. On the other hand, the density of cancer nest-infiltrating CD8+ T-cells in tumors expressing both XAGE-1b and HLA class I was higher than that in other groups, although the difference was not statistically significant ($P = 0.079$; Figure 3A).

Median counts of cancer stroma-infiltrating CD8+ T-cells in adenocarcinomas expressing XAGE-1b and those not expressing XAGE-1b were 1081.0 cells/mm^2 (range 382.8-2409.0 cells/mm^2) and 786.1 cells/mm^2 (range 312.3-1852.0 cells/mm^2), respectively ($P = 0.38$). Median counts of these cells in tumors expressing HLA class I and those with down-regulated HLA class I expression were 920.9 cells/mm^2 (range 421.0-1533.0 cells/mm^2) and 973.5 cells/mm^2 (range 312.3-2409 cells/mm^2), respectively ($P = 0.76$). The densities were not significantly different. No increase in the number of these cells was observed in tumors expressing both XAGE-1b and HLA class I compared to other groups (Figure 3B).

Discussion

In the present study, we show the correlation between XAGE-1b expression and postoperative survival of patients with lung adenocarcinoma when stratified according to the HLA class I expression status of the tumor. Patients with lung adenocarcinoma expressing both XAGE-1b and HLA class I

Figure 3



CD8+ T-cells in lung adenocarcinoma expressing XAGE-1b and HLA class I antigens. Density of CD8+ cells infiltrating cancer nests (A) and cancer stroma (B) as a function of XAGE-1b and HLA class I expression status. Box-whisker plots displaying median, inter-quartile range, and extreme values.

antigens showed significantly prolonged survival when compared to other groups of patients. The density of cancer nest-infiltrating CD8+ T-cells in both XAGE-1b and HLA class I expressing tumors increased compared with the densities in other groups, although the difference was not significant. The short peptides derived from tumor antigens bind to HLA class I molecules and the peptide-HLA class I antigen complex stimulates CD8+ T-cells, followed by differentiation into cytotoxic T-lymphocytes (4-6). Our findings suggest that the XAGE-1b peptide and HLA class I complex elicited a CD8+ T-cell response and caused prolonged survival in postoperative lung adenocarcinoma patients by eradicating minimal residual disease after surgical resection of the tumor. Furthermore, no difference in survival was observed in patients with tumors expressing HLA class I and those with down-regulated HLA class I expression when tumors did not express XAGE-1b. Down-regulated HLA class I antigen expression itself is a poor prognostic factor in various cancers (19), suggesting that it allows tumor cells to escape from immune recognition. In addition, the finding that HLA class I antigen expression was relevant to patient survival when the tumors expressed XAGE-1b antigen, together with the finding that HLA class I antigen expression was irrelevant when the tumors did not express XAGE-1b antigen, suggests that XAGE-1b is one of the dominant antigens in lung adenocarcinoma which stimulates CD8+ T-cells and causes eradication of minimal residual disease after surgery. Concomitant expression of CT antigens has frequently been observed in NSCLC (9, 11), hence other cancer antigens concomitantly expressed with XAGE-1b might also be relevant to patient survival.

A population of lung adenocarcinomas has been shown to express MHC class II antigens (20, 21). It is intriguing to analyze whether, in addition to class I antigen expression, HLA class II expression results in prolonged survival of patients with tumors expressing XAGE-1b. *In vitro* CD8+ and CD4+ T-cell immune responses against XAGE-1b in lung cancer patients with tumors expressing XAGE-1b are currently under study using XAGE-1b overlapping peptides with PBMCs from patients.

The expression of CT antigens has been shown to correlate with poor prognosis (10, 11). Zendman *et al.* (13) detected *XAGE-1* mRNA in 23 of 61 metastatic melanomas and 0 of 8 primary melanomas, suggesting that XAGE-1b is associated with malignant potential in melanoma. The present study indicates that patients with adenocarcinoma expressing XAGE-1b and with down-regulated HLA class I expression showed even shorter survival compared to other groups of patients. XAGE-1b might be associated with the malignant phenotype in lung adenocarcinoma if not efficiently presented on HLA class I, consistent with the above notion.

Groeper *et al.* (9) reported that the detection of specific cytotoxic lymphocytes against MAGE-A1, -A2, -A3, -A4, -A10, -A12, and NY-ESO-1 antigens was rare in tumor-infiltrating lymphocytes from resected NSCLC tissues despite diffuse expression of these CT antigens in the tumors. In their study, HLA expression in the tumors was not examined and the detection of a low frequency of infiltrating T-cells might be due to the down-regulation of HLA expression.

Expression of XAGE-1b in NSCLC showed no correlation with any clinicopathological feature, except for dominant expression in adenocarcinoma which was consistent with previous reports (15, 17). Staining was observed in the nuclei of cancer cells and in the normal testis tissue used as control. In the testis, positive staining was observed in the nuclei of spermatogonia and spermatocytes. The XAGE-1b protein consists of 81 amino acids harboring a functional bipartite nuclear localization signal and a C-terminal acidic transcription-activation-like domain (13).

In conclusion, we showed that XAGE-1b and HLA class I expression results in prolonged postoperative survival in lung adenocarcinoma patients. Co-expression of XAGE-1b and HLA class I may elicit efficient CD8+ T-cell responses and eradicate minimal residual disease after surgery in these patients. The present results indicate that XAGE-1b is a promising target for immunotherapy against NSCLC in an adjuvant setting.

Abbreviations

CT, cancer/testis

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Materials and methods

Study population

We conducted a retrospective analysis of patients diagnosed with NSCLC who underwent surgery as initial treatment at Hokkaido University Hospital between 1982 and 1994. The present study included 121 patients for whom adequate archival primary tissues were available for analysis. Histological diagnosis and grade of differentiation were determined in accordance with World Health Organization criteria for histopathological classification (22). Pathological stage was based on the American Joint Committee on Cancer guidelines for postoperative Tumor-Node-Metastasis (TMN) (23). Survival data was available for 77 of 83 adenocarcinoma patients and median duration of follow-up was 3287 days (range 1184-5830 days). Informed consent was obtained from all patients. The study protocol was approved by the Institutional Review Board of Hokkaido University Hospital.

Immunohistochemistry

Resected primary tumor specimens fixed in formalin and embedded in paraffin were cut into sequential 5 μm-thick sections and then deparaffinized. Antigen retrieval was performed by autoclave heating at 121°C for 20 min in 10 mM citrate buffer (pH 6.0). After inactivation of endogenous peroxidases with 0.3% hydrogen peroxide for 30 min, tissue slides were preincubated with serum-free blocking solution (DakoCytomation, Kyoto, Japan). USO9-13 anti-XAGE-1b monoclonal antibody (15-17) was added at a concentration of 2 μg/ml and incubated at 4°C overnight. After washing, the tissue slides were stained immunohistochemically using streptavidin-biotin complex (SimpleStain MAX-PO kit; Nichirei, Tokyo, Japan), followed by reaction with 3,3'-diaminobenzidine tetrahydrochloride-hydrogen peroxide as the chromogen and counterstaining using hematoxylin solution.

HLA class I expression in these specimens had previously been examined by immunohistochemical means using EMR8-5 anti-pan HLA class I monoclonal antibody (18). Tumor-infiltrating CD8+ cells had been examined immunohistochemically using anti-CD8 mAb (clone C8/144b, DAKO, Glostrup, Denmark) as described previously (18).

XAGE-1b and HLA class I expression

Expression of XAGE-1b was assessed by two investigators (E.K. and K.Y.) blinded to other immunohistological and clinical data. The expression level of XAGE-1b in the tumor cells was defined as described previously (15). Briefly, expression was defined as diffuse when >50% of the nuclei of cancer cells were stained, intermediate when 11-50% were stained, focal when 5-10% were stained, and negative when <5% were stained. Sections of normal human testis were used as positive tissue controls.

HLA class I expression on the tumor cell membrane was classified as positive and down-regulated compared with stromal lymphocytes as an internal control. Expression was defined as positive when >80% of tumor cells stained for the membrane at the same level as stromal lymphocytes, and down-regulated when 80% or less cells were stained.

Quantification of tumor-infiltrating CD8+ cells

Tumor-infiltrating CD8+ cells in cancer cell nests and adjacent cancer stroma were counted. The mean cell density in the highest 6 microscopic fields under high magnification (200x) was determined as the number of cancer nest- or stroma-infiltrating CD8+ cells. Tumors showing no tumor cell nests and replacing growth of pulmonary alveolar structure were excluded from the evaluation. The details have been reported previously (18).

Statistical analysis

The chi-square test was used to assess the significance of correlations between expression of XAGE-1b and clinicopathological features. Densities of tumor-infiltrating cells were compared using the nonparametric Mann-Whitney *U* test. Overall patient survival was calculated from the date of surgery to the date of last follow-up (censored) or the date of patient death by any cause (event). Survival probabilities were calculated using the Kaplan-Meier method. Differences in survival between patient subgroups were analyzed using the log-rank test. Univariate analysis and multivariate analysis using Cox proportional hazards regression model were performed to assess the association of clinicopathological variables with overall survival. Independent variables with P values <0.1, as determined by univariate analysis, and XAGE-1b expression were included in the multivariate analysis. P values <0.05 were considered statistically significant in all tests.

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