



Relationship between Kita-Kyushu Lung Cancer antigen-1 expression and prognosis of cases with lung squamous cell carcinoma

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Background: Previously, we identified the highly immunogenic cancer testicular antigen named Kita-Kyushu Lung Cancer antigen-1 (KK-LC-1). In this study, we analyzed the effect of KK-LC-1 expression on the prognosis of patients with resected squamous cell lung cancer.

Methods: Fifty squamous cell lung cancer patients, who received complete resection, were enrolled in this study. The expressions of KK-LC-1, CD8, human leukocyte antigen (HLA) class I, and programmed cell death protein ligand-1 (PD-L1) were assessed via immunohistochemistry staining using the specimens obtained from the participants. The association between the expression of the abovementioned molecules and patient prognosis was investigated.

Results: KK-LC-1 expression was observed in 21 of 50 recruited cases (42%). However, no significant correlation was found between KK-LC-1 expression and patient prognosis. The prognosis was significantly better in lung cancer cases with KK-LC-1 expression in which CD8⁺ T cells infiltrated the tumor. Regardless of the HLA class I expression or the PD-L1 expression, the KK-LC-1 expression in squamous cell lung cancer could not be detected as a significant prognostic factor. Furthermore, considering the polarity of the cancer tissue as epithelium, staining of KK-LC-1 tended to be strong in the area corresponding to the basal side of the tumor tissue. The Ki-67 expression was frequently observed in cancer cells on the basal side, which was consistent with the KK-LC-1 expression in representative four cases with KK-LC-1-positive squamous cell lung cancer.

Conclusions: Our results indicated that lung squamous cell cancer patients with KK-LC-1 expression and the tumor infiltrating CD8⁺ T cells might exhibit better prognosis. KK-LC-1 might be highly expressed in cancer cells with high proliferative capacity. Larger cohort analysis is still required for further elucidation and validation of the results of this study.

Keywords: Lung cancer; Kita-Kyushu Lung Cancer antigen-1 (KK-LC-1); cytotoxic T lymphocyte (CTL); programmed cell death protein ligand-1 (PD-L1); tumor immunology

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Introduction

Previously, we identified the cancer testis antigen Kita-Kyushu Lung Cancer antigen-1 (KK-LC-1) via cDNA expression cloning method using tumor-specific cytotoxic T lymphocyte (CTL) clone (1). KK-LC-1 is mapped to chromosome Xq22 and is not expressed in normal tissues except the testis; 33% of non-small cell lung cancers expressed it (1). In addition, we isolated T cell receptor (TCR) from KK-LC-1-specific CTL clones and transferred them into gd T cells. KK-LC-1-specific TCR-introduced gd T cells exhibited KK-LC-1-specific activity both *in vivo* and *in vitro* (2). A dominant KK-LC-1-specific immune response was previously observed in human papillomavirus-positive cervical cancer patients who completely regressed after adoptive immunotherapy (3). KK-LC-1 is a cancer testis antigen that is specifically expressed in cancer cells and has high immunogenicity, but its application to immunotherapy is still awaited.

Immune checkpoint inhibitors (ICIs) have made it possible to prolong the survival of lung cancer patients and have become a standard treatment for lung cancer. However, the response rate of ICI monotherapy is about 20% to 40% (4-8), and it is considered, to this end, that there are many aspects that still need to be improved. Applying the KK-LC-1-specific immune response to ICI might lead to better results.

Herein, the significance of the KK-LC-1 expression on the immunological microenvironment and prognosis of lung squamous cell cancer patients were analyzed. We present the following article in accordance with REMARK reporting checklist (available at <https://dx.doi.org/10.21037/tcr-21-1581>).

Methods

Patients and samples

From August 2009 to June 2019, we analyzed 50 lung squamous cell cancer patients who received complete resection at our hospital. The pathological stage (p-stage) was determined according to the latest tumor, lymph node, and metastasis (TNM) classification (9). The relationship between these clinicopathologic factors and the KK-LC-1 expression in cancer cells was retrospectively analyzed.

This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Saitama Hospital Ethics Committee (R2019-01) and informed consent was taken from all the patients.

Immunohistochemistry (IHC)

The Benchmark XT autostainer (Ventana Medical System, Tucson, AZ, USA) with anti-CD8 (SP57), anti-programmed cell death protein ligand-1 (anti-PD-L1) (SP263), anti-human leukocyte antigen (anti-HLA) class I HLA-A, B, C (D370-3H) (EMR8-5.1) MBL and anti-Ki-67 (30-9) was used for immunohistochemistry analysis (IHC) by using serial sections from paraffin-embedded tumors. Two pathologists counted the proportion of tumor stromal areas where IHC-positive lymphocytes existed to evaluate tumor infiltration of CD8⁺ lymphocytes. The median of the obtained values was applied for the analysis. We counted the proportion of cancer cells expressing the HLA class I among the viable cancer cells to assess the expression of HLA class I. To evaluate the PD-L1 expression in tumor stromal area, the proportion of IHC-positive immune cells among the immune cells in tumor stromal area was calculated. The proportion of IHC-positive immune cells in the immune cells in the tumor stromal area was counted to assess the PD-L1 expression in tumor infiltrating immune cells. The ratio of the area of IHC-positive cancer cells to the total area of viable cancer cells was then calculated to evaluate the PD-L1 expression in cancer cells, as we previously reported (10). The KK-LC-1 protein expression was evaluated in lung cancer tissue using the original monoclonal antibody against KK-LC-1 (Krab34B3), which was constructed in collaboration with Dr. Fukuyama and CLEA Japan, Inc. (Tokyo, Japan). Cytoplasm and/or nucleus in cancer cells appearing as brown granules were defined as immunoreactivities for positive KK-LC-1 expression.

Statistical analyses

Correlation between KK-LC-1 and clinicopathological factors was analyzed using Fisher's Exact test. The Kaplan-Meier method was used for the survival analysis, the log-rank test was used for the univariate analysis, and a Cox proportional hazard model was used for the multivariate analysis. The survival analysis, univariate analysis, and multivariate analysis were calculated using the Kaplan-Meier method, log-rank test, and Cox proportional hazard model, respectively.

A receiver operating characteristic (ROC) curve analysis was performed using the obtained clinicopathological data, and the cut-off values was calculated. All P values were two-sided. Statistical significance was defined by P value was <0.05. The statistical analysis software used in this study was

Table 1 The patients' characteristics are shown

Factor	KK-LC-1		P-value
	Positive	Negative	
Age (years), n (%)			
<70	6 (46.2)	7 (53.8)	0.75
≥70	15 (40.5)	22 (59.5)	
Sex, n (%)			
Male	14 (37.8)	23 (62.2)	0.35
Female	7 (53.8)	6 (46.2)	
Brinkman index, median (range)			
<1,000	7 (29.2)	17 (70.8)	0.09
≥1,000	14 (53.8)	12 (46.2)	
Performance status, n (%)			
0	19 (45.2)	23 (54.8)	0.44
1	2 (25.0)	6 (75.0)	
p-T, n (%)			
1–2	18 (41.9)	25 (58.1)	1.00
3–4	3 (42.9)	4 (57.1)	
p-N, n (%)			
0–1	21 (42.9)	28 (57.1)	1.00
2	0 (0)	1 (100)	

KK-LC-1, Kita-Kyushu Lung Cancer antigen-1.

EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface of R (The R Foundation for Statistical Computing, Vienna, Austria). EZR is a modified version of the R commander with the addition of statistical functions frequently used in biostatistics (11).

Results

Patient characteristics

Table 1 shows the patients' characteristics. Thirty-seven men (74%) and 13 women (26%) were included, with a median age at surgery of 74 years (range, 57–88 years). The numbers of cases with Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0 and 1 cases were 42 (84%) and 8 (16%), respectively. The numbers of cases with p-stages I, II, and III were 36, 11, and 3, respectively. KK-LC-1 expression was observed in 21 of 50 recruited

cases (42%). There were no significant differences in clinicopathological factors between KK-LC-1-positive and -negative cases. The observation period was 16–3,643 days (median: 1,226 days). The median observation period was 1,226 days (16–3,643 days). The 5-year cancer-specific survival rate and disease-free survival rate were 76.7% and 71.5%, respectively. None of the survivors reached the median survival period (Figure 1).

Pathological analyses

Figure 2 shows a statistical analysis using the data derived from IHC. The sensitivity and specificity were calculated from the ROC curve, and the maximum point was used as the cut-off value. Representative positive and negative cases of the PD-L1 and HLA class I expression of cancer cells (Figure 3), and the CD8 and PD-L1 expression of tumor infiltrating immune cells (Figure 4) by using an immunohistochemistry staining are shown.

Cancer-specific survival

As shown in the Figure 3, the specimens that exhibited staining in the cytoplasm and nucleus of lung cancer cells were evaluated as positive (Figure 5A), and those without staining were evaluated as negative (Figure 5B). Differences in prognosis were evaluated on the basis of KK-LC-1 expression status of lung cancer cells, but no significant difference was observed (Figure 5C). As shown in Figure 6A,6B, the prognosis was significantly better in cases in which lung cancer cells expressed KK-LC-1 and CD8⁺ T cells infiltrated into the tumor. On the other hand, even if the lung cancer cells that expressed KK-LC-1 also expressed HLA-class I or PD-L1, it did not affect patient prognosis (Figure 6C–6E).

Distribution of the KK-LC-1 expression

During the KK-LC-1 expression analysis, we observed strong staining in the region corresponding to the basal side of the tumor tissue. Therefore, the relationship between the proliferative ability of cancer cells and the KK-LC-1 expression in cancer cells was confirmed via IHC staining for the Ki-67 expression in four representative cases with KK-LC-1-positive lung squamous cell carcinoma. The Ki-67 expression in cancer cells was frequently observed on the basal side, which was consistent with the KK-LC-1 expression in all four cases (Figure 7).

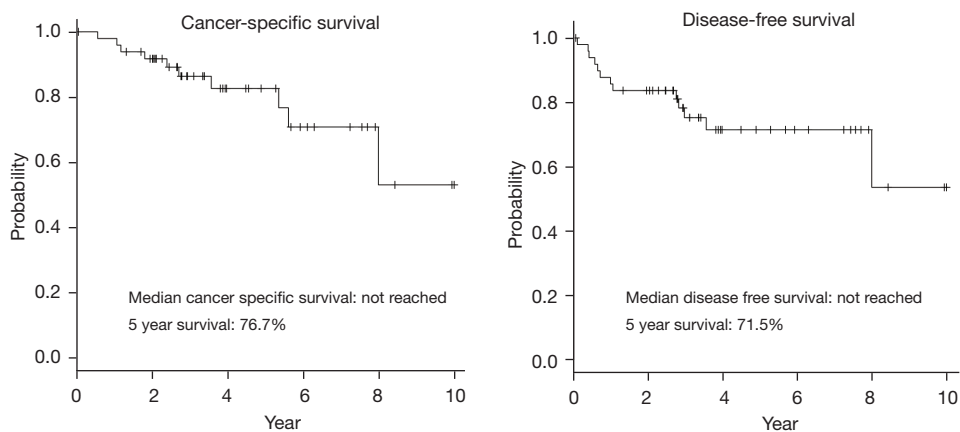


Figure 1 Kaplan-Meier curves for the cancer-specific and disease-free survival of lung squamous cell carcinoma patients are shown. The 5-year cancer-specific survival rate and disease-free survival rate were 76.7% and 71.5%, respectively.

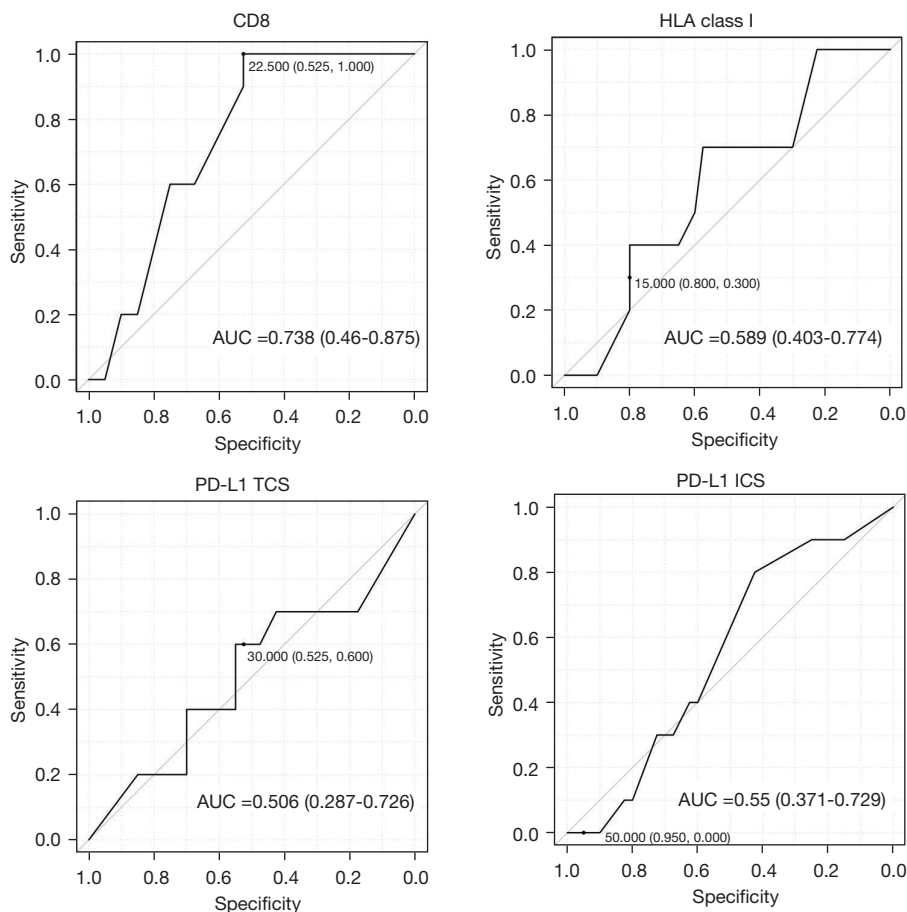


Figure 2 A ROC curve using which the sensitivity and the specificity to the cancer-specific survival were calculated, and the cut-off was determined. The cut-off values for CD8, HLA class I, PD-L1 TCS, and PD-L1 ICS were 22.5, 15.0, 30.0, and 50.0, respectively. ROC, receiver operating characteristics. HLA, human leukocyte antigen; PD-L1, programmed cell death protein ligand-1; TCS, tumor cells; ICS, immune cells.

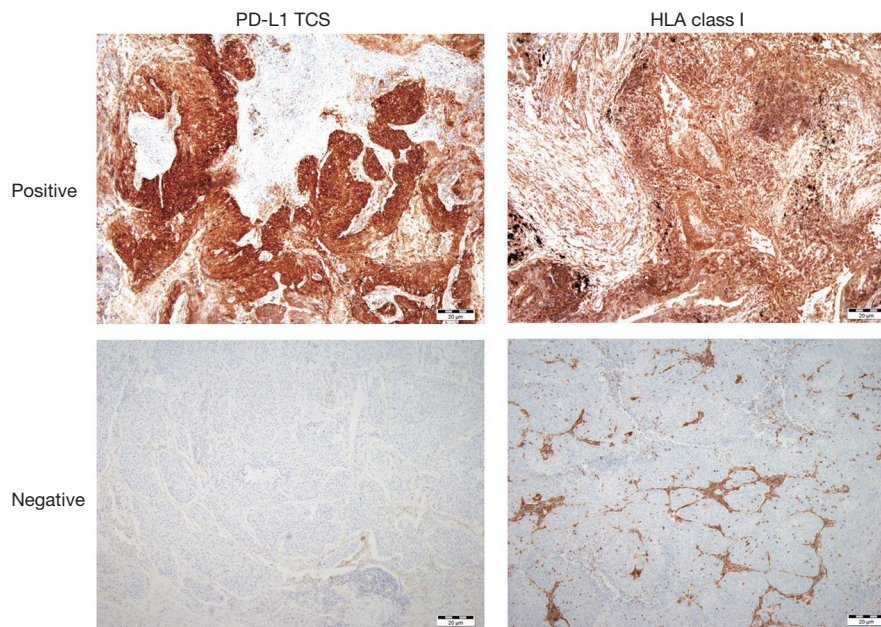


Figure 3 Representative positive and negative cases of the PD-L1 and HLA class I expression of cancer cells by using an immunohistochemistry staining are shown. PD-L1, programmed cell death protein ligand-1; TCS, tumor cells; HLA, human leukocyte antigen.

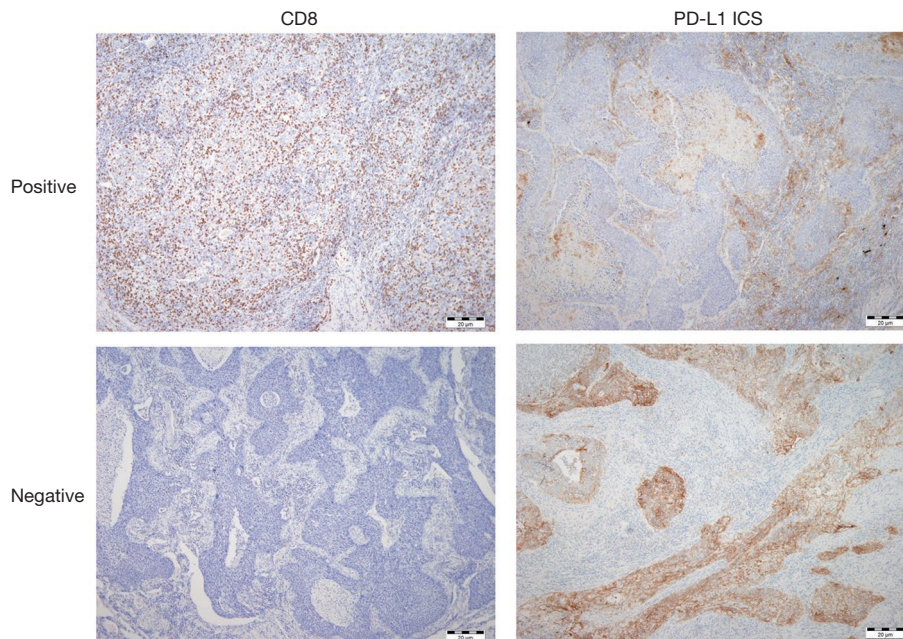


Figure 4 Representative positive and negative cases of the CD8 and PD-L1 of tumor infiltrating immune cells by using an immunohistochemistry staining are shown. PD-L1, programmed cell death protein ligand-1; ICS, immune cells.

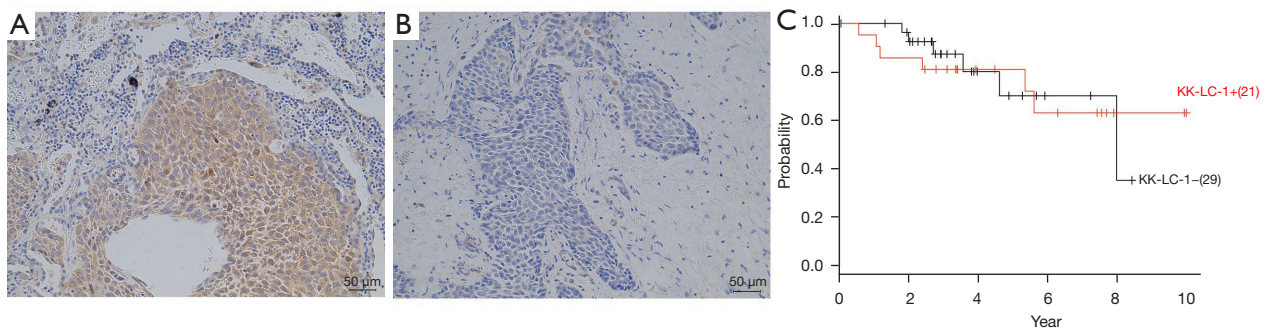


Figure 5 Analyses of the KK-LC-1 expression. (A) An immunohistochemistry staining image of KK-LC-1 in a case of KK-LC-1-positive lung squamous cell carcinoma. Nucleus and/or cytoplasm of lung squamous cell carcinoma cells are stained. (B) An immunohistochemistry staining image of KK-LC-1 in a case of KK-LC-1-negative lung squamous cell carcinoma. Neither the cell membrane nor the cytoplasm is stained. (C) Kaplan-Meier curves for the cancer-specific survival of lung squamous cell carcinoma patients with or without the KK-LC-1 expression. There was no significant difference in prognosis depending on the intensity of KK-LC-1 expression in patients with lung squamous cell carcinoma. KK-LC-1, Kita-Kyushu Lung Cancer antigen-1.

Discussion

ICIs provide a survival advantage over conventional therapies for lung cancer treatment; however, more than 30–40% of patients receiving ICI monotherapy have progressive disease (PD) (4–8). We previously reported that 19 of 44 (43.2%) non-small cell lung cancer (NSCLC) patients who received ICI monotherapy developed PD (12). It is also important to establish a new combined immunotherapy in order to further amplify the effects of ICI.

We have identified a shared cancer antigen, KK-LC-1, recognized by the cancer-specific CTL clone. KK-LC-1 is frequently expressed in many carcinomas (1). In the current analysis on the resected cases of lung squamous cell carcinoma, the expression of KK-LC-1 was observed in 21 of 50 cases (42%), which corroborated our previous analysis. Furthermore, previous studies have reported positive expression of KK-LC-1 in 40 of 49 gastric cancers (81%) (13) and 9 of 17 triple-negative breast cancer cases (53%) (14). Additionally, dominant KK-LC-1-specific immune response has also been observed in the cancer patients with complete regression after adoptive immunotherapy (3). KK-LC-1 is a powerful immunogenic antigen. We have measured various anti-KK-LC-1 polyclonal and monoclonal antibodies and have validated that Kmab34B3 is more precise and more sensitive to detect KK-LC-1 protein than other antibodies (15,16).

However, the relationship between KK-LC-1 expression in cancer cells and patient prognosis remains unclear. In our previous investigation, the relationship between

the expression of the cancer testis antigens, including MAGE-A3, MAGE-A4, NY-ESO-1 and KK-LC-1, and prognosis in 239 cases of NSCLC was analyzed. None of the cancer testis antigens have been found to significantly affect prognosis. However, lung cancer cases in which these four cancer antigens were not expressed at all exhibited a significantly poorer prognosis than those in which they were expressed (17). Jin *et al.* reported that there was no significant association between KK-LC-1 expression and patient prognosis after analysis of 38 resected lung cancer specimens (18). On the other hand, Chen *et al.* reported that high KK-LC-1 expression was an independent poor prognostic factor in hepatocellular carcinoma (HCC). KK-LC-1 regulated the Notch1/Hes1 pathway and exacerbated HCC progression by physical interaction with presenilin-1 (19). In our analysis, the KK-LC-1 expression tended to be high in cancer cells with active cell proliferation on the basal side of the cancer tissue, suggesting a possibility of having a correlation between the proliferation ability of cancer cells and the KK-LC-1 expression in all four representative KK-LC-1 positive cases examined. Further analysis is needed to reach a conclusion.

We also observed that the prognosis might improve significantly if there was intratumoral infiltration of CD8⁺ T cells in cases with KK-LC-1-positive lung squamous cell cancer. Our finding corroborated those of previous studies that reported a good prognosis in cancer cases in which CD8⁺ T cells infiltrated the tumor (20,21). Naito *et al.* reported that various numbers of T cells with cytotoxic phenotype infiltrate in human colorectal cancer tissue,

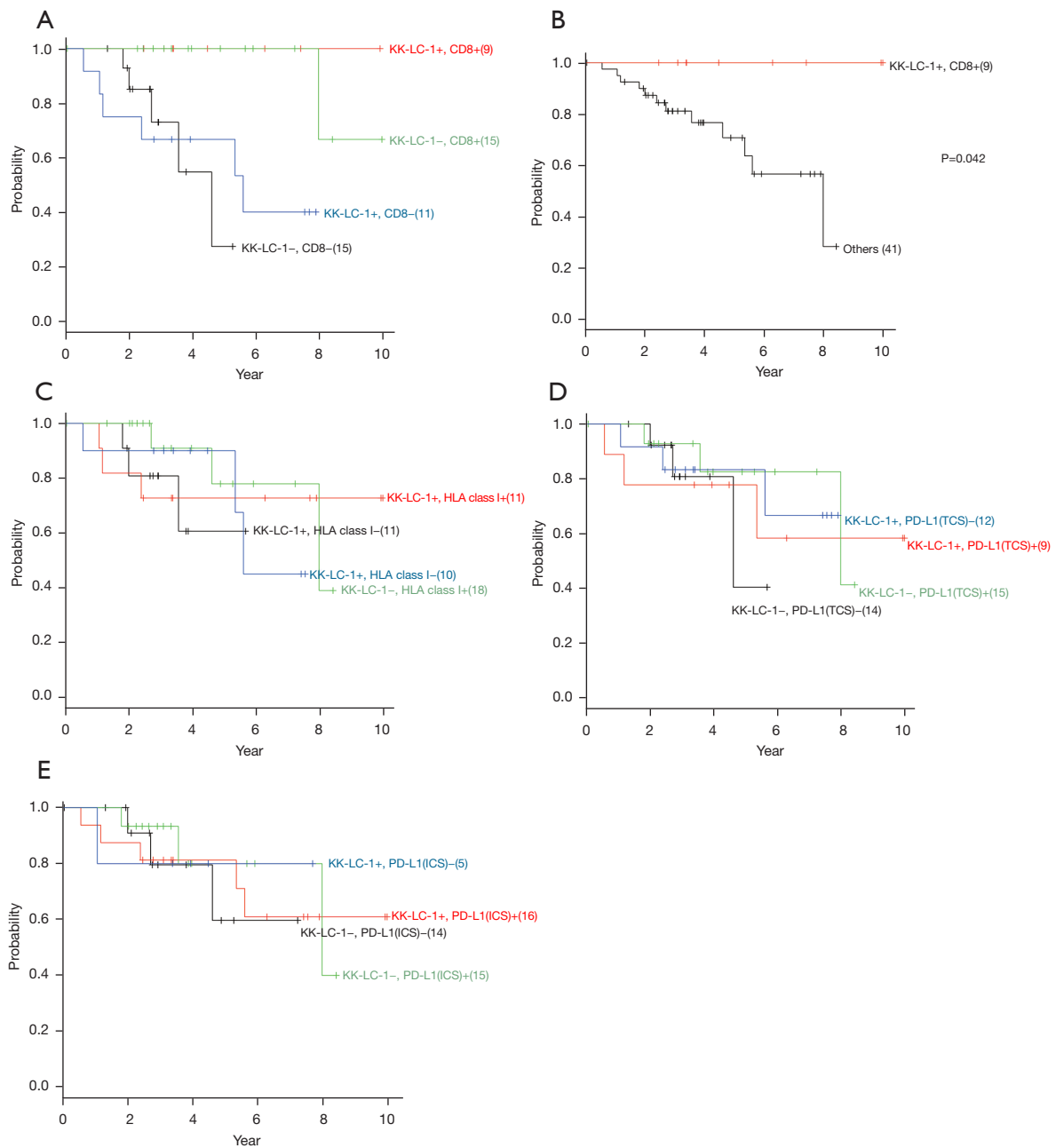


Figure 6 Survival curve with KK-LC-1 and each factor. (A) Kaplan-Meier curves for the cancer-specific survival of lung squamous cell carcinoma patients based on the KK-LC-1 expression and CD8⁺ T cell infiltration. No significant difference was observed. (B) Kaplan-Meier curves for the cancer-specific survival of lung squamous cell carcinoma patients with the KK-LC-1 expression and CD8⁺ T cell infiltration, and others. The cancer-specific survival of lung squamous cell carcinoma patients with the KK-LC-1 expression and CD8⁺ T cell infiltration was significantly better than others. (C) Kaplan-Meier curves for the cancer-specific survival of lung squamous cell carcinoma patients based on expression of KK-LC-1 and HLA class I. No significant difference was observed. (D) Kaplan-Meier curves for the cancer-specific survival of lung squamous cell carcinoma patients based on the expression of KK-LC-1 and PD-L1 in the cancer cells. No significant difference was observed. (E) Kaplan-Meier curves for the cancer-specific survival of lung squamous cell carcinoma patients based on the expression of KK-LC-1 in cancer cells and PD-L1 in the tumor infiltrating immune cells. No significant difference was observed. KK-LC-1, Kita-Kyushu Lung Cancer antigen-1; HLA, Human leukocyte antigen; PD-L1, programmed cell death protein ligand-1; TCS, tumor cells; ICS, immune cells.

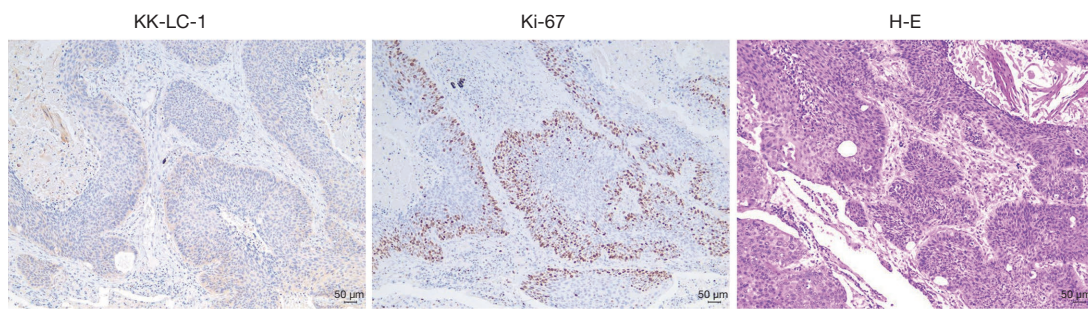


Figure 7 The left panel is KK-LC-1 and the center panel is Ki-67 stained during immunohistochemistry staining. The right panel shows HE staining. KK-LC-1, Kita-Kyushu Lung Cancer antigen-1; HE, Hematoxylin Eosin.

contributing to better patient survival (20). Among T lymphocytes, CD8⁺ T lymphocytes have a central effect of killing cancer cells and may be a sensitive biomarker. In a previous study (10), we found that the ratio of the area of CD8⁺ T lymphocytes to the area in the tumor stroma was more reflected in the prognosis, so we adopted this method in this study. This method is based on the literature (22).

We previously reported that mutated p53-specific CTLs and B cells infiltrated to tumor microenvironment and induced the cancer immune response (23). Functional analysis of tumor infiltrating CD8⁺ T cells is still required, since there is a possibility that the subset of CD8⁺ T cells may also include KK-LC-1-specific CD8⁺ T cells. In fact, we reported that HLA-B*1501- or 1507-restricted KK-LC-1-specific CTL clone was present in the regional lymph nodes of lung cancer cases, killed KK-LC-1-positive lung cancer cells, and contributed to cellular cancer immune response (1). It has also been reported that HLA-A*0101-restricted KK-LC-1-specific CTL clones are also present in cervical cancer patients (3). KK-LC-1 harbors multiple epitopes recognized by CTLs and is known to be a cancer antigen with high immunogenicity regardless of HLA type. We also reported that the transfer of the KK-LC-1-specific CTL-derived TCR into $\gamma\delta$ T cells acquires KK-LC-1-specific cytotoxic activity. A marked T cell infiltration was observed in the tumors that exhibited a tumor regression effect (2). If there is no CD8⁺ T cell infiltration in KK-LC-1-positive cancer cases, administration of KK-LC-1-specific TCR-transferred $\gamma\delta$ T cells may elicit a cancer immune response. To the best of our knowledge, this is the first report showing CD8⁺ T cell infiltration as a potentially good prognostic factor in KK-LC-1-positive lung cancer.

The HLA class I molecule is an important molecule for antigen presentation to CTL. It was thought that cancer cells escape from the cell-mediated immune response via

downregulation of the HLA class I molecule expression (24,25). However, according to our previous report, the relationship between the HLA class I expression and prognosis in 403 NSCLC surgical cases was analyzed, and no significant difference was found between the intensity of expression and prognosis (26). In the present study, even if KK-LC-1 was expressed, the HLA-class I expression did not have any significant effect on patient prognosis.

Expression of PD-L1 in cancer cells suppresses the T cell immune function and induces the escape of cancer cells from the immune response (27). High expression of PD-L1 in tumor infiltrating immune cells also suggests an environment that suppresses the cancer immune response. The PD-1/PD-L1 signaling pathway was shown to transduce suppressive signals to T lymphocytes and promote immune evasion of cancer cells (28). We previously investigated the relationship between prognosis and the PD-L1 expression of tumor cells in 105 surgical patients with pulmonary neuroendocrine tumors. However, the PD-L1 expression status was not significantly correlated with the prognosis (29). Whether the PD-L1 expression in cancer cells affects prognosis remains controversial.

It has previously been reported that anti-CTLA-4 antibody enhances immune responses to NY-ESO-1, which is one the cancer testis antigens similar to KK-LC-1 (30,31), and could induce NY-ESO-1-reactive CD4⁺ T cells (32). In a phase I study involving a combination of NY-ESO-1 vaccine and anti-CTLA-4 Ab for treatment of NY-ESO-1-positive melanoma, enhanced intratumoral CD8⁺ T cell proliferation was enhanced. In most cases, a cellular immune response against NY-ESO-1 was confirmed. It was reported that anti-CTLA-4 Ab might exhibit enhanced cell-mediated immune response against NY-ESO-1 (33). A more effective immunotherapy may be established by combining the cancer testis antigen-specific cancer immune response with ICI.

This study has a few limitations. First, the sample size of the present study was small. Second, our study included some old specimens that were resected more than 10 years ago. However, it was confirmed that the cells of the old specimens were also stained by IHC. Although the possibility of poor staining with older specimens could not be ruled out, the adverse effects of older specimens were ignored. Larger cohort analysis is still required for further elucidation and validation of the results of this study.

In conclusion, our results indicated that a good prognosis might be expected in lung squamous cell cancer patients with the KK-LC-1-positive expression and the tumor infiltrating CD8⁺ T cells. There is a possibility that KK-LC-1 expression might be highly expressed in cancer cells with high proliferative capacity. Further analysis is needed to conclude.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Saitama Hospital Ethics Committee (R2019-01) and informed consent was taken from all the patients.

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