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Prognostic impact of LY6K and CDCA1 expression for patients with esophageal squamous cell carcinoma

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Abstract

Aim: In the present study, we investigated the relationship between the expressions of two cancer testis antigens (CTA), LY6K (lymphocyte antigen 6 complex locus K) and CDCA1 (cell division cycle associated 1), in esophageal squamous cell carcinoma (ESCC) tumors and the long-term outcomes of patients with ESCC to clarify the clinical significance of LY6K and CDCA1 expression in ESCC tumors.

Methods: A total of 175 patients with thoracic ESCC who had undergone a thoracic esophagectomy with three-field lymphadenectomy without neoadjuvant therapy were retrospectively reviewed in this study. LY6K and CDCA1 expressions were evaluated in tumor tissues using immunohistochemical (IH) staining.

Results: Median patient age was 63 years; 159 patients (90.9%) were men. Ninety-four patients (55.3%) were LY6K-positive, and 85 patients (48.6%) were CDCA1-positive. The LY6K-positive group had a significantly worse overall survival (OS) than the LY6K-negative group ($P = 0.012$), and the CDCA1-positive group had a significantly worse OS than the CDCA1-negative group ($P = 0.010$). A multivariate analysis suggested that pathological N stage, venous invasion, LK6Y-positive and CDCA1-positive were independent prognostic factors. The patients were classified into four groups according to the staining pattern combinations of the two CTA. The LY6K-positive and CDCA1-positive group was found to have a significantly poorer outcome than the other groups.

Conclusion: ESCC patients with a combination of LY6K and CDCA1 expression in their tumor tissues had a worse prognosis than all the other ESCC patients and it was an independent factor associated with prognosis for patients with ESCC.

KEYWORDS
cancer testis antigen, cell division cycle associated 1, esophageal squamous cell carcinoma, lymphocyte antigen 6 complex locus K

1 | INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is one of the most malignant gastroenterological cancers,[1] and a definitive treatment for patients with ESCC is difficult because lymph node metastasis or distant metastasis occurs at a relatively early stage.[2] An analysis of prognostic factors and the response to chemoradiotherapy among patients with ESCC is now in progress.[3,4] However, in a comprehensive registry of esophageal cancer performed in Japan in 2012, the five-year survival rate of ESCC patients after surgery was 55.6%, indicating a poor prognosis.[5] Various biomarkers thought to be related to the prognosis of ESCC are known, including vascular endothelial growth factor (VEGF),[6] the vasohibin family,[7] epidermal growth factor receptor (EGFR),[8,9] cyclin D1,[10] the *p16* gene,[11] and so on. However, novel biomarkers should continue to be investigated not only for diagnosis, but also the development of treatments, including molecular targeting treatments.

Cancer testis antigens (CTA) are defined as proteins that are highly expressed in cancer cells but not in normal cells, except for the cells in reproductive tissues such as the testis, ovary and placenta.[12] CTA are considered to be both immunotherapeutic targets and good biomarkers for the diagnosis of cancer or the monitoring of recurrences. We previously reported that an expression pattern of IMP-3 (insulin-like growth factor II m-RNA binding protein 3), which is a CTA, was related to the long-term outcome of patients with ESCC.[13] The immunohistochemical (IH) expression of LY6K (lymphocyte antigen 6 complex locus K), another CTA, was thought to be related to the prognosis of patients with ESCC.[14] Accordingly, a serological antibody against LY6K was suggested as a diagnostic biomarker for ESCC.[15] Moreover, a phase II clinical trial of immunotherapy using peptides derived from CTA including IMP-3, LY6K and TTK (tyrosine/threonine kinase) for the treatment of ESCC has been reported.[16] Moreover, a phase II clinical trial of immunotherapy using IMP-3, LK6Y and CDCA1 (cell division cycle associated 1) for patients with head and neck cancer has also been reported.[17] Several reports have described CDCA1 overexpression in gastric cancer,[18] lung cancer[19] and head and neck cancer,[20] but no report has mentioned CDCA1 expression in ESCC. These reports suggest that CTA are potential biomarker candidates for the diagnosis and monitoring of patients with ESCC and that they might also be targets for the development of novel molecular-targeted drugs and immunotherapy. However, the clinicopathological significance of LY6K and CDCA1 expression in ESCC tumors remains unclear, and the relationship of expression in ESCC among multiple CTA was also unclear. Although the present study was a classic IH analysis, it used a novel approach to evaluate the relationships among combinations of the expressions of multiple CTA and prognosis.

The aim of the present study was to investigate the relationship between the expressions of LY6K and CDCA1 in ESCC tumors and the clinicopathological features and long-term outcomes of patients with ESCC to clarify the clinical significance of these expressions in ESCC tumors.

2 | METHODS

2.1 | Patients

In the present study, a total of 175 patients with thoracic ESCC who had undergone thoracic esophagectomy with three-field lymphadenectomy without neoadjuvant therapy at Tokai University Hospital between January 2003 and December 2005 were retrospectively reviewed. Adjuvant chemotherapy with cisplatin and 5-fluorouracil was performed after surgery for patients with pathological lymph node metastasis. We excluded patients with synchronous or metachronous multi-organ primary cancers and tissue types other than squamous cell carcinoma. The patients were followed up using endoscopy, computed tomography (CT), ultrasonography (US), and blood tests every 6 months for 5 years after surgery. The esophageal cancers were mainly classified according to the Japanese Classification of Esophageal Cancer.[21,22] Pertinent clinicopathological information was collected from the medical records of each patient. This study was approved by the Ethics Committee of Tokai University Hospital (registration No.13R-058).

2.2 | Immunohistochemical staining

Surgically resected tumor specimens and metastatic lymph nodes were fixed in 10% formalin for 24 hours and embedded in paraffin. Four-micrometer-thick paraffin sections were mounted on silane-coated glass slides and deparaffinized in xylene (5 minutes, 3 times) and ethyl alcohol (3 minutes, 4 times). Antigen retrieval was performed using the following process. After washing with 0.01 mol/L phosphate buffered saline (PBS), the slides were incubated in 0.01 mol/L Tris-buffered saline at 98℃ for 20 minutes and then left at room temperature for 60 minutes. After washing with 0.01 mol/L PBS once again, the endogenous peroxidase activity was abolished in 0.3% H_2O_2 in methanol for 30 minutes. This reaction was then blocked with 10% normal sheep serum for 10 minutes. The slides were incubated with a rabbit polyclonal antihuman LY6K antibody (Imgenex, San Diego, CA, USA) as the first antibody and biotinylated anti-rabbit IgG antibody (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA, USA) was used as the second antibody. A mouse monoclonal antihuman Nuf2 (CDCA1) antibody (Upstate, Lake Placid, NY, USA) as a first antibody and biotinylated anti-mouse IgG antibody (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA, USA) was used as the second antibody. After washing with 0.01 mol/L PBS, the labeled antigen was visualized using the diaminobenzidine reaction. The sections were counterstained with hematoxylin. The placenta was used as a positive control. Cancerous tissue from an esophageal cancer was used as a negative control after the addition of 0.01 mol/L PBS instead of a rabbit polyclonal antihuman LY6K antibody and a mouse monoclonal antihuman CDCA1 antibody.

2.3 | Expression of LY6K and CDCA1

Immunohistochemical staining results were assessed by two independent investigators with no knowledge of the clinicopathological data. If the interpretation of the IH staining results differed between two pathologists, a final decision was made after a review and discussion. Kappa statistics of IH staining of two CTA were 86.3% in LY6K and 91.4% in CDCA1. The expression of LY6K was evaluated based on the intensity of staining in the tumor cells and was classified into three groups: strong, moderate and weak. Strong or moderate staining was regarded as LY6K-positive, and weak staining was regarded as LY6K-negative (Figure 1). These criteria for LY6K expression were similar to those of previous reports.[14,15] The expression of CDCA1 was evaluated based on the area of staining in the tumor tissues using the following criteria: diffuse, dark brown staining in more than 60% of the tumor cells; focal, brown staining in 30% to 60% of tumor cells; and sporadic, brown cells in less than 30% of the tumor cells. The cutoff value for the area of dark brown staining indicating positive CDCA1 expression was defined using a receiver operating characteristic curve analysis (cutoff value: 60%, area under the curve: 0.576); Figure S1. Diffuse staining was regarded as CDCA1-positive, and focal or sporadic staining was regarded as CDCA1-negative (Figure 2).

2.4 | Statistical analysis

Correlations between each group and the clinicopathological variables were analyzed using the chi-squared test and Fisher's exact test for categorical variables and the Mann-Whitney U test for continuous variables. Overall survival (OS) was defined as the time from surgery until death from any cause. Relapse-free survival (RFS) was defined as any disease recurrence, but deaths were censored. The survival curves were estimated using the Kaplan–Meier method. A Cox proportional hazards model was used for the univariate and multivariate analyses to determine associations between clinicopathological factors and survival. A multivariate analysis was carried out for variables for which the P value was <0.05 in univariate analysis. The number of factors selected in multivariate analysis was determined based on the number of events in each analysis. P values of <0.05 were considered statistically significant. SPSS Statistics software, version 23 (IBM Corp., Armonk, NY, USA) was used for all the statistical analyses.

3 | RESULTS

3.1 | Patient characteristics

The clinicopathological factors of the 175 patients in our cohort are shown in Table 1. The median age was 63 years; 159 patients (90.9%) were men, and 16 patients (9.1%) were women. Ninety-four patients (53.7%) were LY6K-positive, and 85 patients (48.6%) were CDCA1-positive. Regarding LY6K staining, the numbers of patients with pathological lymph node metastasis or an infiltrative type of infiltrative growth pattern (INFc) were higher in the LY6K-positive group than in the LY6K-negative group. Regarding CDCA1 staining, the number of patients with poorly differentiated ESCC was higher in the CDCA1-negative group than in the CDCA1-positive group. The median follow-up period was 50 months.
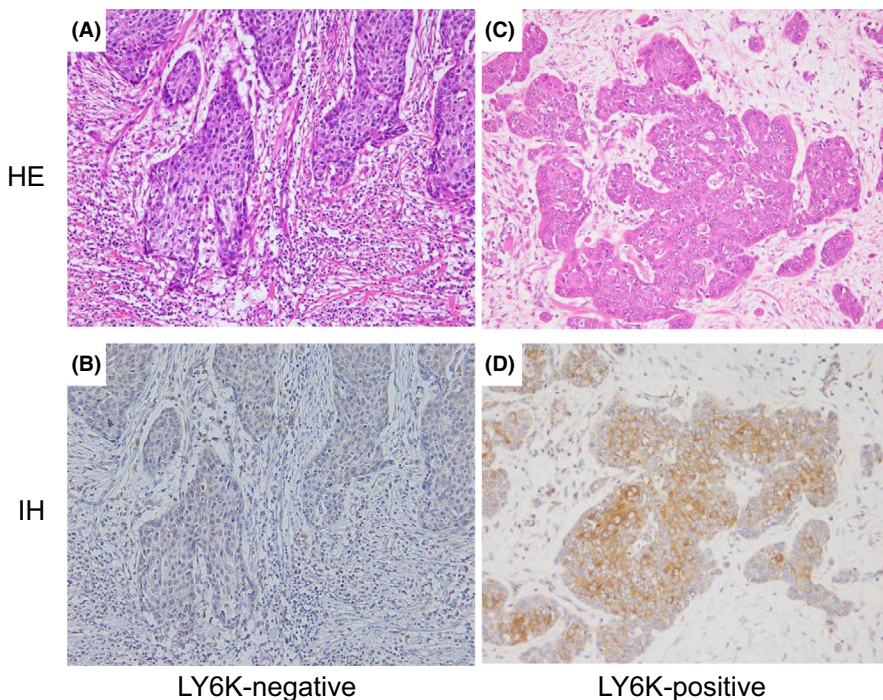

FIGURE 1 Immunohistochemical (IH) staining for LY6K expression in a resected esophageal squamous cell carcinoma (ESCC) specimen. A and B; Representative LY6K-negative specimen, magnification ×200 (A, hematoxylin and eosin [HE] staining, B, LY6K IH staining). C and D, Representative LY6K-positive specimen, magnification ×200 (C, HE staining, D, LY6K IH staining)

FIGURE 2 Immunohistochemical (IH) staining for CDCA1 expression in a resected esophageal squamous cell carcinoma (ESCC) specimen. A and B; Representative CDCA1-negative specimen, magnification ×200 (A, HE staining, B, CDCA1 IH staining). C and D, Representative CDCA1-positive specimen, magnification ×200 (C, HE staining, D, CDCA1 IH staining)
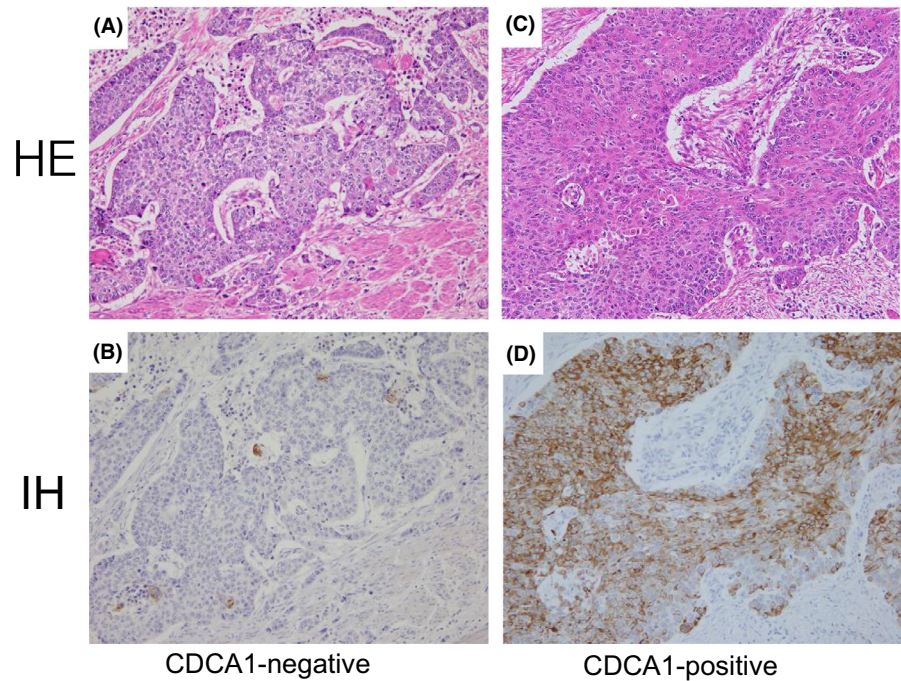
3.2 | Survival analysis

The OS rate and the RFS rate were compared using a log-rank test according to the staining patterns for each IH staining. Regarding LY6K staining, the LY6K-positive group had a significantly worse OS ($P = 0.012$; Figure 3A) and RFS ($P = 0.015$) than the LY6K-negative group. Regarding CDCA1 staining, the CDCA1-positive group had a significantly worse OS ($P = 0.010$; Figure 3B) and RFS ($P = 0.029$) than the CDCA1-negative group.

3.3 | Prognostic factors in survival analysis

In the survival analysis using the Cox proportional hazard model, univariate analyses revealed relationships between the pathological T stage (HR = 2.416, $P < 0.001$), pathological N stage (HR = 3.785, $P < 0.001$), INF (HR = 1.641, $P = 0.003$), lymphatic invasion (HR = 2.235, $P = 0.025$), venous invasion (HR = 3.581, $P < 0.001$), LY6K-positive (HR = 1.758, $P = 0.014$), CDCA1-positive (HR = 1.750, $P = 0.012$) and OS. A multivariate analysis of OS suggested that the pathological N stage (HR = 2.772, $P = 0.004$), venous invasion (HR = 3.372, $P < 0.001$), LY6K-positive (HR = 1.890, $P = 0.011$) and CDCA1 positive (HR = 1.987, $P = 0.003$) were independent factors associated with OS (Table 2). Univariate analyses revealed relationships between the pathological T stage (HR = 2.056, $P < 0.001$), pathological N stage (HR = 4.086, $P < 0.001$), INF (HR = 1.980, $P = 0.001$), lymphatic invasion (HR = 2.242, $P = 0.016$), venous invasion (HR = 3.386, $P < 0.001$), LY6K-positive (HR = 1.667, $P = 0.017$), CDCA1-positive (HR = 1.562, $P = 0.032$) and RFS. A multivariate analysis of RFS suggested that the pathological N stage (HR = 3.184, $P < 0.001$), venous invasion (HR = 3.091, $P < 0.001$), LY6K positivity (HR = 1.739, $P = 0.016$)

and CDCA1 positivity (HR = 1.613, $P = 0.027$) were independent factors associated with RFS. Moreover, a multivariate analysis of OS that included the combination of LY6K positivity and CDCA1 positivity as a factor suggested that the pathological N stage (HR = 2.925, $P = 0.002$), venous invasion (HR = 3.453, $P < 0.001$), and LY6K and CDCA1 positivity (HR = 2.981, $P < 0.001$) were independent factors associated with OS (Table 3). A multivariate analysis of RFS that included the combination of LY6K positivity and CDCA1 positivity as a factor suggested that the pathological N stage (HR = 3.322, $P < 0.001$), venous invasion (HR = 3.092, $P < 0.001$), and LY6K and CDCA1 positivity (HR = 2.401, $P < 0.001$) were independent factors associated with RFS.

3.4 | Relationship between LY6K and CDCA1 expression

To evaluate the relationship between LY6K expression and CDCA1 expression in ESCC tissues, the frequency of each expression was analyzed using a chi-squared test. A significant relationship was not observed between the expressions of the two CTA ($P = 0.880$; Table 4).

3.5 | Relationship between recurrence pattern and LY6K and CDCA1 expression

The recurrence pattern according to the expressions of LY6K and CDCA1 is shown in Table S1. The recurrence rate after surgery was higher among patients with LY6K positivity regardless of the recurrence pattern, but it was not related to CDCA1 expression. The recurrence rate was much higher in patients with a combination of LY6K positivity and CDCA1 positivity, regardless of the recurrence pattern.

TABLE 1 Association between expression of two CTA and clinicopathological factors of patients with ESCC

Characteristics	No. of all patients (n = 175) (%)	LY6K			CDCA1		
		Positive (n = 94) (%)	Negative (n = 81) (%)	P-value	Positive (n = 85) (%)	Negative (n = 90) (%)	P-value
Age (median; range)	63 (41-82)	63 (47-82)	62 (41-82)	0.766	63 (45-82)	63 (41-82)	0.720
Gender							
Male	159 (91)	85 (90)	74 (91)	1.000	74 (87)	85 (94)	0.117
Female	16 (9)	9 (10)	7 (9)		11 (13)	5 (6)	
Location of tumor							
Upper	17 (10)	10 (11)	7 (9)	0.974	9 (10)	8 (9)	0.554
Middle	92 (52)	49 (52)	43 (53)		44 (52)	48 (53)	
Lower	66 (38)	35 (37)	31 (38)		32 (38)	34 (38)	
Depth of tumor invasion (pT)							
T1a	21 (12)	8 (8)	13 (16)	0.112	14 (16)	7 (8)	0.112
T1b	49 (28)	21 (22)	28 (34)		21 (25)	28 (31)	
T2	27 (15)	16 (17)	11 (13)		9 (11)	18 (20)	
T3	71 (41)	44 (47)	27 (33)		36 (42)	35 (39)	
T4	7 (4)	5 (6)	2 (4)		5 (6)	2 (2)	
Lymph node metastasis (pN)							
Positive	108 (62)	67 (71)	41 (51)	0.008	54 (63)	54 (60)	0.644
Negative	67 (38)	27 (29)	40 (49)		31 (37)	36 (40)	
Lymphatic invasion (ly)							
Positive	149 (85)	83 (88)	66 (81)	0.286	70 (82)	79 (88)	0.341
Negative	26 (15)	11 (12)	15 (19)		15 (18)	11 (12)	
Venous invasion (v)							
Positive	104 (59)	60 (64)	44 (54)	0.220	49 (58)	55 (61)	0.648
Negative	71 (41)	34 (36)	37 (46)		36 (42)	35 (39)	
Differentiation							
Well	55 (31)	28 (30)	27 (33)	0.838	35 (41)	20 (22)	<0.001
Moderate	92 (53)	50 (53)	42 (52)		45 (53)	47 (52)	
Poor	28 (16)	16 (17)	12 (15)		5 (6)	23 (26)	
INF							
a	27 (15)	10 (11)	17 (21)	0.031	17 (20)	10 (11)	0.150
b	96 (55)	49 (52)	47 (58)		41 (48)	55 (61)	
c	52 (30)	35 (37)	17 (21)		27 (32)	25 (28)	
pStage							
0	19 (11)	7 (7)	12 (15)	0.268	12 (14)	7 (8)	0.462
I	30 (17)	14 (15)	16 (20)		11 (13)	19 (21)	
II	38 (22)	19 (20)	19 (23)		20 (23)	18 (20)	
III	57 (32)	34 (36)	23 (28)		27 (32)	30 (33)	
IVa	31 (18)	20 (22)	11 (14)		15 (18)	16 (18)	
Adjuvant chemotherapy							
+	80 (46)	46 (49)	34 (42)	0.366	31 (36)	49 (54)	0.023
−	95 (54)	48 (51)	47 (58)		54 (64)	41 (46)	

Abbreviations: CTA, cancer testis antigen; ESCC, esophageal squamous cell carcinoma; INF, infiltrative growth pattern (a: expansive type, b: intermediate type, c: infiltrative type).
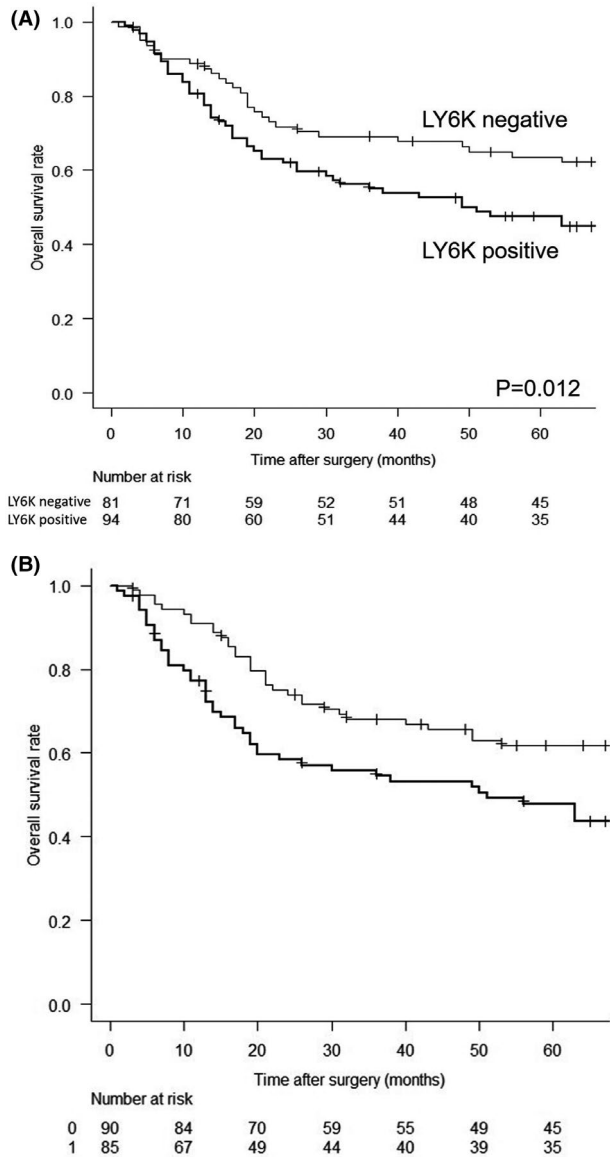
4 | DISCUSSION

In the present study, ESCC patients with the combined overexpression of both LY6K and CDCA1 in their tumor tissues had a worse prognosis than all the other patients, and the combined overexpression of these two CTA was also an independent factor associated with prognosis.

Previous reports have suggested a relationship between LY6K expression and the prognosis of patients with head and neck cancer,[23] breast cancer,[24] and non-small cell lung cancer.[14] Ishikawa et al reported that LY6K was expressed in 95% of ESCC cases and suggested that patients with LY6K expression in their tumor tissues had a poor prognosis.[14] In the present study, the expression of LY6K was evaluated and patients with LY6K positivity had a poorer prognosis than those with LY6K negativity. Our results were consistent with the above-mentioned previously reported findings. We also evaluated CDCA1 expression in ESCC patients and the CDCA1 expression in patients with ESCC has never been reported before. Patients with CDCA1-positive staining had a significantly worse prognosis; CDCA1 positivity was also an independent factor associated with survival.

Regarding the differences in the staining patterns of the two CTA, these differences may have been caused by the different functions of the two CTA and/or tumor heterogeneity. The IH staining findings in our cohort supported this hypothesis, as the positive areas of IH staining were not consistent between the two CTA (data not shown). Our study suggested that multiple CTA are correlated with the prognosis of ESCC patients. The combination of the two CTA expressions was also evaluated in the present cohort, and the synchronous expression of LY6K and CDCA1 in tumor tissues was associated with a significantly worse prognosis compared with that of the other patient groups. Nevertheless, the expressions of the two CTA were not associated with each other. Moreover, our results suggested that these two CTA had differing influences on recurrence after surgery. A previous report suggested that LY6K and the LY6 family may have functions related to cell signaling and cell adhesion, and the overexpression of LY6K was associated with a highly malignant phenotype of ESCC.[14] In contrast, CDCA1 is thought to play a role in the regulation of mitosis, and the overexpression of CDCA1 might be primarily caused by an epigenetic mechanism.[25] Our results suggested that the synchronous activation of multiple CTA may further promote the progression of cancer. The reason was thought to be tumor heterogeneity, rather than the interaction of multiple CTA. However, additional investigation is needed to clarify the relationship between tumor heterogeneity and the biological malignancy of ESCC. ESCC patients with the expressions of multiple CTA may benefit from aggressive immunotherapeutic intervention.

Sato et al suggested that the presence of multiple biomarkers was more strongly correlated with the prognosis of ESCC patients than that of a single biomarker.[26] Regarding the development of novel therapeutic procedures, single-target therapy might have a lower tumor specificity, and its influence on other tissues and organs might be a concern. In contrast, multiple-target therapy would increase the tumor specificity, enabling a stronger therapeutic effect and greater

FIGURE 3 Overall survival curves for all patients according to LY6K (A) and CDCA1 (B) expression. The curves were calculated using the Kaplan-Meier method. Differences between two groups were evaluated using a log-rank test

3.6 | Survival analysis in the combination of LY6K and CDCA1 expression

The patients were classified into four groups according to the combinations of the two CTA staining patterns: Group A (n = 41), patients with LY6K-negative and CDCA1-negative expression; Group B (n = 49), patients with LY6K-positive and CDCA1-negative expression; Group C (n = 40), patients with LY6K-negative and CDCA1-positive expression; and Group D (n = 45), patients with LY6K-positive and CDCA1-positive expression. The OS rates were compared among these four groups using the log-rank test, and Group D had a significantly poorer outcome than the other groups (Figure 4).

TABLE 2 Univariate and multivariate analysis of overall survival for patients in the present study by Cox proportional hazards model

Characteristics	No. of patients (n = 175)	Univariate analysis			Multivariate analysis		
		HR	95% CI	P-value	HR	95% CI	P-value
Age (years)		0.980	0.635-1.513	0.929			
≥64	78						
≤63	97						
Gender		1.081	0.471-2.483	0.854			
Male	159						
Female	16						
Location of tumor		0.965	0.445-2.095	0.929			
Upper	17						
Middle and Lower	158						
Adjuvant chemotherapy		1.280	0.854-1.920	0.233			
+	80						
−	95						
pT		2.416	1.491-3.915	< 0.001	1.188	0.669-2.109	0.556
2, 3 and 4	105						
1a and 1b	70						
pN		3.785	2.212-6.474	< 0.001	2.772	1.387-5.539	0.004
1	108						
0	67						
Differentiation		0.807	0.427-1.525	0.509			
Poor	28						
Well and Moderate	147						
INF		1.641	1.047-2.571	0.003	0.916	0.563-1.488	0.722
c	52						
a and b	123						
ly		2.235	1.106-4.519	0.025	0.419	0.150-1.171	0.097
Positive	149						
Negative	26						
v		3.581	2.137-6.001	< 0.001	3.372	1.699-6.694	< 0.001
Positive	104						
Negative	71						
LY6K		1.758	1.120-2.758	0.014	1.890	1.160-3.079	0.011
Positive	94						
Negative	81						
CDA1		1.750	1.131-2.709	0.012	1.987	1.264-3.123	0.003
Positive	85						
Negative	90						

Abbreviations: INF, infiltrative growth pattern (a: expansive type, b: intermediate type, c: infiltrative type), ly, lymphatic invasion, v, venous invasion.

feasibility in patients who meet the criteria for such treatments. Although several reports have suggested that combinations of multiple CTA expression are observed in other cancers,[27,28] no report has suggested that the presence of these combinations is correlated with the long-term outcome of ESCC patients. Recently, immunotherapy with cancer vaccination, which is derived from peptides expressed in cancer tissues, has been aggressively developed for

the treatment of several cancers.[29,30] Cancer vaccines derived from multiple antigens may have more clinical benefits than those derived from a single antigen, as they are expected to overcome problems associated with the heterogeneity of tumor cells and the escape of tumor cells from peptide-specific immunoresponses because of the loss of expression of a particular antigen.[31] A phase II clinical trial for ESCC patients using a combination of three peptide vaccines has

TABLE 3 Multivariate analysis of overall survival for patients in the present study including the combination of LY6K positivity and CDCA1 positivity as an explanatory variable by Cox proportional hazards model

Characteristics	No. of patients (n = 175)	HR	95% CI	P-value
pT				
2, 3, and 4	105	1.275	0.714-2.219	0.427
1a and 1b	70			
pN				
1	108	2.925	1.468-5.828	0.002
0	67			
INF				
c	52	0.835	0.509-1.370	0.475
a and b	123			
ly				
Positive	149	0.351	0.125-0.986	0.050
Negative	26			
v				
Positive	104	3.453	1.735-6.873	<0.001
Negative	71			
LY6K and CDCA1 expression				
Group D	45	2.981	1.822-4.876	<0.001
Group A, B, and C	130			

Abbreviations: INF, infiltrative growth pattern (a: expansive type, b: intermediate type, c: infiltrative type); ly, lymphatic invasion; LY6K and CDCA1 expression, (Group A: LY6K-negative and CDCA1-negative, Group B: LY6K-positive and CDCA1-negative, Group C: LY6K-negative and CDCA1-positive, Group D: LY6K-positive and CDCA1-positive). v, venous invasion.

TABLE 4 Relationship between LY6K expression and CDCA1 expression

Variable	CDCA1 positive (n = 85)	CDCA1 negative (n = 90)	P-value
LK6Y positive (n = 94)	45	49	0.880
LK6Y negative (n = 81)	40	41	

demonstrated a therapeutic effect.[32] Moreover, a phase II clinical trial for head and neck cancer using cancer vaccines derived from a combination of IMP-3, LY6K and CDCA1, which we have already evaluated, also showed a survival benefit.[33] Immunotherapy associated with these CTA is expected to contribute to the development of treatments for patients with ESCC in the future.

A limitation of the present study was first its single-center, retrospective design. Second, sample size of the present study was small for evaluation of clinicopathological significance of CTA in patients with ESCC. Third, the decisions regarding the expressions of the CTA evaluated in this study were not objective, as they were based on the pathologists' judgements of the histopathological findings. However, standardized criteria for histopathological decisions regarding the expressions of CTA do not yet exist.

To our knowledge, the present study is the first report to suggest that the combined expression of two CTA, LK6Y and CDCA1, is a predictor of a poor prognosis among patients with ESCC. The present results suggest that the expression of multiple CTA in ESCC might be a stronger biomarker for the diagnosis and targeting of novel molecular drugs or immunotherapy than the expression of a single CTA. Tumor-specific therapies targeting specific CTA should be developed for patients with ESCC in the future.

FIGURE 4 Overall survival curves according to the combined classification of LY6K and CDCA1 expression in four groups: Group A, patients with LY6K-negative and CDCA1-negative type; Group B, patients with LY6K-positive and CDCA1-negative type; Group C, patients with LY6K-negative and CDCA1-positive type; and Group D, patients with LY6K-positive and CDCA1-positive type. The curves were calculated using the Kaplan-Meier method. Differences between two groups were evaluated using a log-rank test
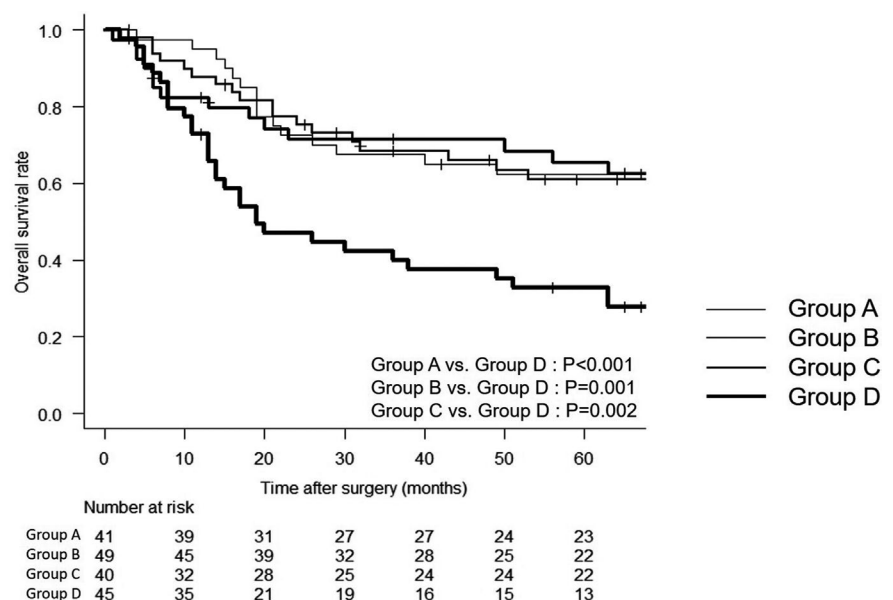
Group A vs. Group D : P<0.001
Group B vs. Group D : P=0.001
Group C vs. Group D : P=0.002

Number at risk

	0	10	20	30	40	50	60
Group A	41	39	31	27	27	24	23
Group B	49	45	39	32	28	25	22
Group C	40	32	28	25	24	24	22
Group D	45	35	21	19	16	15	13

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.