Original article

Serum levels of melanoma-associated antigen D4 in patients with squamous cell carcinoma of the esophagus predict disease-specific and recurrence-free survival

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Running title: Serum MAGE-D4 in esophageal cancer

ABSTRACT

Despite improvements in surgical techniques, perioperative management, and multidisciplinary therapy, treatment outcomes of patients with esophageal squamous cell carcinoma (ESCC) remain poor. Therefore, development of novel molecular biomarkers which either predict patient survival or become therapeutic targets are urgently required. In the present study, to facilitate early detection of ESCC and predict its clinical course, we investigated the relationship of the serum level of melanoma-associated antigen (MAGE)-D4 to clinicopathological characteristics of the patients. Using quantitative real-time reverse transcription-polymerase chain reaction and enzyme-linked immunosorbent assays, we determined the levels of *MAGE-D4* mRNA and protein in cell lysates and conditioned media of cultures, respectively, using nine ESCC cell lines. Further, we determined MAGE-D4 levels in serum samples collected from 44 patients with ESCC who underwent radical esophagectomy without neoadjuvant therapy and from 40 healthy volunteers. Samples of conditioned media and cell lysates contained comparable levels of MAGE-D4 that correlated closely with the levels of *MAGE-D4* mRNA. Preoperative MAGE-D4 levels in the sera of 44 patients with ESCC varied from 0 to 2,354 pg/mL (314 ± 505 pg/mL, mean ± standard deviation) and were significantly higher compared with those of healthy volunteers. By setting the cutoff at the highest value for healthy volunteers (50 pg/mL), the MAGE-D4-positive group of patients was more likely to have shorter disease-specific survival and recurrence-free survival compared with those of the MAGE-D4-negative group, although the differences

were not statistically significant. Our results indicate that the elevation of preoperative serum MAGE-D4 levels in some patients with ESCC was possibly caused by excess production by the tumor cells and release into the circulation. Serum MAGE-D4 levels may serve as a biomarker to aid early detection and prediction of the postoperative outcomes of patients with ESCC.

KEY WORDS: MAGE-D4, esophageal cancer, serum, diagnostic marker.

INTRODUCTION

Esophageal cancer is the eighth most common form of cancer worldwide, and it is the seventh most frequent cause of cancer-related deaths in Japan.[1-3] Esophageal squamous cell carcinoma (ESCC) represents more than 90% of esophageal cancers in Japan.[4, 5] Despite improvements in surgical techniques, reduced perioperative mortality, and development of multidisciplinary therapy, treatment outcomes of patients with ESCC remains poor even after radical esophagectomy.[4] In addition, several patients are deemed incurable because the disease is diagnosed at an advanced stage.[1, 6] Various tumor markers are used to detect early-stage esophageal cancer. For example, carcinoembryonic antigen (CEA), carbohydrate antigen 19-9, and squamous cell carcinoma (SCC) antigen are commonly used to manage patients with esophageal cancer.[7] However, role of tumor markerss in the early detection is severely limited because of their unsatisfactory sensitivities.[8, 9] Even the TNM staging has limited roles in predicting survival, because outcomes may differ dramatically for patients within the same clinical stage.[10, 11] Thus, new biomarkers are required to predict patient survival and to select the patients who will benefit from specific treatments such as adjuvant therapies.

We recently reported that overexpression of melanoma-associated antigen (MAGE)-D4, one of tumor-associated antigens, influences tumor progression and can serve as a prognostic marker of ESCC as well as a potential target for therapy.[12] MAGE-D4 is a unique member of the type II MAGE family

that was discovered in 2001 and is specifically expressed in the brain and ovary.[13] MAGE-D4 is overexpressed in the tumor tissues of malignancies including glioma, non-small-cell lung cancer, breast cancer, oral SCC, hepatocellular carcinoma, colorectal cancer, and ESCC.[14-18] However, to our knowledge, there are no reports on the clinical significance of serum MAGE-D4 levels in patients with ESCC.

In the present study, we investigated the association between the serum levels of MAGE-D4 and clinicopathological characteristics of the tumors and whether the serum levels of MAGE-D4 facilitate early detection of ESCC and predict its clinical course. In addition, secretion of MAGE-D4 by ESCC cell lines into the medium was evaluated to confirm that the MAGE-D4 produced in the cells is actually released into the circulation.

MATERIALS AND METHODS

Cell lines and conditioned medium

Nine ESCC cell lines (TE1, TE2, TE3, NUEC1, NUEC2, NUEC3, TT, TTn, and WSSC) were obtained from the American Type Culture Collection (Manassas, VA, USA), stored at −80°C using the cell preservative Cell Banker (Mitsubishi Chemical Medience Corporation, Tokyo, Japan), and cultured in RPMI-1640 supplemented with 10% fetal bovine serum at 37°C in an atmosphere containing 5% CO_2.[2,]

[19, 20] Cultured cells (10^6) were washed twice with phosphate-buffered saline and lysed using RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA). Levels of the cell lysates were measured using a NanoDrop 1000 (Thermo Fisher Scientific). Conditioned medium harvested from cultures of ESCC cells was prepared as follows: ESCC cells (10^5/mL) were added to 10-cm dishes with 10 mL of Dulbecco's essential minimal medium (DMEM) containing 2% fetal bovine serum and incubated for 3 days. To obtain conditioned medium, ESCC cells were washed twice with phosphate-buffered saline and then incubated for 3 days in 3 mL of DMEM. Conditioned medium was collected from each dish, centrifuged at 1,000 *g* for 5 min and stored at −30°C until use.

Study population

We studied 44 patients who underwent radical esophageal resection for ESCC at Nagoya University Hospital between December 2001 and October 2010. Subjects were excluded from the study if they had any malignancies other than ESCC or underwent preoperative treatment that included chemotherapy and radiation. Histological staging was determined according to the Union for International Cancer Control (7^{th} edition) staging system for esophageal cancer.[10] Demographics, tobacco and alcohol consumption, preoperative serum tumor markers (CEA and SCC), tumor size, tumor differentiation, tumor depth, vascular invasion, and lymph node metastasis were acquired from patients' medical records. Tobacco

consumption was estimated using the Brinkman index defined as numbers of cigarettes smoked per day multiplied by the number of years smoked. Questionnaires were used to acquire data on alcohol consumption from patients. Excessive alcohol consumption was defined as >210 g/week for ≥3 years.[21] The median duration of patient follow-up was 42.5 months (range, 1.5–125 months). Postoperative follow-up included physical examination, measurement of serum tumor markers every 3 months, and enhanced computed tomography scan (chest and abdominal cavity) every 6 months. Adjuvant chemotherapy was administered to selected patients according to the patient's condition and the physician's discretion.[12] Forty healthy volunteers (age ranged 32-64, 32 males and eight females) were included in this study as the control group. Serum samples were collected within 7 days of surgery and immediately preserved at −80°C. This study conforms to the ethical guidelines of the World Medical Association Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects. We obtained written informed consent from all patients for using clinical samples and data, as required by the Institutional Review Board of Nagoya University, Japan.[22, 23]

Enzyme-linked immunosorbent assay (ELISA)

Levels of MAGE-D4 in cell lysates, conditioned media, and serum samples were determined using a human MAGE-D4 ELISA Kit (CSB-EL088646HU; Cusabio, Wuhan, China) according to the manufacturer's protocol. All samples were tested in duplicate, and mean values were calculated. Serum samples with MAGE-D4 levels higher than the highest value among 40 healthy volunteers were defined as positive.

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Levels of *MAGE-D4* mRNA in ESCC tissues determined using qRT-PCR were taken from our previous study.[12] The qRT-PCR assays were performed according to our previously published work.[24-26]

Statistical analysis

The qualitative χ^2 test and quantitative Mann–Whitney test were used to compare the two groups. The strength of a correlation between two variables was assessed using Spearman's rank correlation coefficient. Disease-specific and recurrence-free survival rates were calculated using the Kaplan–Meier method, and the difference in survival curves was analyzed using the log-rank test. All statistical analyses were performed using JMP 10 software (SAS Institute Inc., Cary, NC, USA). $P < 0.05$ was considered significant.

RESULTS

MAGE-D4 levels in ESCC cells and conditioned media

MAGE-D4 levels in the lysates and condition media of nine ESCC cells and the corresponding *MAGE-D4* mRNA levels are shown in Fig. 1A. The MAGE-D4 levels (range, 380–2340 pg/mL) in conditioned media were similar to those of cell lysates (range, 527–3200 pg/mL), and there was a positive correlation of MAGE-D4 levels in the media with mRNA levels of the corresponding cell lines (Spearman's correlation coefficient 0.750, $P = 0.020$; (Fig. 1B).

Detection of MAGE-D4 in serum

MAGE-D4 levels in the sera of 40 healthy volunteers varied from 0 to 49.2 pg/mL (10.0 ± 13.2 pg/mL, mean ± standard deviation (SD)) (Fig. 2A). Preoperative serum MAGE-D4 levels in 44 patients with ESCC varied from 0 to 2,354 pg/mL (314 ± 505 pg/mL, mean ± SD) and were significantly higher compared with those of healthy volunteers ($P < 0.001$, Fig. 2A).There was a weak correlation between *MAGE-D4* mRNA levels in ESCC tissues and the corresponding serum MAGE-D4 levels (Spearman's correlation coefficient 0.450, $P = 0.002$, Fig. 2B).

Evaluation of the clinical utility of the serum levels of MAGE-D4

By setting the cutoff at the highest value for healthy volunteers (50 pg/mL), we categorized 22 of 44 patients with ESCC as the serum MAGE-D4-positive group. Patients in the MAGE-D4-positive group tended to suffer from shorter disease-specific survival (5-year survival rates, 47% vs 68%, $P = 0.086$, Fig. 3A) and recurrence-free survival (1-year survival rates, 55% vs 75%, $P = 0.235$, Fig. 3B) compared with those in the MAGE-D4-negative group, although the differences were not statistically significant. In contrast, neither preoperative CEA nor SCC levels were predictive for postoperative outcome (Fig. 4). There was no significant association between preoperative MAGE-D4 serum levels and clinicopathological factors such as macroscopic tumor size, T factor, N factor, and tumor differentiation and.

DISCUSSION

To improve treatment outcomes, the levels of several tumor markers in the sera of patients with ESCC have been analyzed to determine whether they are useful for staging, establishing prognosis, monitoring treatment, and detecting relapse in patients with ESCC.[7, 27, 28] Although the tumor markers CEA and SCC antigen are commonly used to manage patients with ESCC, their sensitivities are less than 10% for

detecting patients with early-stage esophageal cancer.[29] Moreover, the preoperative CEA and SCC levels were unsatisfactory for predicting prognosis.[29]

Recently, we discovered that MAGE-D4 is a candidate novel biomarker of ESCC.[12] However, the need for invasive procedure to obtain esophageal cancer tissue samples for evaluation of MAGE-D4 mRNA levels renders this metnod unsuitable for large-scale screening and routine postoperative examination.[30] Serum samples are an ideal source for overcoming these problems and are more accessible in clinical practice. Accordingly, we investigated the association of the serum levels of MAGE-D4 with the clinicopathological features of patients with ESCC. To the best of our knowledge, this is the first report looking at clinical relevance of preoperative serum levels of MAGE-D4 in patients with ESCC.

We show here that the levels of MAGE-D4 in patients' sera correlated with the expression levels of MAGE-D4 mRNA in tumor tissues, although the correlation was not strong. The mean level of serum MAGE-D4 was significantly higher in patients with ESCC compared with those of healthy volunteers. Moreover, serum MAGE-D4 levels were associated with shorter patient survival, although not significantly so, suggesting that measurement of preoperative MAGE-D4 levels may be useful for predicting long term outcome. Similar to our results, Zhang et al. demonstrated that an aberrant increase in *MAGE-D4* mRNA and protein expression occurs frequently in patients with advanced colorectal

cancer, and humoral immunity to MAGE-D4 was detected in 18.8% of serum samples from patients with colorectal cancer but not those from healthy volunteers.[31] Thus, determining MAGE-D4 levels is gaining importance in the field of gastrointestinal malignancies.

Another important finding of the present study is that MAGE-D4 was detected in conditioned media harvested from ESCC cell lines. Scatter-plot analysis reveals close correlation between the levels of *MAGE-D4* mRNA and those of cellular and secreted MAGE-D4. Moreover, levels of cellular and secreted MAGE-D4 were comparable. These results suggest that MAGE-D4 detected in the circulation originated from ESCC tissues, although further investigations are needed to understand the mechanism by which the MAGE-D4 produced within cancer cells enter the circulation.

This study was limited by its small sample size, since surgical specimens were used to evaluate the expression of MAGE-D4 in tissue samples at this time. Given that neoadjuvant chemotherapy is the current standard of care for Stage II/III ESCC in Japan, the availability of tumor tissues that were not pretreated is severely limited. To establish the place of MAGE-D4 as a biomarker for ESCC, analysis of a larger cohort will be necessary to decide on optimal cutoff values, to evaluate the assay's ability to distinguish ESCC from precursor lesions and other malignancies, and to determine how the MAGE-D4 serum levels change with treatments through longitudinal acquisition of the blood samples. Moreover, external validation of reproducibility and standardization across laboratories of the ELISA and mRNA

data are required. Although patients without preoperative therapy were exclusively enrolled here, changes of serum MAGE-D4 levels before and after neoadjuvant therapy should also be evaluated, because the data may expand the utility of MAGE-D4 serum levels as a predictor of response.

In summary, pretreatment levels of circulating MAGE-D4 were elevated in 50% of patients with ESCC, which was possibly caused by excess production by tumor cells and subsequent release into the circulation. Our results suggest that the levels of circulating MAGE-D4 may serve as a biomarker for early detection and prediction of postoperative outcome of patients with ESCC.

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Figure legends

Fig. 1 (A) *MAGE-D4* mRNA levels and MAGE-D4 levels in cell lysates and conditioned medium prepared from ESCC cell lines. MAGE-D4 levels are represented as the mean value of four replicates. Error bars indicate SD. **(B)** Comparison of *MAGE-D4* mRNA levels and MAGE-D4 levels in cell lysates and conditioned media.

Fig. 2 Measurement of serum *MAGE-D2* levels using an ELISA. **(A)** The mean value of serum MAGE-D4 levels was significantly higher in patients with ESCC compared with those of healthy volunteers. **(B)** MAGE-D4 levels in serum samples correlated positively with mRNA expression in the corresponding ESCC tissues.

Fig. 3 Patients in the MAGE-D4-positive group had shorter disease-specific survival (A) and recurrence-free survival (B) compared with those of the MAGE-D4-negative group.

Fig. 4 Elevated preoperative serum CEA **(A)** and SCC **(B)** levels did not correlate with postoperative outcomes.

Table 1 Association between serum MAGE-D4 level and clinicopathological parameters of 44 patients with squamous cell carcinoma of the esophagus

Clinico-pathological Parameters	Serum MAGE-D4 (pg/ml, mean ± SD)		*P* value
Age	≤ 60 year	> 60 year	1.000
	430 ± 655	234 ± 361	
Gender	Male	Female	0.793
	305 ± 534	348 ± 396	
Preoperative symptom	Absent	Present	0.915
	374 ± 459	301 ± 519	
Brinkman index	< 1000	≥ 1000	0.942
	366 ± 582	232 ± 350	
Excessive alcohol consumption	Absent	Present	0.843
	273 ± 356	329 ± 554	
CEA (ng/ml)	≤ 5 ng/ml	> 5 ng/ml	0.567
	212 ± 339	327 ± 524	
SCC (ng/ml)	≤ 1.5 ng/ml	> 1.5 ng/ml	0.425
	161 ± 264	359 ± 551	
Tumor size	< 5.0 cm	≥ 5.0 cm	0.896
	232 ± 334	433 ± 674	
UICC T factor	T1-2	T3-4	0.537
	269 ± 516	410 ± 484	
Differentiation	Moderate to well	Poor	0.839
	269 ± 385	490 ± 833	
Lymphatic involvement	Absent	Present	0.726
	235 ± 328	331 ± 538	
Vessel invasion	Absent	Present	0.806
	382 ± 417	375 ± 536	
Intraepithelial spread	Absent	Present	0.0933
	173 ± 356	455 ± 594	
Lymph node metastasis	Absent	Present	0.389
	253 ±459	460 ± 595	

Figure 1

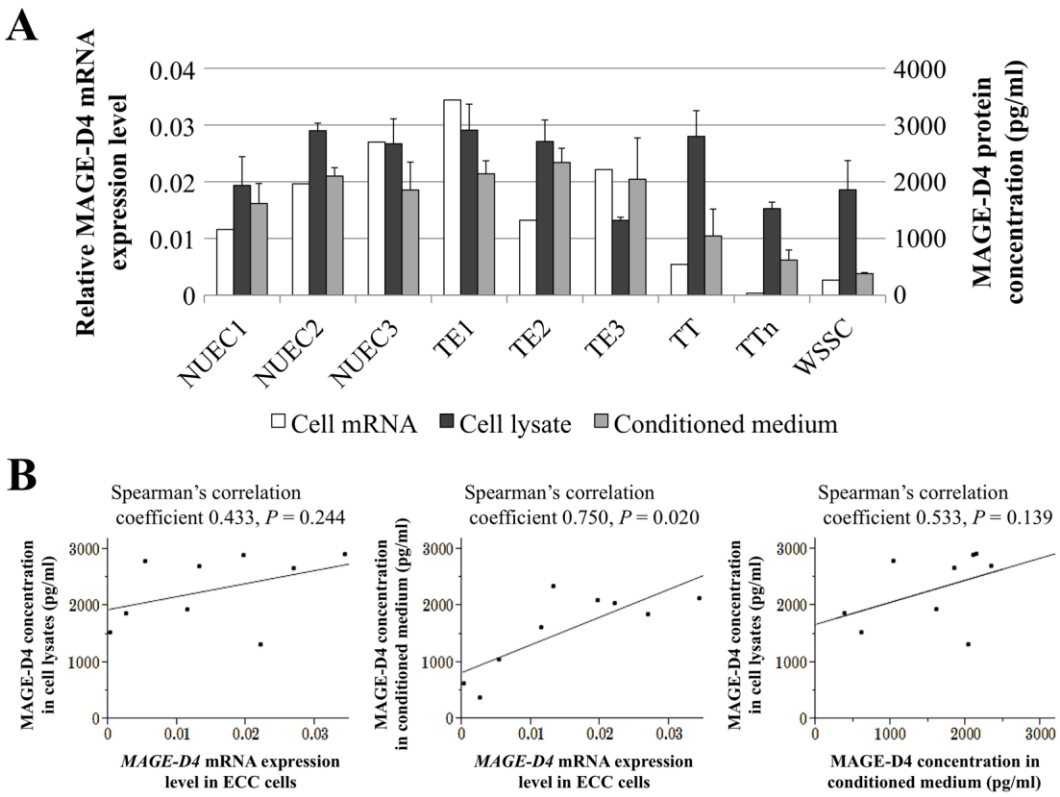
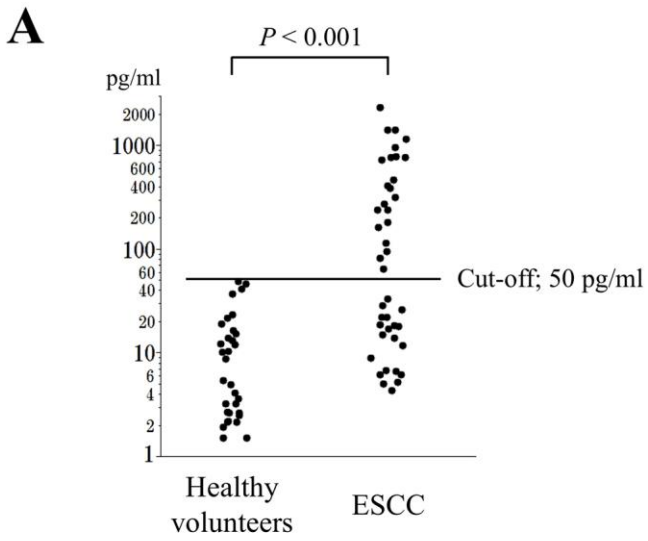

A
Relative MAGE-D4 mRNA expression level
MAGE-D4 protein concentration (pg/ml)
0.04
0.03
0.02
0.01
0
4000
3000
2000
1000
0
NUEC1
NUEC2
NUEC3
TE1
TE2
TE3
TT
TTn
WSSC
Cell mRNA
Cell lysate
Conditioned medium
B
Spearman's correlation coefficient 0.433, P = 0.244
MAGE-D4 concentration in cell lysates (pg/ml)
MAGE-D4 mRNA expression level in ECC cells
Spearman's correlation coefficient 0.750, P = 0.020
MAGE-D4 concentration in conditioned medium (pg/ml)
MAGE-D4 mRNA expression level in ECC cells
Spearman's correlation coefficient 0.533, P = 0.139
MAGE-D4 concentration in cell lysates (pg/ml)
MAGE-D4 concentration in conditioned medium (pg/ml)

Figure 2


A
P < 0.001
pg/ml
2000
1000
600
400
200
100
60
40
20
10
6
4
2
1
Cut-off; 50 pg/ml
Healthy volunteers
ESCC

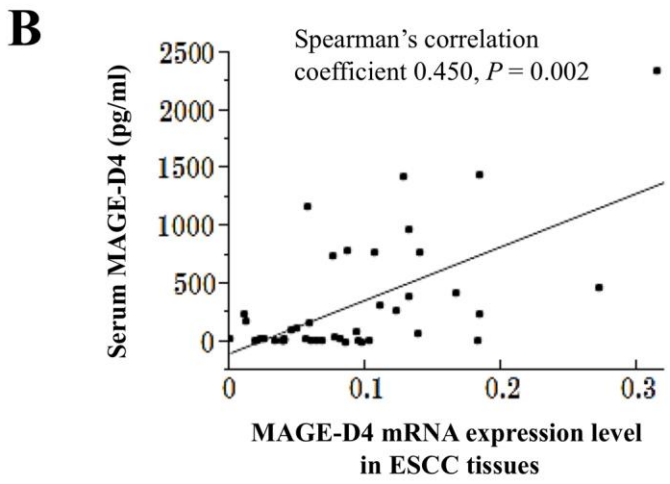

B
Spearman's correlation coefficient 0.450, P = 0.002
Serum MAGE-D4 (pg/ml)
2500
2000
1500
1000
500
0
0
0.1
0.2
0.3
MAGE-D4 mRNA expression level in ESCC tissues

Figure 3

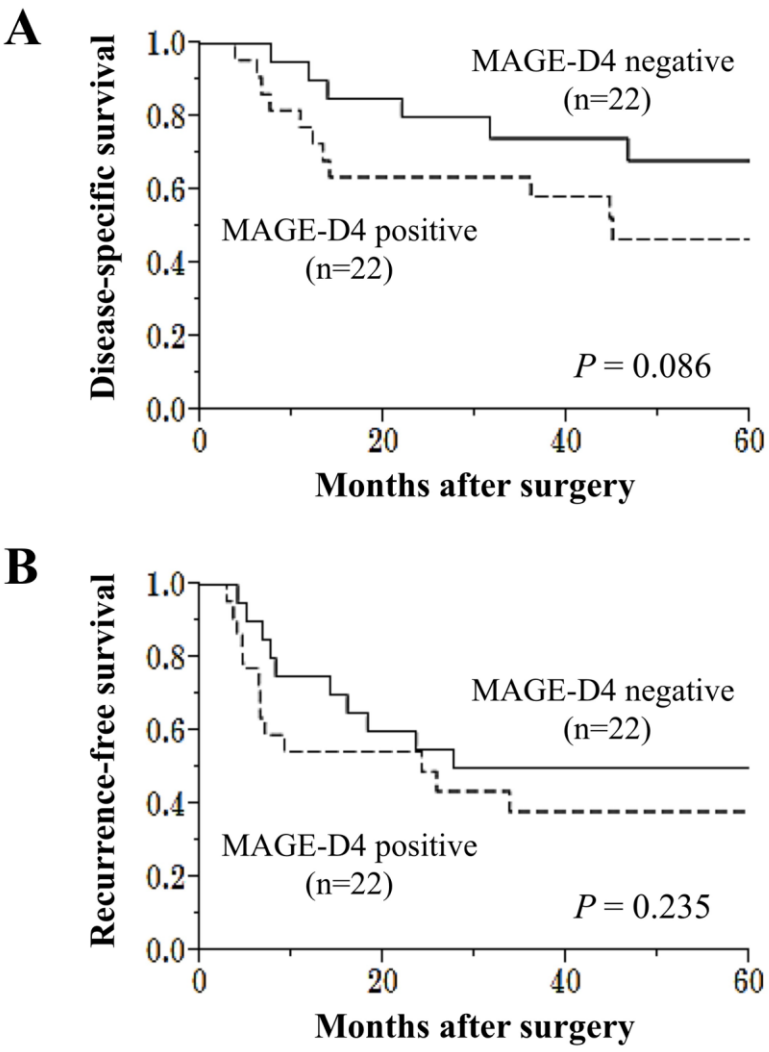
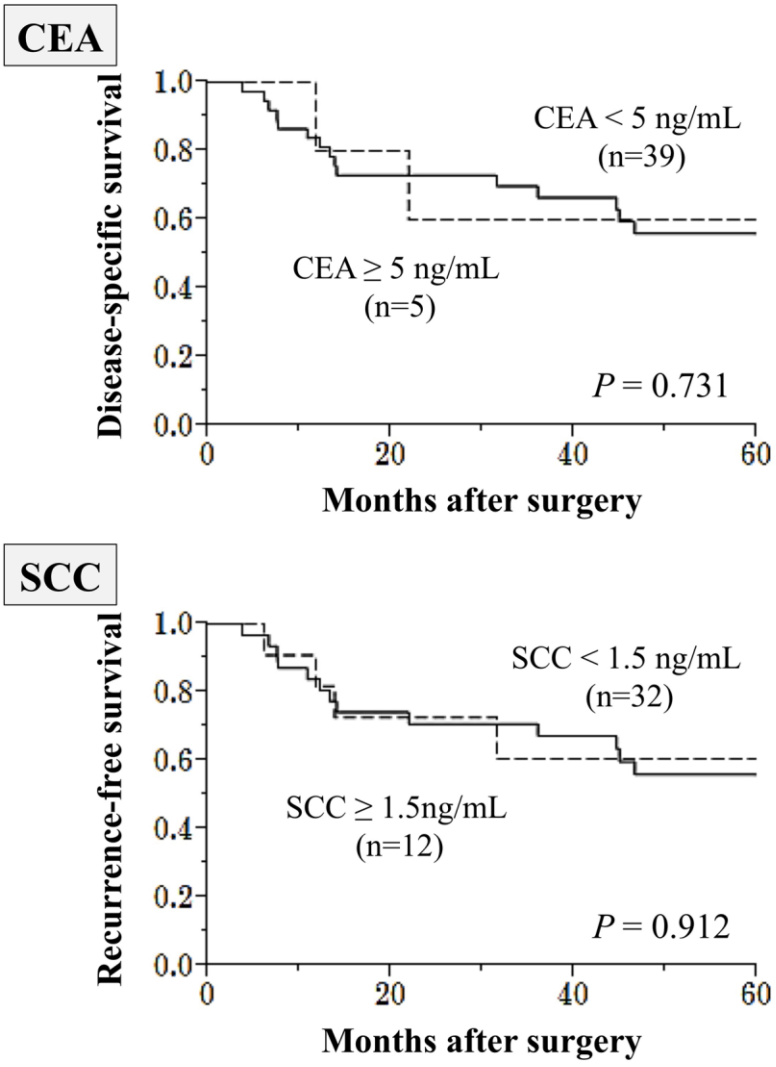

A
Disease-specific survival
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
Months after surgery
MAGE-D4 negative
(n=22)
MAGE-D4 positive
(n=22)
P = 0.086
B
Recurrence-free survival
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
Months after surgery
MAGE-D4 negative
(n=22)
MAGE-D4 positive
(n=22)
P = 0.235

Figure 4


CEA
Disease-specific survival
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
CEA < 5 ng/mL
(n=39)
CEA ≥ 5 ng/mL
(n=5)
P = 0.731
Months after surgery
SCC
Recurrence-free survival
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
SCC < 1.5 ng/mL
(n=32)
SCC ≥ 1.5ng/mL
(n=12)
P = 0.912
Months after surgery