Squamous cell carcinoma-antigen messenger RNA level in peripheral blood predicts recurrence after resection in patients with esophageal squamous cell carcinoma

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Background. *The aim of this study was to clarify whether preoperative squamous cell carcinoma-antigen messenger RNA (SCC-Ag mRNA) level in peripheral blood can be used to predict tumor recurrence after curative resection for esophageal squamous cell carcinoma.*

Methods. *A prospective analysis was conducted for 46 consecutive patients who underwent curative esophagectomy and who had no residual tumor. The SCC-Ag mRNA level in the peripheral blood of each patient was measured preoperatively by using quantitative reverse transcriptase-polymerase chain reaction. Median follow-up period was 34 months.*

Results. *Receiver operating characteristic analysis demonstrated that the optimal cutoff level of SCC-Ag mRNA was 40. Patients were divided into the high SCC-Ag mRNA level group (n = 14) and the low SCC-Ag mRNA level group (n = 32). The cumulative probabilities of tumor recurrence were higher in the high SCC-Ag mRNA level group (probability of recurrence was 71% at 2 years) than in the low group (22% at 2 years; P = .0005). SCC-Ag mRNA level (relative risk, 3.00; 95% confidence interval, 1.05-8.54; P = .040) was the strongest independent predictor of recurrence by multivariate analysis.*

Conclusions. *Preoperative SCC-Ag mRNA levels in the peripheral blood are the best predictive factor for recurrence in patients with esophageal squamous cell carcinoma who undergo curative resection (R0). (Surgery 2006;139:678-85.)*

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Circulating tumor cells (CTCs) in the peripheral blood or bone marrow of patients with various types of cancer have been documented.[1–19] CTCs in peripheral blood usually are detected by reverse transcriptase-polymerase chain reaction (RT-PCR) with the use of tumor marker messenger ribonucleic acid (mRNA) for specific tumor markers.[1–15] The reported detection rates of CTCs in peripheral blood by RT-PCR range from 17% to 69%.[1–15] Some authors demonstrated a significant association between CTCs detected by RT-PCR and outcome,[1–8] whereas others argue against this association.[9–12] Thus, controversy remains regarding the risk association between CTCs and tumor recurrence.[20,21]

Although RT-PCR, especially nested RT-PCR, is a very sensitive procedure for detecting CTCs, it is difficult to quantify exactly the levels of expressed tumor marker mRNA.[22] Recently, quantitative RT-PCR (QRT-PCR) has been developed to measure mRNA levels.[22,23]

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Squamous cell carcinoma-antigen (SCC-Ag) is a tumor antigen derived from cervical squamous cell carcinoma tissue.[24] Serum SCC-Ag has been proposed as a useful tumor marker for predicting disease progression in patients with esophageal squamous cell carcinoma (SCC), lung SCC, or cervical SCC.[25–29] An abnormal increase in serum SCC-Ag predicts poor survival with greater sensitivity than 2 other tumor markers, carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9, in patients with esophageal SCC.[30]

This study tested the hypothesis that the level of SCC-Ag mRNA in peripheral blood measured preoperatively by QRT-PCR predicts tumor recurrence and poor survival after curative resection in patients with esophageal SCC.

PATIENTS AND METHODS

Patients. The current study was approved by the ethics committee of Niigata University Graduate School of Medical and Dental Sciences. Informed consent to participate in the current study was obtained in writing from all patients and healthy donors. A total of 61 consecutive patients with esophageal SCC were admitted in the department of Surgery at Niigata University Medical and Dental Hospital from April 2000 to May 2003. The current study was designed to evaluate the value of measuring preoperative SCC-Ag mRNA levels in peripheral blood to predict patient outcome after resection of esophageal SCC. To avoid any influence of residual tumor cells or epithelial cells on the SCC-Ag mRNA levels, we enrolled patients in the study on the basis of the following criteria: (1) no history of malignant disease, (2) no history of severe dermatologic disease, and (3) history of resection with no residual neoplasm. All 61 patients had no history of malignancy or severe dermatologic disease. Five patients who had received chemoradiotherapy with no operation and 10 patients who had undergone resection with macroscopic or microscopic residual neoplasm were excluded. The remaining 46 patients formed the basis of the current prospective study, which comprised 41 men and 5 women aged 50 to 83 years (median, 66) years. All patients were Japanese.

In the department of Surgery at Niigata University Medical and Dental Hospital, esophagectomy is the standard treatment for esophageal carcinoma whenever the neoplasms are considered resectable. 5-Fluorouracil, nedaplatin, and doxorubicin were administered as neoadjuvant chemotherapy before operation in 11 patients. Nine patients received adjuvant chemotherapy with intravenous administration of 5-fluorouracil and cisplatin.

Patient follow-up after resection. Patients were followed every 1 to 3 months in outpatient clinics and monitored for recurrence by measuring the serum concentrations of SCC-Ag, CEA, and soluble cytokeratin-19 fragments and/or imaging studies. The follow-up after resection ranged from 12 to 55 months (median, 34 months).

Pathologic examination. Resected specimens were submitted to the Department of Surgical Pathology in our hospital. Each specimen was examined to determine the anatomic site of the primary neoplasm, histopathologic grading (G), pathologic extent of primary neoplasm (pT), pathologic regional lymph node metastasis (pN), pathologic distant metastasis (pM), lymphatic invasion (L), venous invasion (V), and classification of residual neoplasm. Histopathologic findings were described according to the *TNM Classification of Malignant Tumours* by the International Union Against Cancer (6th ed, 2002).[31]

Blood sampling. Peripheral blood was collected from each patient immediately after the induction of general anesthesia in 56 patients who underwent esophagectomy. To prevent contamination by epithelial cells, we inserted an indwelling catheter into a vein and discarded the first 10 mL of blood drawn. Subsequently, 3 mL of blood was drawn through the intravenous catheter into 2 vacuum tubes containing sodium citrate and stored immediately at 4°C. RNA extraction was performed as soon as possible after the blood was drawn, generally within 30 minutes. Peripheral blood samples obtained similarly from 42 healthy donors with no malignant disease were treated as controls.

RNA extraction. To avoid contamination from erythrocytes and serum protein, Lymphoprep (AXIS-SHIELD PoC AS, Oslo, Norway) was used according to the protocol provided by the manufacturer. Peripheral blood (3 mL) was diluted with an equal volume of 154 mmol/L NaCl and then laid over 3 mL of Lymphoprep in a centrifuge tube. Samples were centrifuged at 800*g* for 30 minutes, and the mononuclear cell layer was extracted. These cells were diluted further in 154 mmol/L NaCl and then pelleted by centrifugation at 400*g* for 10 minutes. The cell pellet was dissolved completely in TRIZol LS (Invitrogen Corp, Carlsbad, Calif) for RNA extraction according to the protocol provided by the manufacturer. The extracted RNA was dissolved in 20 μL of water treated with diethyl pyrocarbonate stored at −80°C. The optical density of total RNA was measured at 260/280 nm to quantify the yield and to exclude poor RNA quality; only specimens having an absorption ratio within the

range of 1.8 to 2.2 were used. The RNA extraction process took approximately 90 minutes.

Quantitative reverse transcriptase-polymerase chain reaction. Reverse transcriptase-polymerase chain reaction was carried out in accordance with the protocol provided by the manufacturer Invitrogen Corp; all reagents were purchased from the manufacturer. Two micrograms of total RNA was denatured for 2 minutes at 80°C, then cooled immediately on ice. After adding 8 μL of 5× First Strand Buffer, 8 μL of 2.5 mmol/L dNTP mixture, 2.0 μL of oligo dT primer, and 4 μL of 0.1 mol/L dithiothreitol , DEPC-treated water was added to a solution containing 80 U of RNase OUT Recombinant Inhibitor and 400 U of Superscript II and brought to a final volume of 40 μL. After incubating for 60 minutes at 42°C, the reaction was stopped by heating for 10 minutes at 80°C. The synthesized complementary DNA (cDNA) was stored at –20°C. The cDNA synthesis process took approximately 80 minutes.

A PRISM 7700 System (Applied Biosystems, Foster City, Calif) was used for QRT-PCR.[12,13] The specific primers and the TaqMAN probe (Applied Biosystems) were designed on the basis of the sequence of SCC-Ag mRNA and spanned exon 7 or 8 with an intron placed approximately 1 kbp between the sequences to prevent genomic amplification.[32] The primer and probe sequences were as follows: sense primer, 5'-CTG CCA AAT GAA ATC GAT GGT-3'; antisense primer, 5'-TGC AAA CTT GTC CAT TCC ATC A-3'; and TaqMAN probe, 5'-FAM-CAG AAG CTT GAA GAG AAA CTC ACT GCT GA-Tamra-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as the internal control. Commercially available GAPDH control reagents (Applied Biosystems) were used as described by the manufacturer. The SCC-Ag mRNA sense and antisense primers and TaqMAN probe were added to the QRT-PCR solution to make a final concentration of 100 nmol/L each, followed by the addition of 12.5 μL of 2× Universal Master Mix (Applied Biosystems), 1 μL of cDNA solution, and distilled water. The sense and antisense primers of GAPDH were added to the QRT-PCR solution to make a final concentration of 200 nmol/L. The TaqMAN probe of GAPDH was added and adjusted to make a final concentration of 100 nmol/L, followed by the addition of 12.5 μL of 2× Universal Master Mix and 1 μL of cDNA solution and distilled water. The cycling conditions for SCC-Ag and GAPDH QRT-PCR were as follows; 35 cycles of 15 seconds at 95°C and 60 seconds at 62.2°C, and 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C, respectively. The amplification curves were analyzed with the SDS Analysis System supplied with the PRISM 7700 System. The levels of SCC-Ag mRNA were normalized to GAPDH for each target mRNA sequence, and each sample was repeated 5 times. The mean of 3 quantitative values excluding the maximum and minimum was used to represent the quantitative level for each blood sample. QRT-PCR was performed in approximately 70 minutes.

Factors influencing recurrence and survival. To elucidate factors influencing recurrence and survival after resection, we analyzed 13 conventional variables (Table I) together with SCC-Ag mRNA levels by univariate and multivariate analyses. Serum concentrations of SCC-Ag were determined preoperatively.

Statistical analysis. Survival data were obtained from medical records for all patients. The end points of the current study were the detection of recurrence on imaging studies and death from all causes as determined from the medical records. Deaths from other causes were treated as uncensored cases. The Kaplan-Meier method was used to estimate the cumulative incidences of events; differences in these incidences were evaluated with the use of the log-rank test. The Cox proportional hazards regression model, in which stepwise selection is used for variable selection with removal limits of $P < .1$ and $P > .15$, respectively, was performed to identify factors that were associated independently with recurrence after resection. The stability of each model was confirmed by using step-backward and step-forward fitting procedure. The variables identified as having an independent influence on recurrence and death were identical according to both procedures. Fisher exact test was used to test the association between the 2 groups. The Mann-Whitney *U* test was used to compare SCC-Ag mRNA levels between patients with recurrence and those without evidence of recurrence. An analysis by receiver operating characteristic (ROC) curve was applied to determine the optimal cutoff level of SCC-Ag mRNA to discriminate patients with future recurrence after resection. All statistical evaluations were performed with the SPSS 11.5J software package (SPSS Japan Inc, Tokyo, Japan). All tests were 2-sided and differences with P values $< .05$ were considered statistically significant.

RESULTS

SCC-Ag mRNA levels in patients with esophageal SCC, compared with a control group. The SCC-Ag mRNA levels in patients with esophageal SCC ranged from 0 to 5862 (median, 0.09), compared

Table I. Clinicopathologic characteristics of 46 patients with esophageal squamous cell carcinoma by SCC-Ag mRNA level

Variable	*No. of patients*		P *value*
	Low SCC-Ag mRNA level	*High SCC-Ag mRNA level*	
Age (y)			.76
<65	16	6	
≥65	16	8	
Gender			.16
Male	30	11	
Female	2	3	
Primary tumor site			>.99
Upper-middle	27	12	
Lower	5	2	
pT			.34
pT1–T2	15	4	
pT3–T4	17	10	
pN			.016
pN0	15	1	
pN1	17	13	
pM			>.99
pM0	27	12	
pM1	5	2	
pStage			.19
pStage I–II	15	3	
pStage III–IV	17	11	
Lymphatic invasion (L)			.35
L0	19	6	
L1	13	8	
Venous invasion (V)			.08
V0	25	7	
V1–V2	7	7	
Histopathologic grading (G)			.27
G1–G2	26	9	
G3	6	5	
Serum SCC-Ag			>.99
≤1.5 ng/mL	20	9	
>1.5 ng/mL	12	5	
NAC			.27
No	26	9	
Yes	6	5	
Adjuvant chemotherapy			>.99
No	26	11	
Yes	6	3	

SCC-Ag mRNA, Squamous cell carcinoma-antigen messenger ribonucleic acid; *pT,* pathologic extent of primary neoplasm; *pN,* pathologic regional lymph node metastasis; *pM,* pathologic distant metastasis; *pStage,* pathologic stage grouping; *NAC,* neoadjuvant chemotherapy.

with levels in the control group that ranged from 0 to 3.8 (median, 0.04; Fig 1). The cutoff level of 2.7 was determined by the 95th percentile value of SCC-Ag mRNA levels in a control group. The sensitivity and specificity of the test were 46% (21/46) and 95% (40/42), respectively.

Association between SCC-Ag mRNA level and recurrence. At the time of assessment of disease status, 14 patients had died of recurrence. An additional 4 patients had died of other causes with no evidence of disease. Three patients were alive with recurrent disease; the remaining 25 patients were alive with no evidence of disease. The SCC-Ag mRNA level in the patients with recurrence ranged from 0 to 1689 (median, 83.0), whereas the SCC-Ag mRNA level in the patients with no evidence of tumor recurrence ranged from 0 to 5862 (median, 0.01; Fig 1). The SCC-Ag mRNA level was therefore higher in patients with tumor than in those with no evidence of recurrence ($P = .002$).

Cutoff level of SCC-Ag mRNA. An ROC curve for predicting recurrence was constructed for 46 patients with esophageal SCC to evaluate sensitivity and specificity. The area under the ROC curve was 0.771, compared to the area under the diagonal line ($P = .002$; 95% confidence interval [CI], 0.631-0.910). The maximum level of sensitivity × specificity was 0.51 (sensitivity = 59%, specificity = 86%). The cutoff level of SCC-Ag mRNA leading to the above results was 40; therefore, the cutoff level for SCC-Ag mRNA was set at 40 in the current study.

Clinicopathologic characteristics of 46 patients undergoing operative resection for esophageal SCC according to SCC-Ag mRNA level. Patients were divided into those with a low SCC-Ag mRNA level (n = 32) and those with a high SCC-Ag mRNA level (n = 14) on the basis of the cut-off level of 40. Clinicopathologic characteristics were comparable between the 2 groups except that regional lymph nodes metastasis was more frequent in the high SCC-Ag mRNA level group ($P = .016$;Table I). The pattern of recurrence in the high SCC-Ag mRNA group included hematogenous metastasis (brain, n = 2; liver, lung, and bone, n = 1 each), regional lymph node metastasis (n = 4), and locoregional recurrence (n = 1), whereas the pattern in the low SCC-Ag mRNA group comprised hematogenous metastasis (bone, n = 1), regional lymph node metastasis (n = 3), locoregional recurrence (n = 2), and hematogenous with regional lymph node metastasis (lung, n = 1). The incidence of hematogenous metastasis after resection was higher in the high SCC-Ag mRNA group (36%, 5/14) than in the low SCC-Ag mRNA group (6%, 2/32) ($P = .020$).

Factors influencing recurrence after resection. The overall cumulative probabilities of recurrence after resection were 35% at 1 year and 37% at 2

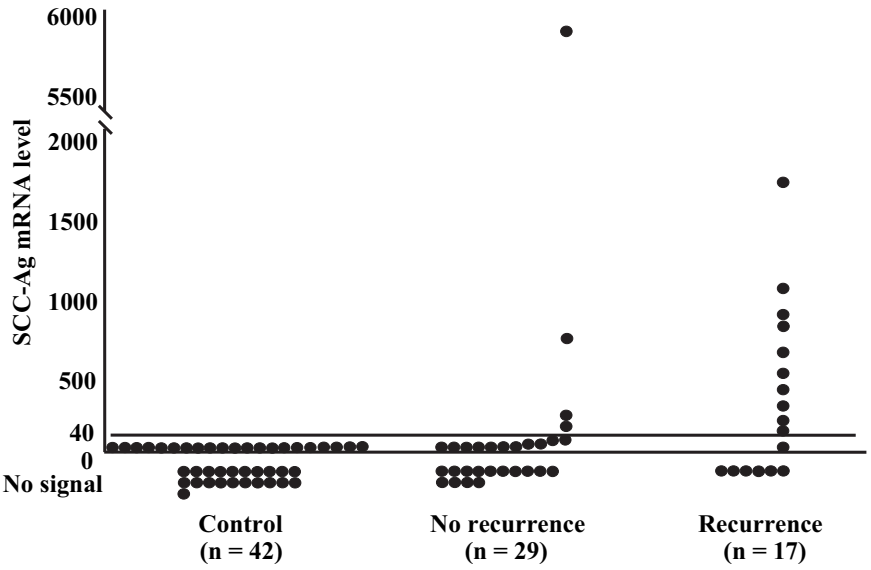

Fig 1. SCC-Ag mRNA levels in control, patients with no evidence of recurrence, and patients with recurrence. *SCC-Ag mRNA,* Squamous cell carcinoma-antigen messenger ribonucleic acid.

years in the current series. Univariate analysis revealed that venous invasion, SCC-Ag mRNA level, extent of primary neoplasm, and lymphatic invasion were significantly associated with tumor recurrence (Table II). In contrast, age ($P = .67$), gender ($P = .49$), site of primary neoplasm ($P = .67$), regional lymph node metastasis ($P = .19$), distant metastasis ($P = .66$), stage ($P = .09$), histopathologic grading ($P = .95$), serum SCC-Ag concentration ($P = .47$), neoadjuvant chemotherapy ($P = .12$), and adjuvant chemotherapy ($P = .78$) were not significant variables.

Variables that were significant by the univariate analyses were entered into multivariate analyses, which showed that SCC-Ag mRNA level (relative risk [RR], 3.00; $P = .040$), venous invasion (RR, 2.97; $P = .058$), and extent of primary neoplasm (RR, 4.13; $P = .078$) remained independently associated with recurrence (Table II).

Impact of preoperative SCC-Ag mRNA level on recurrence after resection. The cumulative probabilities of recurrence were higher in the high SCC-Ag mRNA level group than in the low group ($P = .0005$; Fig 2). The median duration of detecting recurrence was four months for the patient group with high SCC-Ag mRNA levels.

Factors influencing survival after resection. Overall cumulative survival rates after resection were 78% at 1 year and 63% at 2 years in the current series. Univariate analysis suggested that the extent of the primary neoplasm ($P = .0001$), lymphatic invasion ($P = .003$), venous invasion ($P = .008$), SCC-Ag mRNA level ($P = .032$; Fig 3), and regional lymph node metastasis ($P = .03$) were prognostic factors. These 5 significant variables were entered into multivariate analyses, which showed only the extent of the primary neoplasm (RR, 16.9; 95% CI, 2.22-128; $P = .010$) as an independent variable.

DISCUSSION

This is the first study to demonstrate that the SCC-Ag mRNA level in peripheral blood is the strongest independent predictor of recurrence after resection in patients with esophageal SCC. A high SCC-Ag mRNA level in peripheral blood, compared with that preoperatively measured by QRT-PCR, may predict recurrence after resection, because it represents the number of CTCs, which has been associated with the development of recurrence. Koike[33] documented a significant correlation between the amount of CTCs in blood samples and the number of pulmonary metastases in mice. Mayhew and Glaves[34] also demonstrated that when the number of Lewis carcinoma cells in the blood was 10^6 or greater, pulmonary metastases developed in a mice model, while cell levels of 10^5 or less caused no development of metastases. In the current study, the authors found a close association between the quantity of SCC-Ag mRNA and recurrence in patients with esophageal SCC. Taken together, these data suggest that the development of metastasis is highly dependent on the number of CTCs.

The measurement of preoperative SCC-Ag mRNA levels in peripheral blood with the use of

Table II. Factors influencing recurrence after resection

Variable	No. of patients	Probability of recurrence (%)		Univariate analysis	Multivariate analysis	
		1 year	2 year	P value	Relative risk (95% CI)	P value
pT				.002		.08
pT1–T2	19	6	11		1.00	
pT3–T4	27	56	56		4.13 (0.85–19.9)	
Lymphatic invasion (L)				.013		
L0	25	20	20			
L1	21	52	58			
Venous invasion (V)				.0001		.06
V0	32	19	19		1.00	
V1–V2	14	71	81		2.97 (0.96–9.18)	
SCC-Ag mRNA level				.0005		.04
Low	32	19	22		1.00	
High	14	71	71		3.00 (1.05–8.54)	

CI, Confidence interval; *pT,* pathologic extent of primary neoplasm; *SCC-Ag mRNA,* squamous cell carcinoma-antigen messenger ribonucleic acid.

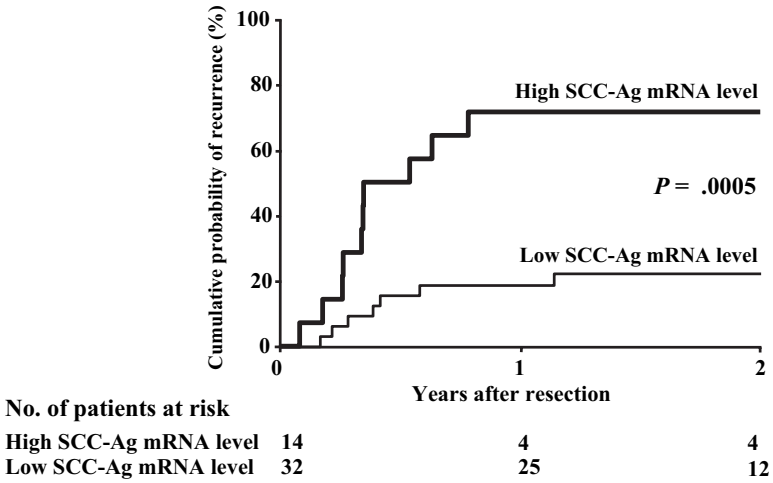

Fig 2. The cumulative probability of recurrence according to preoperative SCC-Ag mRNA level. *SCC-Ag mRNA,* Squamous cell carcinoma-antigen messenger ribonucleic acid.

QRT-PCR was shown to be a significant predictor of recurrence after resection in patients with esophageal SCC in the current study. Kaganoi[4] and Kano[35] both reported similar results using nested RT-PCR, although the technique was noted to be overly "laborious" for use in clinical examinations.[35] However, QRT-PCR is more feasible for clinical use to measure SCC-Ag mRNA levels, as the test can be conducted in approximately 5 hours (RNA extraction, 90 minutes; cDNA synthesis, 80 minutes; QRT-PCR for SCC-Ag mRNA and GAPDH, 140 minutes).

Serum SCC-Ag is a novel test for monitoring recurrence of esophageal SCC. Earlier evidence suggested that the sensitivity and specificity of preoperative serum SCC-Ag for recurrence were approximately 40% to 50% and 60% to 80%, respectively.[25,36] In the current study, the sensitivity and specificity of preoperative SCC-Ag mRNA levels were 59% and 86%, respectively. These findings suggest that the SCC-Ag mRNA level is more sensitive than a serologic test. In the current series, SCC-Ag mRNA, but not serum SCC-Ag, levels was a significant independent predictor for recurrence in patients with esophageal SCC who underwent a curative resection.

The current study has 2 primary limitations. First, the number of patients tested was relatively small. Second, the follow-up period was less than 36 months for 19 of the 46 patients. However, the authors believe that these limitations did not influence markedly the outcome of the study, because the differences be-

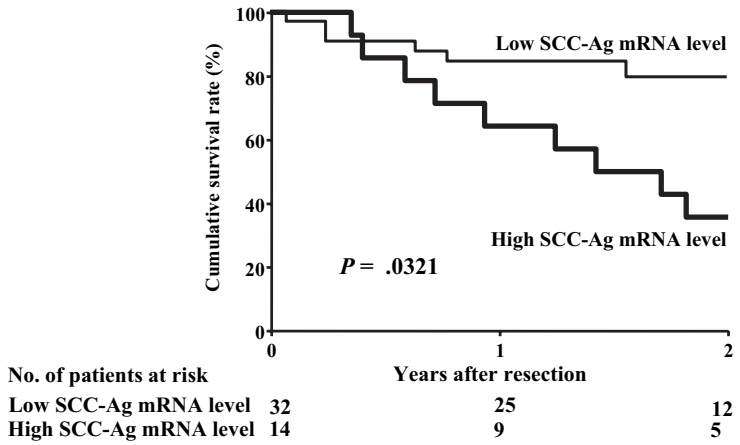

Fig 3. Kaplan-Meier survival estimates according to preoperative SCC-Ag mRNA level. *SCC-Ag mRNA*, Squamous cell carcinoma-antigen messenger ribonucleic acid.

tween the groups were too marked to have resulted from these biases. The current study has several clinical implications. First, the SCC-Ag mRNA level in peripheral blood measured by QRT-PCR could be used to predict recurrence in patients with esophageal SCC, because the measurement can be done before operation. Second, a high SCC-Ag mRNA level appears to be associated with hematogenous metastasis; hence, it is recommend that patients with a high SCC-Ag mRNA level in preoperative peripheral blood undergo a detailed chest-abdominal evaluation and systemic evaluation (e.g. positron emission tomographic scanning, brain computed tomographic scanning) at the time of preoperative assessment or during follow-up after curative esophagectomy.[37–44]

In conclusion, the SCC-Ag mRNA level in peripheral blood is one of the best current predictive factors for recurrence in patients with esophageal SCC who undergo curative, R0 resection.

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