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Original Article

Serum p53 antibody as a potential tumor marker in extrahepatic cholangiocarcinoma

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Abstract

Purpose Only a few studies have evaluated the clinicopathological significance of the p53 protein expression and s-p53-Abs level in patients with cholangiocarcinoma. We therefore analyzed the clinicopathological and prognostic significance of s-p53-Abs in patients with extrahepatic cholangiocarcinoma.

Methods We prospectively evaluated s-p53-Abs levels before and after surgery in 61 patients with extrahepatic cholangiocarcinoma to determine the relationship between clinicopathological factors and the prognostic significance of s-p53-Abs.

Results Among a total of 61 primary extrahepatic cholangiocarcinoma cases, 23% were positive for s-p53-Abs. Combination of s-p53-Abs with the conventional serum markers carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) significantly increased the rate of positive extrahepatic cholangiocarcinoma cases (57% for CEA and/or CA19-9 vs. 75% for CEA and/or CA19-9 and/or s-p53-Abs, P= 0.035). There were no significant differences in clinicopathological factors between the p53-seropositive and p53-seronegative patients. An immunohistochemical analysis showed the presence of significant associations between the intensity (P = 0.003) and extent (P = 0.001) of p53 immunoreactivity and p53-seropositivitly. Although s-p53-Abs was not a significant prognostic factor for the survival in either univariate or multivariate analyses, p53 immunoreactivity was independently associated with a poor survival. Among patients

positive for s-p53-Abs before surgery, the s-p53-Abs levels were reduced after surgery in most.

Conclusion These findings suggested that s-p53-Abs might be associated with p53 immunoreactivity. In addition, s-p53-Abs may be useful for a diagnosis but was not useful for predicting tumor recurrence or the survival. This study was registered as UMIN000014530.

Keywords: autoantibody・cholangiocarcinoma・enzyme-linked immunosorbent assay・p53・tumor marker

Introduction

The early diagnosis of extrahepatic cholangiocarcinoma (ECC) is difficult; therefore, the majority of cases are detected beyond the point of potentially curative therapy [1]. While ultrasonography and/or computed tomography are utilized for the early detection of ECC, serum markers provide substantial clinical benefit for an early diagnosis, follow-up, and the prediction of outcomes in ECC patients. However, carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9), which are currently used as the main serum markers of cholangiocarcinoma [2], are inadequate for early detection.

Studies investigating the molecular pathways underlying ECC have demonstrated the significance of a number of proto-oncogenes and tumor suppressor genes, including p53, in the development of ECC [3-5]. Specifically, we and others have shown that serum p53 antibody (s-p53-Abs) was useful for the early detection of several tumor types, including ECC [6, 7]. Changes in the s-p53-Ab levels were also shown to be useful for follow-up of patients after radical surgery [8, 9]. The presence of s-p53-Abs was reported to correlate with a poor prognosis in a number of tumors with different origins [10-12]. The percentage of cholangiocarcinoma cases positive for s-p53-Abs ranges between 7.3% and 16.7% [6, 13, 14]. However, the clinicopathological significance of s-p53-Abs and/or changes in its levels following surgery in patients with ECC is not well known.

In this study, we prospectively evaluated s-p53-Abs levels before and after surgery in

patients with ECC to determine the relationship between clinicopathological factors and the prognostic significance of s-p53-Abs.

Methods

Collection of serum samples and follow-up after surgery

This study was designed prospectively and registered as UMIN000014530. Of the 61 patients with primary ECC included in this study, 43 (70%) were male, and 18 (30%) were female. The median age was 68 (range, 42–88) years. All patients underwent surgical treatment at Omori Medical Center, Toho University School of Medicine, between January 2010 and October 2015. The TNM classification of patients was determined based on the pathologic evaluation of resected specimens [15]. All patients were considered curable with surgery. Serum samples were obtained before and one month after surgery. Patient recruitment and sample collections were performed within the guidelines of protocols approved by the institutional review board (#22–112, #22–047). Written informed consent was obtained from all patients whose identifying information was included in this study.

After surgery, all patients were followed regularly by a clinical examination and imaging studies either until the end of December 2015 or until death. Postoperative recurrence was defined as positive clinical findings of tumors on successive monthly clinical examinations, by 6-month ultrasonography, and/or by annual computed tomography scans. Recurrent disease was treated according to the standard approaches described previously [16].

Detection of serum p53 antibody and conventional tumor markers by ELISA

S-p53-Abs was assessed using a highly specific and quantitative p53 Ab ELISA kit (MESACUP anti-p53 test; Medical & Biological Laboratories, Nagoya, Japan) [17]. Briefly, samples were added to the wells of a microtiter plate coated with either wild-type human p53 or control protein and were incubated for 1 h. A peroxidase-conjugated goat anti-human immunoglobulin G binding anti-p53 antibody (Ab) was then added and incubated for another hour. Next, the plate was incubated with the substrate solution for 30 minutes, followed by the addition of the stop solution. The colorimetric measurement was immediately performed by absorption at 450 nm using a spectrophotometer. A calibration curve was constructed from specific signals of standards containing specific amounts of antibody. Levels of s-p53-Abs were then determined from the calibration curve. The cut-off value was set at 1.3 U/ml [6].

Immunohistochemical analyses and scoring for p53 expression in tumor specimens

Paraffin-embedded tissue blocks of formalin-fixed biopsy specimens from three different areas of each case were processed for a conventional histological assessment by hematoxylin and eosin (H&E) staining and an immunohistochemical analysis by the avidin-biotin-peroxidase complex (ABC) method [18]. p53 protein was detected by anti-p53 monoclonal Ab (clone DO-7; Dako, Carpenteria, CA, USA) using the conventional peroxidase method [19]. Briefly, 4-mm-thick sections were deparaffinized in xylene, dehydrated through graded alcohol

concentrations, and incubated in citrate buffer (pH 6.0) for 5 minutes using a microwave oven at 800 W. Slides were then allowed to cool to room temperature before a brief wash with PBS and incubation with 3% H_2O_2 in methanol for 15 minutes to block endogenous peroxidase activity. Biotinylated anti-cocktail of mouse/rabbit antibody (Dako) at a dilution of 1: 500 was used as the secondary antibody. After three washes with PBS, sections were incubated with ABC (Dako), and diaminobenzidine was used for visualization. p53 immunoreactivity was scored as described previously by determining staining intensity as negative (0), weak (1), medium (2), or strong (3) [20]. Stained tissue sections were also reviewed to determine the extent of positively stained areas in relation to the whole carcinoma in a given specimen using the scoring system described by Sheng et al. [7], in which sections were scored as 0 (0%), 1 (1%–25%), 2 (26%–50%), 3 (51%–75%), or 4 (76%–100%). The immunohistochemical analysis was evaluated by the co-authors T. N. and R. O.

Statistical analyses

Comparisons between paired groups were performed with non-parametric analyses. The survival probabilities were calculated by the product limit method of Kaplan and Meier to estimate the survival from the time of surgery. Differences between groups were determined using the log-rank test. Clinicopathological parameters associated with the overall survival were evaluated by univariate and multivariate analyses using the Cox proportional hazards

model. All statistical analyses were performed with the JMP® statistical software program (SAS Institute, Cary, NC, USA), and *P* values <0.05 were considered statistically significant.

RESULTS

Rate of cases with positive serum p53 antibody, CEA, and CA19-9 in extrahepatic cholangiocarcinoma

Among patients with ECC, the rates of tumors that were positive for s-p53-Abs, CEA, and CA19-9 were 23%, 13%, and 54%, respectively. The rate of s-p53-Abs positivity was higher than that of CEA positivity but lower than that of CA19-9 positivity among patients with ECC. Based on these initial observations, we proceeded with the evaluation of the clinicopathological significance of s-p53-Abs in ECC in subsequent analyses.

As s-p53-Abs was usually independent of conventional serum markers, we hypothesized that using s-p53-Abs in combination with conventional serum markers would increase the rate of positivity for these markers. Thus, among patients with ECC, we assessed the percentage of cases positive for any two of the three serum markers tested in this study. As seen in Fig. 1, while combinations including CEA significantly increased the rate of positive tumors from 13% (8/61) to 31% (19/61) (P = 0.016), combinations including CA19-9 did not significantly change the rate of marker positivity, increasing from 54% (33/61) to 62% (38/61) (P = 0.359). Furthermore, the rates of cases that were positive for CEA and/or CA19-9 significantly increased from 57% (35/61) to 75% (46/61) (P = 0.035) when s-p53-Ab was added to the panel of these markers.

In addition, we found that there were no significant differences in any of the

clinicopathological features between p53-seropositive (i.e. detectable s-p53-Ab levels) and p53-seronegative patients with ECC (Table 1). While the rate of cases positive for s-p53-Ab was higher in patients with stage III/IV ECC than that in patients with stage I/II disease (40% vs. 20%, $P = 0.183$), this was not statistically significant.

Associations between p53 immunoreactivity and the presence and levels of serum p53 antibody

A total of 61 ECC cases were divided into two groups based on the intensity scores (0–1 and 2–3) as well as the extent of p53 immunoreactivity (scores, 0–2 and 3–4) in resected specimens to determine their association with p53 seropositivity. Analyses revealed significant associations between intensity scores ($P = 0.003$) as well as the extent ($P = 0.001$) of p53 immunoreactivity and p53 seropositivity. As shown in Table 2, p53 intensity scores and the percentage of p53-positive cells in surgically resected specimens were significantly higher in p53-seropositive patients than in those seronegative for p53 (Table 2). Furthermore, both a high intensity score and high proportional score were significantly associated with higher s-p53-Ab titers. These findings indicated the presence of an association between s-p53-Abs levels and the intensity as well as the extent of tissue p53 immunoreactivity.

Prognostic role of serum p53 antibody and p53 immunoreactivities in patients with

extrahepatic cholangiocarcinoma

The median follow-up of survivors included in this study was 24 months (range from 0.4month to 67 months). Forty (66%) patients were alive at the end of the study in December 2015, whereas the remaining 21 (42%) patients were dead or lost to follow-up. Although none of the clinicopathological factors assessed were significantly associated with the overall survival in a univariate analysis, the lack of any adjuvant chemotherapy or an intensity score of 2-3 or proportion score of 3-4 was associated with a particularly poor prognosis (Table 3). Although s-p53-Abs was not a significant prognostic factor for the survival in either univariate or multivariate analyses, p53 immunoreactivities were independently associated with a poor survival (Table 3). A comparison of the overall survival curves according to the preoperative status of serum markers is shown in Fig. 2.

Perioperative monitoring of serum p53 antibody levels

In the present study, 10 of the 14 p53-seropositive patients were monitored for s-p53-Abs levels 1 month after surgery. We found that the s-p53-Abs levels were decreased in 9 of the patients after surgery. Albeit not to a statistically significant degree, the s-p53-Abs levels tended to be lower in the postoperative period than in the preoperative period (12.6 ± 3.1 U/ml vs. 5.6 ± 5.7 U/ml; $P = 0.128$). Among the 10 patients that were monitored, 4 developed recurrent disease (dotted lines, recurrent cases; solid lines, relapse-free cases; Fig.

3). The mean rate of reduction in s-p53-Abs levels was 43.3% (0%–87%) at 1 month after surgery. The mean rates of reduction in s-p53-Abs levels were 34% (0%–64%) and 57% (36%–87%) among the 6 relapse-free and 4 recurrent patients, respectively.

Among the 10 patients who were followed-up, 5 became p53-seronegative after several months (median 3 months, range from 1 month to 9 months); of these, 4 patients were alive without recurrence at the end of the present study. Three of the five p53-seropositive patients developed recurrent disease, whereas the remaining two survived without recurrence of ECC. In these two patients, the s-p53-Abs levels were consistently decreased even at one month after surgery. In one relapse-free representative case, long-term monitoring revealed that the s-p53-Abs levels decreased from 10.2 to 4.1 U/ml at 1 month after surgery. This patient achieved p53 seroconversion at nine months after surgery and did not have recurrence of disease on evaluation three years after surgery.

Discussion

The aim of the present study was to evaluate the clinical utility of s-p53-Abs as a serological marker in patients with ECC. We therefore evaluated serum samples for s-p53-Abs levels before and after surgery. The overall rate of s-p53-Abs positivity in ECC was 23%. In agreement with the findings of a study by Muller et al., we confirmed that the combination of s-p53-Abs with conventional tumor markers significantly increased the rate of positivity for serum tumor markers [21-23]. Our findings suggested that s-p53-Abs, as a marker independent from conventional tumor markers, was useful for identifying patients with ECC who were negative for conventional tumor markers. We showed that the s-p53-Abs level was significantly associated with the intensity as well as the extent of p53 immunoreactivity in ECC surgically resected specimens. There were several cases in which the serum p53 antibody status and p53 immunoreactivity did not match. Indeed, 11 of 21 patients with p53 protein over-expression did not show any serum antibodies. This discrepancy may be due in part to the host immunity and mutation status. Previous reports have also shown that only two thirds of p53 protein over-expressing tumors induced serum p53 antibodies [24,25].

The prognosis of ECC was generally poor due to an advanced stage at the diagnosis. Wellner et al. reported that perineural invasion, lymph node metastasis, positive resection margin status, and not-well-differentiated adenocarcinoma were associated with a shortened survival for distal cholangiocarcinoma [26]. While previous studies have shown that

s-p53-Abs was associated with the prognosis in esophageal, lung, and colon cancer [9, 27, 28, 29], such associations between the three markers of s-p53-Abs, CEA, and CA19-9 and the disease prognosis were not detected in patients with ECC in the current study. In fact, the small number of studies investigating the utility of s-p53-Abs as a prognostic marker in ECC reported results similar to our present findings [30]. Previous reports have shown that extremely high levels of s-p53-Abs of >10 IU/ml were associated with a high risk of recurrence in patients with esophageal carcinoma [10]. However, in the current cohort of ECC, we detected no such tendency, possibly due to the number of patients with high levels of s-p53-Abs >10 IU/ml. According to an ROC curve predicting patients' outcomes, the cut-off level of s-p53-Ab for prognostic value was 0.44 U/ml (supplemental data). However, the area under the curve was 0.603, the sensitivity 0.808, and the specificity 0.457. Therefore, such cut-off values seemed to be inappropriate for further analyses. Furthermore, when the cut-off value was set at 2, 5, or 10, no prognostic significance of s-p53-Abs was observed.

In this study, although s-p53-Abs was not found to be a risk factor for a reduced survival, p53 immunoreactivities, an intensity score of 2-3, and a proportion score of 3-4 were independently associated with a poor prognosis. Although previous reports described a relationship between the intensity or proportion score and s-p53-Abs, those reports did not note any relationship between p53 immunoreactivities and the survival. Based on our present findings, we suspected that p53 immunoreactivities, but not s-p53-Abs, were associated with

a high risk of recurrence and a poor survival.

In p53-seropositive patients who are negative for other tumor markers, s-p53-Abs might be useful for the monitoring of potential recurrence after surgery. Postoperative p53-seropositive patients may have residual tumor cells and be more likely to develop recurrent disease than seronegative patients. Although we were unable to statistically confirm an association between the relapse-free survival and the rate of reduction in s-p53-Abs levels, a p53 seropositive status at one month after surgery suggested tumor recurrence. Regarding the association between postoperative seropositivity and tumor recurrence [31], half of the s-p53-Abs-positive patients developed recurrent tumors. In contrast, only 25% of the CEA-positive patients and 35% of CA19-9-positive patients developed recurrent tumors. Furthermore, long-term monitoring of s-p53-Abs levels beyond the first month after surgery might be a more precise predictive tool for the identification of patients at high risk for disease recurrence. A previous report of a patient with superficial esophageal adenocarcinoma showed that monitoring s-p53-Abs levels for perioperative changes was useful in the detection of residual tumor cells [32].

In conclusion, the s-p53-Abs level was significantly associated with p53 immunoreactivity of cancer tissue. Because s-p53-Abs was independent of CEA and CA19-9, the overall positive rate increased when s-p53-Abs was used in combination with CEA and CA19-9 for the detection of ECC. S-p53-Abs may be useful as a potential tumor marker for a

diagnosis but was not useful for predicting tumor recurrence or the survival.

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DISCLOSURES

Hideaki Shimada received research grants and technical lecture fees from Medical & Biological Laboratories Co., Ltd., Nagoya, Japan. The other authors declare that they have no conflicts of interest.

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FIGURE LEGENDS

Figure 1. A comparison of the rates of positivity for carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9), alone or in combination with serum p53 antibody, in patients with extrahepatic cholangiocarcinoma.

Figure 2. The overall survival curves of patients with extrahepatic cholangiocarcinoma according to the preoperative serum tumor marker status. (a) Serum p53 antibody, (b) carcinoembryonic antigen (CEA), and (c) carbohydrate antigen 19-9 (CA19-9).

Figure 3. The perioperative changes in serum p53 antibody levels in patients with extrahepatic cholangiocarcinoma. Dotted lines indicate patients with recurrent disease (n = 4), and solid lines represent patients without relapse (n = 6) at the time of the evaluation 1 month after surgery.

Fig. 1.

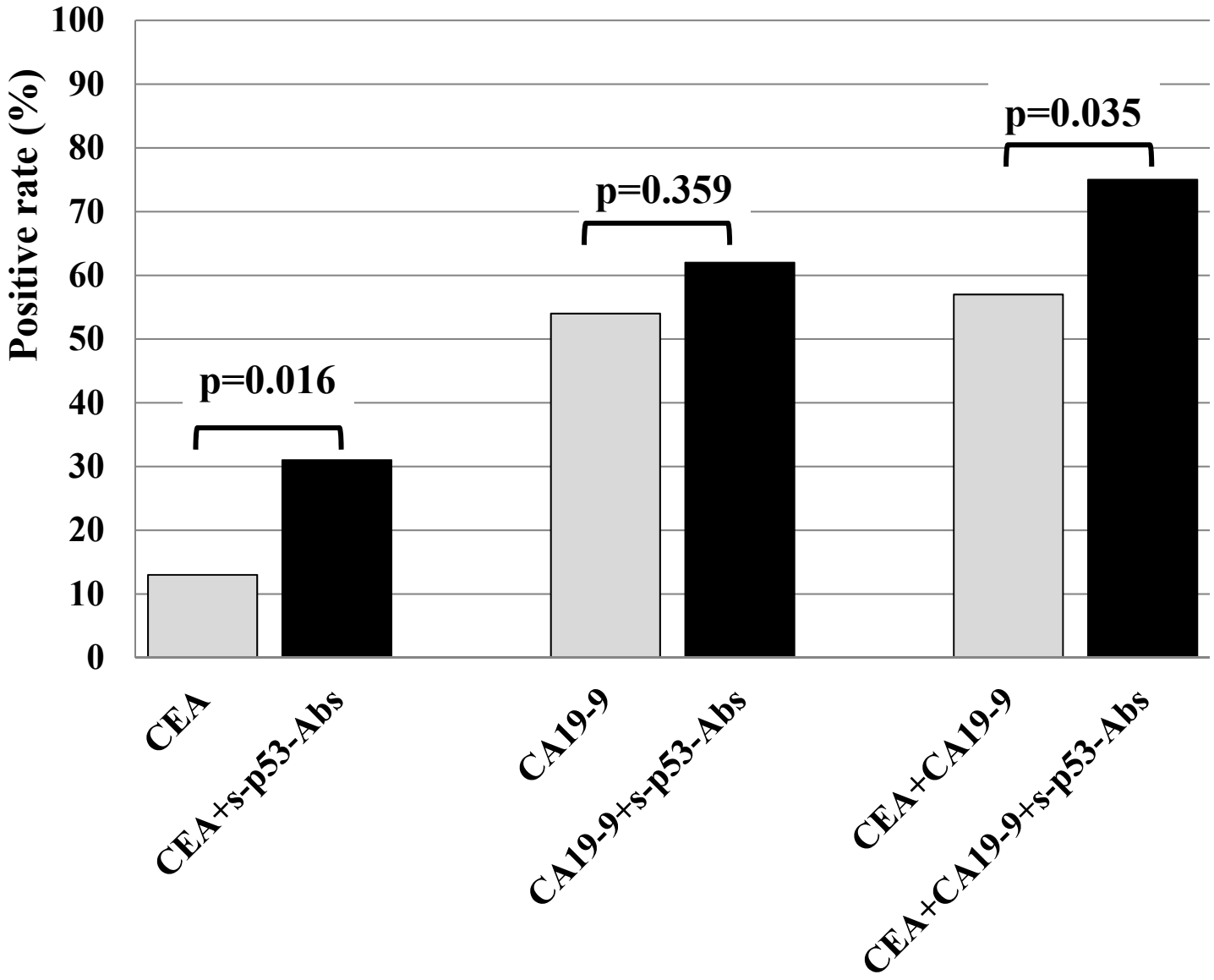

Positive rate (%)
100
90
80
70
60
50
40
30
20
10
0
p=0.016
p=0.359
p=0.035
CEA
CEA+s-p53-Abs
CA19-9
CA19-9+s-p53-Abs
CEA+CA19-9
CEA+CA19-9+s-p53-Abs

Fig. 2.

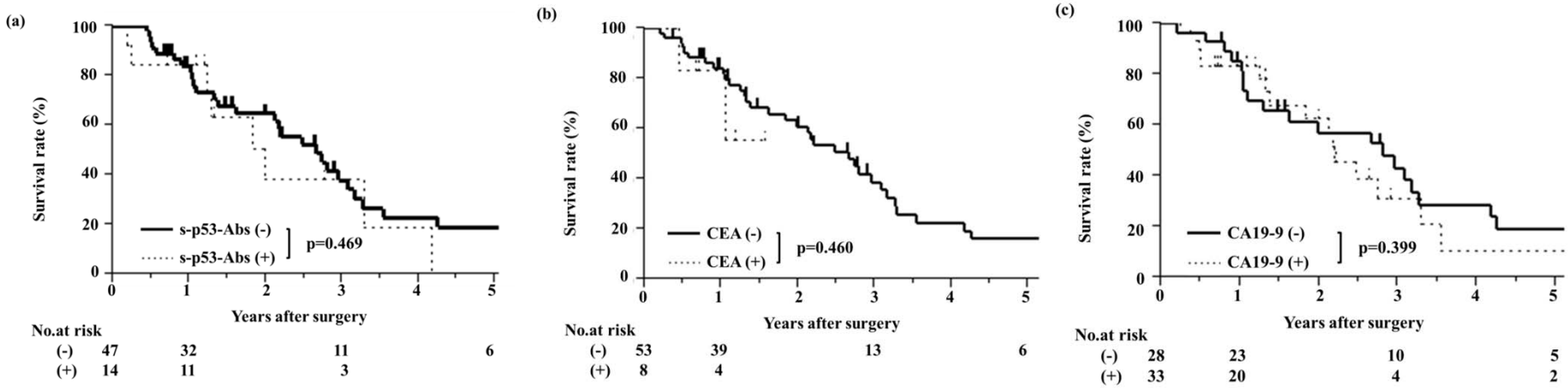
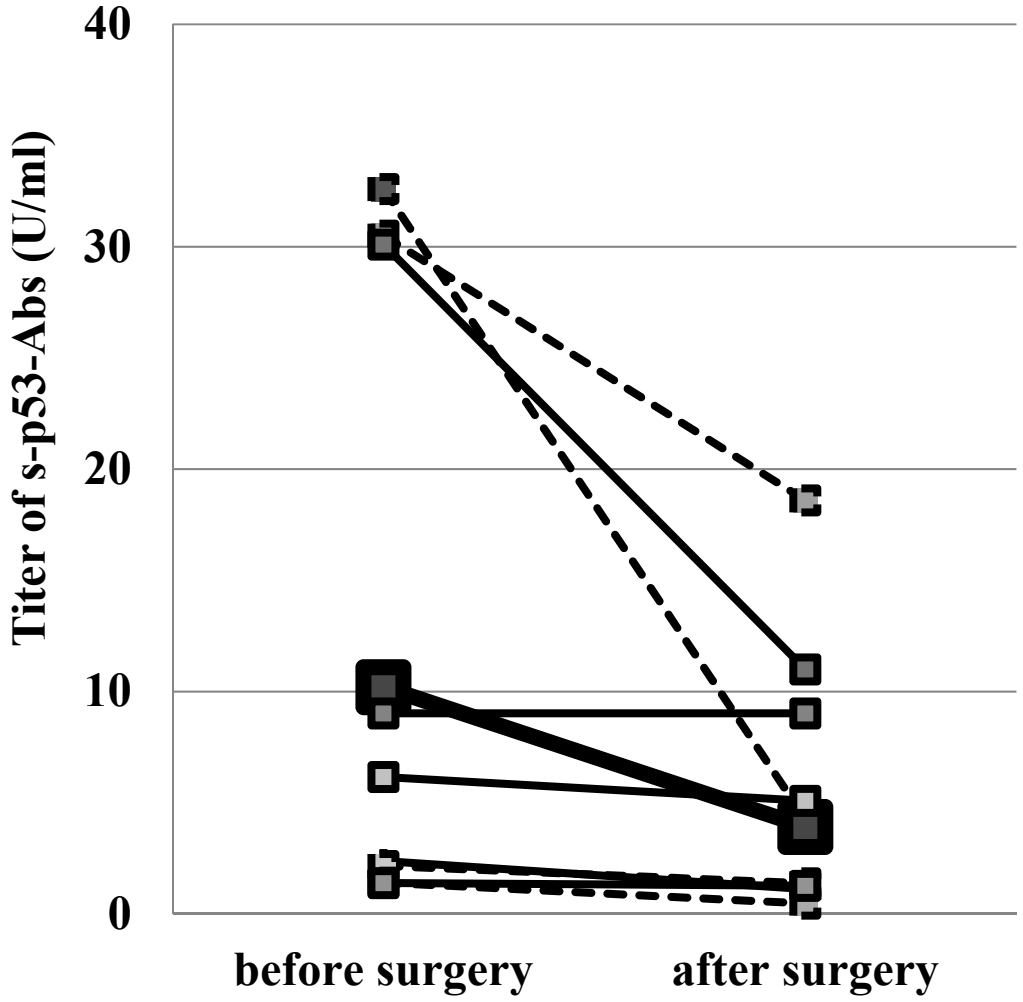
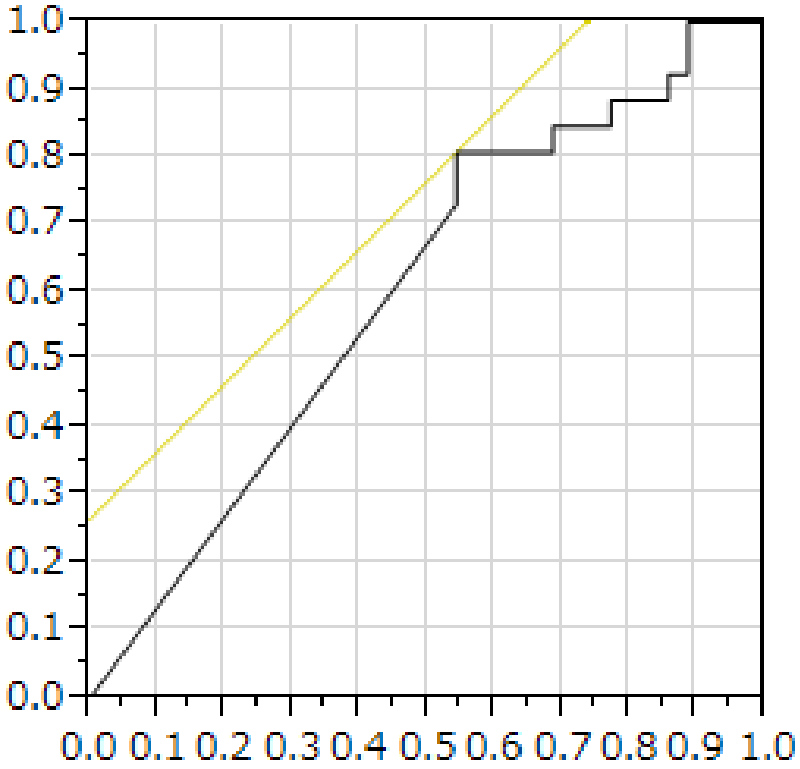

(a)
Survival rate (%)
100
80
60
40
20
0
0
1
2
3
4
5
Years after surgery
s-p53-Abs (-)
s-p53-Abs (+)
p=0.469
No.at risk
(-) 47 32 11 6
(+) 14 11 3
(b)
Survival rate (%)
Years after surgery
CEA (-)
CEA (+)
p=0.460
No.at risk
(-) 53 39 13 6
(+) 8 4
(c)
Survival rate (%)
Years after surgery
CA19-9 (-)
CA19-9 (+)
p=0.399
No.at risk
(-) 28 23 10 5
(+) 33 20 4 2

Fig. 3.


40
30
20
10
0
Titer of s-p53-Abs (U/ml)
before surgery
after surgery

Supplemental data



Area under the Curve: 0.603
Sensitivity: 0.808
Specificity: 0.457

Table 1. Comparisons of the clinicopathological characteristics and conventional serum markers of patients with serum p53 antibodies of extrahepatic cholangiocarcinoma.

Variables	Total number	s-p53-Abs(+) (n=14)	s-p53-Abs (-) (n=47)	P value [a]
Gender	Male (43)	9	34	0.567
	Female (18)	5	13	
Age	≤65 years (27)	6	21	0.904
	≥65 years (34)	8	26	
Operation	PD	8	39	0.152
	Extrahepatic duct resection	1	1	
	Hepatectomy	5	7	
TNM stage	I+II (51)	10	41	0.183
	III+IV (10)	4	6	
Tumor depth	T1T2 (28)	5	23	0.380
	T3T4 (33)	9	24	
Nodal status	Negative (38)	7	31	0.285
	Positive (23)	7	16	
Chemotherapy	Negative (28)	6	22	0.795
	Positive (33)	8	25	
CEA	Negative (53)	11	42	0.317
	Positive (8)	3	5	
CA19-9	Negative (28)	5	23	0.380
	Positive (33)	9	24	

a. Fisher's exact test.

Table 2. The association of p53 protein expression in the tumors and serum p53 antibodies of patients with extrahepatic cholangiocarcinoma.

Variables		Total (n=61)	s-p53-Abs (+) (n=14)	s-p53-Abs (-) (n=47)	P value [a]
Intensity score	0-1	34	3	31	0.003
	2-3	27	11	16	
Proportion score	0-2	40	4	36	0.001
	3-4	21	10	11	

a. Fisher's exact test.

Table 3. Univariate and multivariate analyses of prognostic variables in patients with extrahepatic cholangiocarcinoma.

Variables		Univariate P value [a]	H.R.[b]	95%CI [c]	Multivariate P value [d]
Gender	Female	0.918	0.987	0.438-2.143	0.975
	Male				
Age	≤65 years	0.736	0.858	0.411-1.807	0.683
	>65 years				
Tumor size	≤30 mm	0.808	0.634	0.298-1.369	0.242
	>30 mm				
Tumor depth	T1T2	0.617	0.828	0.382-1.731	0.619
	T3T4				
Lymph node metastasis	Negative	0.348	0.412	0.141-1.086	0.074
	Positive				
Peritoneal metastasis	Negative	0.132	0.163	0.028-1.393	0.090
	Positive				
Chemotherapy	Negative	0.012	2.217	1.174-4.262	0.014
	Positive				
Intensity score	0-1	0.399	0.248	0.039-0.893	0.031e
	2-3				
Proportion score	0-2	0.584	0.235	0.034-0.938	0.039e
	3-4				
s-p53-Abs	Negative	0.469	0.542	0.222-1.415	0.202e
	Positive				
CEA	Negative	0.460	0.840	0.186-6.144	0.841
	Positive				
CA19-9	Negative	0.399	0.525	0.232-1.155	0.110
	Positive				

a. Log-rank test.
b. Adjusted hazards ratio
c. Adjusted 95% confidence interval
d. Cox proportional hazards model
e. These variables were analyzed independently.