Clinical significance of serum anti-p53 antibody expression following curative surgery for colorectal cancer

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Abstract. The aim of the present study was to investigate the usefulness of serum anti-p53 antibody (Ap53Ab) measurement for the diagnosis of colorectal cancer (CRC), and the clinical significance of the association between Ap53Ab expression and survival rate. Ap53Ab, carcinoembryonic antigen (CEA) and carbohydrate antigen (CA)19-9 were measured by ELISA in 674 CRC patients and 115 healthy volunteers (control group). The half-life time of Ap53Ab and CEA was calculated. The association between positive Ap53Ab expression and clinicopathological characteristics, including survival rate, was analyzed. Of the 674 CRC patients, 195 (28.9%) were positive for Ap53Ab expression, while the positive rates of CEA and CA19-9 level were 39.9 and 16.9%, respectively. Positivity for Ap53Ab alone was observed in 94 patients (13.9%), whereas the positivity rate of any markers examined was 58.7%. The mean half-life of Ap53Ab and CEA was 30.7 and 11.3 days, respectively. Positive expression of Ap53Ab was significantly associated with the depth of tumor invasion (P<0.001), lymph node metastasis (P=0.024), stage (P<0.001) and CEA level (P=0.005). No significant correlation between Ap53Ab expression and poor survival rate was observed. The positive rate of Ap53Ab was higher compared with that of CEA and CA19-9 in early-stage CRC. The combination of these markers improved the diagnostic yield of CRC up to ~60%. Furthermore, Ap53Ab expression was associated with lymph node metastasis, but not with shorter survival. These results indicated that the measurement of Ap53Ab may contribute to increased rate of detection of CRC, particularly in patients with early-stage disease, in clinical practice.

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Introduction

Colorectal cancer (CRC) is the most common cancer and the second leading cause of cancer-related mortality in Japan. Tumor markers, including carcinoembryonic antigen (CEA) and carbohydrate antigen (CA)19-9, have been used for the screening of CRC in clinical practice. These markers are useful for monitoring cancer recurrence and the efficacy of chemotherapy in advanced CRC patients with positive expression of CEA or CA19-9. However, the sensitivity of these markers is very low in early-stage CRC. Therefore, the development of novel molecular markers is required, using blood testing as a non-invasive method. The serum anti-p53 antibody (Ap53Ab) has been applied as a novel tumor marker for the detection of several cancers, including esophageal cancer (1,2), breast cancer (1,3) and CRC (1,4,5), from 2007 onwards in Japan. Mutations of the *TP53* gene, which were detected in half of CRC cases (6), were strongly associated with carcinogenesis. The accumulation of mutated-TP53 protein induces the synthesis of Ap53Ab, depending on the condition of the host immune system (7).

Previous reports (1,4-6,8,9) have demonstrated that positive Ap53Ab expression was detected in 24.0-33.1% of CRC patients. These reports suggested that the measurement of Ap53Ab was useful for the screening of CRC patients with early-stage (stage 0 and I) disease, as the positive rate of Ap53Ab expression was higher compared with that of CEA and CA19-9. However, a consensus on the correlation between Ap53Ab expression and poor survival rate was not obtained (4,8,10). The aim of the present study was to investigate the positive rate and clinical significance of Ap53Ab expression in 674 CRC patients, and elucidate the association between Ap53Ab expression and survival.

Patients and methods

Patients and clinical procedures. The subjects included 674 CRC patients (237 women and 437 men), who were

primarily diagnosed with CRC at the Saitama Medical Center (Kawagoe, Japan) between January 2010 and December 2014. A total of 115 healthy volunteers were also selected among patients who had been diagnosed with hemorrhoids or inguinal hernia at the Saitama Medical Center between October 2008 and July 2010. The median age of CRC patients and healthy volunteers was 69 years (range, 25-92 years) and 58 years (range, 16-84 years), respectively. Of the 674 patients investigated, the cancer localization was as follows: Cecum, n=45; ascending colon, n=99; transverse colon, n=56; descending colon, n=24; sigmoid colon, n=173; and rectum, n=277. The histological diagnosis was well-differentiated adenocarcinoma in 153 patients, moderately differentiated adenocarcinoma in 463, poorly differentiated adenocarcinoma in 32, mucinous adenocarcinoma in 17, and other types in 8 patients. The carcinomas at the time of primary tumor resection were staged according to the Union for International Cancer Control classification (11) as follows: Stage 0, n=38; stage I, n=130; stage II, n=206; stage III, n=194; and stage IV, n=106. Of the 194 patients with stage III CRC, 107 (55.2%) received mFOLFOX6 or XELOX chemotherapy and 32 patients (16.5%) received capecitabine or tegafur-uracil and leucovorin (UFT/LV) for 6 months as postoperative adjuvant chemotherapy. The mFOLFOX6 regimen comprises intravenous infusion of oxaliplatin (85 mg/m^2) and LV (200 mg/m^2) for 2 h, followed by rapid intravenous bolus infusion of 5-fluorouracil (5-FU; 400 mg/m^2) for 5 min, and continuous intravenous infusion of 5-FU (2,400 mg/m^2) for 46 h. This regimen was repeated every 2 weeks. The XELOX regimen was administered as follows: Oxaliplatin (130 mg/m^2) was injected intravenously. From day 1 to day 14, capecitabine (2,000 mg/m^2/day) was orally administered. Each cycle was repeated every 3 weeks. The capecitabine or UFT/LV group received 8 cycles of oral capecitabine (2,400 mg/m^2 for 14 days followed by a 7-day rest per cycle), or 5 cycles of adjuvant UFT/LV (UFT 300 mg/m^2 and LV 75 mg/day for 28 days followed by a 7-day rest per cycle), respectively.

The present study was performed in accordance with the ethical guidelines for clinical research with the approval of our Institutional Ethics Committee. Informed consent was obtained from all individuals included in the study.

Measurement of Ap53Ab, CEA and CA19-9. Total blood samples were routinely collected from CRC patients and healthy volunteers at the time of diagnosis. The serum samples were processed using the MESACUP anti-p53Ab Test ELISA kit (MBL, Nagoya, Japan). The cut-off level of the serum Ap53Ab was set to 1.3 U/ml according to the manufacturer's instructions. The minimum value of Ap53Ab was reported at <0.69 U/ml; thus, values <0.69 were set to 0.69 for the analysis. The measurement of serum CEA and CA19-9 was also performed using ELISA. The normal level of CEA and CA19-9 was <6.7 ng/ml and <37.0 U/ml, respectively.

Statistical analysis of Ap53Ab and CEA half-life. The half-life of Ap53Ab and CEA was calculated based on the following formula: T=loge2xt/(loge N0-loge N)=0.693xt/2.303(log N0-log N), where T, half-life time (days); t, days from the operation to next blood testing; N0, preoperative value of Ap53Ab or CEA; and N, postoperative value of Ap53Ab or CEA.

Of the Ap53Ab-positive patients with values >10 U/ml, 39 patients were selected. In addition, 19 patients with a CEA level elevated to >20 ng/ml were selected. These CRC patients underwent curative surgery, with no evidence of recurrence during the 5-year follow-up. The measurement of Ap53Ab and CEA was performed within 60 days after surgery.

Statistical analysis. The Mann-Whitney U-test, Fisher's exact probability test and Chi-squared test were used where applicable. Survival analysis was conducted using the Kaplan-Meier method. The log-rank test was used to determine the significance of the survival curves. The period of disease-free survival (DFS) was calculated from the time of surgery to the time to recurrence and overall survival (OS) was calculated from the time of surgery to death from any cause. DFS and OS were censored at the time of the last visit to our hospital, or December 2015, whichever came first. Differences were considered statistically significant when P<0.05. All statistical analyses were performed using a statistical software package (StatFlex ver.6.0; Artec, Osaka, Japan).

Results

Positive rates of tumor markers, including Ap53Ab, CEA and CA19-9. Of the 674 CRC patients, 195 (28.9%) were positive for Ap53Ab, while 12 positive cases (10.4%) were identified in the control group (Table I). The mean level ± standard deviation (SD) of Ap53Ab in CRC patients and the control group was 26.0±132.0 and 1.24±2.42 U/ml, respectively (Table I). There difference in the Ap53Ab level between CRC patients and the control group was significant (P<0.0001; Table I).

The positive rates of Ap53Ab, CEA, and CA19-9 in each CRC stage were as follows: Stage 0: 5.3, 10.5 and 7.9%, respectively; stage I: 20.0, 13.1 and 12.3%, respectively; stage II: 31.6, 38.3 and 12.1%, respectively; stage III: 34.0, 43.8 and 12.4%, respectively; and stage IV: 34.0, 79.2 and 43.4%, respectively (Table II). Positivity for Ap53Ab alone was observed in 94 patients (13.9%; Table II). The positive rate of any examined markers was 58.7% (Table II).

Half-life time of Ap53Ab and CEA. The mean ± SD half-life of Ap53Ab was 30.7±27.2 days (range, 8.5-118.1 days) in 39 CRC patients, while that of CEA was 11.3±4.4 days (range, 4.4-21.8 days) in 19 CRC patients. Of the 39 patients with elevated Ap53Ab level, the level returned to normal within 1 year in only 4 patients (10.3%).

Association between Ap53Ab expression and clinicopathological characteristics and prognosis. Positive expression of Ap53Ab was significantly associated with the depth of tumor invasion (P<0.001), lymph node metastasis (P=0.024), stage (P<0.001) and CEA level (P=0.005) (Table III). There was no association of Ap53Ab expression with gender, age, tumor location, histology, lymphatic invasion, venous invasion, liver metastasis or recurrence (Table III).

Regarding DFS in CRC patients with stage I-III disease, no significant difference was observed between patients with

Table I. Ap53Ab level and positive rate in colorectal cancer patients and healthy control group.

Groups	n	Positive expression, n (%)	Mean ± SD (U/ml)	P-value
Control	115	12 (10.4)	1.24±2.42	<0.0001
Colorectal cancer	674	195 (28.9)	26.0±132.0	

Ap53Ab, anti-p53 antibody; SD, standard deviation.

Table II. Positive rate of tumor markers in 674 colorectal cancer patients.

	Stage, n (%)					
Markers	0 (n=38)	I (n=130)	II (n=206)	III (n=194)	IV (n=106)	Total (n=674)
Ap53Ab	2 (5.3)	26 (20.0)	65 (31.6)	66 (34.0)	36 (34.0)	195 (28.9)
CEA	4 (10.5)	17 (13.1)	79 (38.3)	85 (43.8)	84 (79.2)	269 (39.9)
CA19-9	3 (7.9)	16 (12.3)	25 (12.1)	24 (12.4)	46 (43.4)	114 (16.9)
Ap53Ab alone	1 (2.6)	20 (15.4)	33 (16.0)	32 (16.5)	8 (7.5)	94 (13.9)
Any marker	7 (18.4)	49 (37.7)	120 (58.3)	123 (63.4)	94 (88.7)	393 (58.7)

Ap53Ab, anti-p53 antibody; CEA, carbohydrate antigen; CA19-9, carbohydrate antigen 19-9.

positive Ap53Ab expression (n=157) and those with negative Ap53Ab expression (n=373) (P=0.86, Fig. 1). Moreover, no association between Ap53Ab expression and OS was observed (P=0.44, Fig. 2).

Discussion

In the present study, positive Ap53Ab expression was detected in 28.9% of 674 CRC patients. Previous studies (1,4-6,8,9) reported a positive Ap53Ab expression rate of 24.0-33.1% in CRC patients. Our data were consistent with those reports in terms of the positive Ap53Ab rate. Previous reports (4,5) suggested that the measurement of Ap53Ab was useful for the screening of CRC patients with early-stage (stage 0 and I) disease, since the positive rate of Ap53Ab expression was higher compared with that of CEA and CA19-9. There was an advantage of Ap53Ab for the detection of CRC patients with stage I disease, while the positive rate was low in CRC patients with stage 0 disease. When stage 0 and I patients were collectively analyzed, the positive rates of Ap53Ab, CEA and CA19-9 expression were 16.7, 12.5 and 11.3%, respectively. The number of studies investigating Ap53Ab expression in a large population of stage 0 and I CRC patients is limited. A recent report (4) demonstrated that positive Ap53Ab expression was detected in 72 (15.1%) of 478 CRC patients with stage 0 and I disease. Another study (5) reported that 9 of 38 CRC patients (23.7%) with stage I disease exhibited positive Ap53Ab expression. In our series, the positive rate of Ap53Ab expression in stage 0 and I and only stage I cases was 16.7 and 20.0%, respectively, while that of CEA in stage 0 and I and only stage I cases was 12.5 and 13.1%, respectively. Therefore, the positive rate of Ap53Ab expression was higher compared with that of CEA in CRC patients with early-stage disease. Of note, one in three CRC patients with stage 0 and I disease may be detected using the combination of Ap53Ab, CEA and CA19-9. Of 168 patients with stage 0 and I disease, any one of these markers was positive in 56 patients (33.3%). Since measurement of CEA alone was able to detect 12.5% of CRC cases, the detection rate with measurement of all markers is approximately three times higher compared with that of CEA alone.

The positive rate of Ap53Ab expression in stage II, III, and IV CRC is similar (31.6, 34.0 and 34.0%, respectively). A similar result was also reported by Yamaguchi *et al* (4). Thus, it was hypothesized that repeated exposure to the p53 antigen may have reduced the production of Ap53Ab in the serum via induction of immunological tolerance (12). Positive expression of Ap53Ab alone was observed in 94 patients (13.9%). This rate was similar with previously reported rates of 13.6 and 15% (4,5). Consequently, the combined measurement of Ap53Ab, CEA and CA19-9 improved the diagnosis of CRC up to ~60%.

Based on a previous study (1), the cut-off value was defined as <1.3 U/ml. The upper values of Ap53Ab were 4.39 and 16.9 U/ml in 205 healthy control donors and 189 patients with benign disease, respectively (1). In the present study, positive Ap53Ab expression was observed in 12 (10.4%) of 115 healthy volunteers using the same detection method. The range of the Ap53Ab level in those volunteers was 1.5-19.5 U/ml. The 12 volunteers with positive Ap53Ab expression were followed up for ~5 years, and there was no cancer occurrence during that time. Previous reports indicated that false-positives may occur as a result of a severely dysplastic gastric mucosa (13), or lung tissues with squamous metaplasia and dysplasia (14). When the Ap53Ab value is as high as 20 U/ml, a thorough physical examination should be

Table III. Clinicopathological correlation of Ap53Ab expression in CRC.

Characteristics	Ap53Ab expression, n (%) Positive (n=195)	Negative (n=479)	P-value
Gender			0.33
Male	121 (62.1)	316 (66.0)	
Female	74 (37.9)	163 (34.0)	
Age (years)			0.42
<70	104 (53.3)	239 (49.7)	
≥70	91 (46.7)	240 (50.3)	
Tumor location			0.53
Right	55 (28.2)	145 (30.3)	
Left	63 (32.3)	134 (28.0)	
Rectum	77 (39.5)	200 (41.8)	
Differentiation			0.16
High	34 (17.4)	119 (24.8)	
Moderate	144 (73.9)	319 (66.6)	
Poor	11 (5.6)	21 (4.4)	
Mucinous	6 (3.1)	11 (2.3)	
Others	0 (0.0)	8 (1.9)	
Depth			**<0.001**
Tis	2 (1.0)	36 (7.5)	
T1	13 (6.7)	64 (13.4)	
T2	24 (12.3)	59 (12.3)	
T3	103 (52.8)	207 (43.2)	
T4	8 (4.1)	103 (21.5)	
Unknown	4 (2.1)	010 (2.1)	
Lymphatic invasion			0.1
Absent	79 (40.5)	227 (47.4)	
Present	111 (56.9)	240 (50.1)	
Unknown	5 (2.6)	12 (2.5)	
Venous invasion			0.15
Absent	59 (30.2)	173 (36.1)	
Present	131 (67.2)	295 (61.6)	
Unknown	5 (2.6)	11 (2.3)	
Lymph node metastasis			**0.024**
Negative	99 (50.8)	289 (59.8)	
Positive	93 (47.7)	184 (38.1)	
Unknown	3 (1.5)	10 (2.1)	
Liver metastasis			0.28
Absent	169 (86.7)	429 (89.6)	
Present	26 (13.3)	50 (10.4)	
UICC stage			**<0.001**
0	2 (1.0)	36 (7.5)	
I	26 (13.3)	104 (21.8)	
II	65 (33.3)	141 (29.4)	
III	66 (33.9)	128 (26.7)	
IV	36 (18.5)	70 (14.6)	
CEA			**0.005**
≤6.7	101 (51.8)	303 (63.3)	
>6.7	94 (48.2)	175 (36.5)	
Unknown	0 (0.0)	1 (0.2)	
Recurrence			0.45
Absent	139 (87.4)	368 (90.0)	
Present	18 (11.3)	38 (9.3)	
Unknown	2 (1.3)	3 (0.7)	

Bold print indicates statistical significance. CRC, colorectal cancer; Ap53Ab, anti-p53 antibody; UICC, Union for International Cancer Control; CEA, carbohydrate antigen.

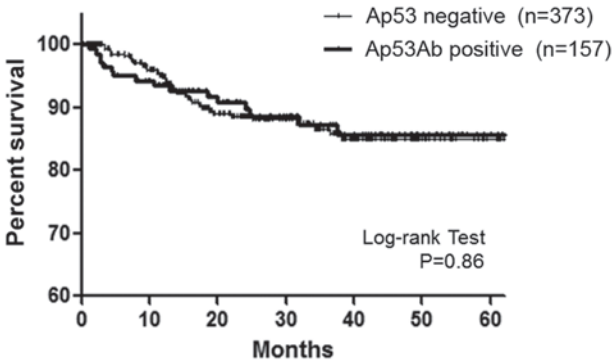

Figure 1. Comparison of disease-free survival between colorectal cancer patients with positive (n=157) and those with negative (n=373) anti-p53 antibody (Ap53Ab) expression.

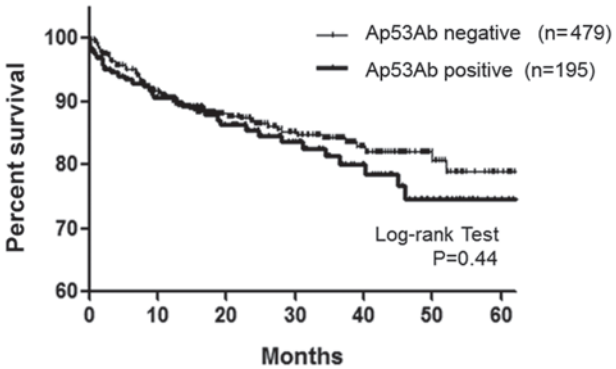

Figure 2. Comparison of overall survival between colorectal cancer patients with positive (n=195) and those with negative (n=479) anti-p53 antibody (Ap53Ab) expression.

performed, bearing in mind the possibility of false-positive readings. Of note, when positive Ap53Ab expression is detected in clinical practice, physical examination should be performed to rule out the possibility of various cancers, including head and neck, esophageal, uterine, breast,

prostatic, biliary tract, lung, bladder, gastric and pancreatic cancer (1).

In the present study, the Ap53Ab level decreased slowly following curative resection. Ap53Ab is an IgG antibody and the half-life of IgG is considered to be ~21 days (15). The mean half-life of CEA was reported to be 4-12 days, depending on the status of recurrence (16,17). In our results, the mean ± SD half-life of Ap53Ab was 30.7±27.2 days in 39 CRC patients, while that of CEA was 11.3±4.4 days in 19 CRC patients. A previous study (18) reported that the Ap53Ab level decreased to normal following curative resection within at least 6 months in almost all Ap53Ab-positive CRC patients. Recently, Kawahara *et al* (19) reported data supporting our results, as the positive Ap53Ab rate was 75% at 6 months, 70.8% at 12 months, and 54.2% at 24 months after curative operation in 24 CRC patients with no recurrence. In our results, the Ap53Ab level had returned to normal within 12 months in only 4 (10.3%) of the 39 CRC patients with elevated Ap53Ab level, whereas in some cases without recurrence it took ~5 years to return to the normal range. Therefore, the measurement of Ap53Ab should be limited to prior to surgery. When the elevated Ap53Ab level decreases slowly, even if it requires a long time, it does not necessarily indicate that the Ap53Ab level reflects the presence of residual tumor and/or recurrence.

It has been reported that positive expression of Ap53Ab was significantly associated with lymph node metastasis and lymphatic invasion (4,5,20). Furthermore, Yamaguchi *et al* (4) reported that other factors, including tumor location, histology, depth of tumor invasion, vessel invasion, distant metastasis, CEA and recurrence, were also significantly associated with positive Ap53Ab expression. Other studies (9,10) reported that no significant correlation was observed between positive Ap53Ab expression and clinicopathological factors. In the present study, several factors, including depth of tumor invasion, lymph node metastasis, stage and CEA, were found to be significantly associated with positive Ap53Ab expression. The association between elevated Ap53Ab and lymph node metastasis was consistent with previous data from an Asian population (4,5,7). In gastric cancer, an elevated Ap53Ab level tended to be associated with lymph node metastasis (21), but positive Ap53Ab expression was not found to be correlated with lymph node metastasis in esophageal squamous cell carcinoma (22). Therefore, the elevated Ap53Ab level may be consistently associated with deeper depth of invasion and lymph node metastasis in CRC, that is to say that deeper depth of invasion and lymph node metastasis may be involved in the production of Ap53Ab.

In the present study, no correlation was observed between Ap53Ab expression and poor survival rate, including DFS and OS. Although previous reports (8,23) demonstrated that positive Ap53Ab expression was associated with poor prognosis in CRC patients, there appears to be no consensus (4,6,10). Recently, Yamaguchi *et al* (4) reported that no association between Ap53Ab expression and OS was found in 1384 CRC patients, although Ap53Ab expression was associated with relapse-free survival in 1212 CRC patients who underwent curative surgery. Of those 1212 CRC patients, 339 (28%) received adjuvant chemotherapy. In our series, >70% of CRC patients with stage III disease were treated with adjuvant chemotherapy. Of those patients, 77% underwent oxaliplatin-based adjuvant chemotherapy, including mFOLFOX6 and XELOX. It may be hypothesized that oxaliplatin-based chemotherapy may improve the prognosis of CRC patients regardless of Ap53Ab expression. Several researchers have analyzed the prognosis of cancer patients with TP53 expression using immunohistochemical examination and genetic testing (6,24-26). Since the association between TP53 expression and prognosis using has not yet reached a firm conclusion, even with these methods, it is difficult to predict prognosis using Ap53Ab expression. Chang *et al* (6) investigated genetic alteration of *TP53*, overexpression of intratumoral p53 protein and Ap53Ab expression in CRC, and found that the positive rates of each examination were 56.3, 44.9 and 28.1%, respectively; they concluded that only genetic alterations of *TP53* were significantly associated with poor prognosis, while intratumoral TP53 and Ap53Ab expression were not. Moreover, they mentioned that *TP53* mutations at exons 6 and 7 were associated with the presence of Ap53Ab. The frequency of CRC patients with positive Ap53Ab expression was estimated at ~50% among CRC patients with genetic alterations of *TP53*. Further studies are required to validate the association between Ap53Ab expression and survival.

Taken together, our results indicate that the measurement of Ap53Ab may contribute to the detection of early-stage CRC in clinical practice. The use of Ap53Ab with CEA and CA19-9 may increase the diagnostic yield for CRC up to ~60%. Furthermore, the time to normalization of the Ap53Ab level was longer than expected in CRC patients with elevated Ap53Ab level preoperatively. Ap53Ab expression was associated with lymph node metastasis, although the association between Ap53Ab expression and poor prognosis could not be fully elucidated.

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