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Increased Serum Levels of Mutant p53 Proteins in Patients with Colorectal Cancer

We have examined the serum levels of the mutant p53 protein in patients with colorectal cancer preoperatively (n=50), and in patients with adenomatous polyp (n=13). Mutant p53 protein in patients after curative surgical resection of colorectal cancer (n=26, part of the fifty preoperative patients) was also measured. Serum samples were stored frozen at -70℃ until the time of analysis. We used the p53 mutant ELISA (QIA03, CALBIOCHEM) system. Serum levels of the mutant p53 protein in patients with colorectal cancer (mean=0.97±0.14 ng/ml, ranged from 0.7 ng/ml to 1.37 ng/ml, n=50) were significantly greater than those in patients with adenomatous polyp (mean=0.73±0.06 ng/ml, ranged from 0.69 ng/ml to 0.83 ng/ml) ($p<0.001$). There was a significant correlation between serum p53 levels and CA19-9 levels ($p<0.01$). Serum levels of the mutant p53 protein prior to surgery (mean=0.97±0.13 ng/ml, n=26) significantly decreased after surgical resection of tumor (mean=0.82±0.07 ng/ml) ($p<0.001$, paired t-test). These results suggest that mutant p53 protein might be used as a potential biomarker in the management of patients with colorectal cancer. Further study is warranted to establish its clinical significance.

Key Words : *Colorectal cancer, Mutant p53 protein, Serum, ELISA*

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INTRODUCTION

Molecular biologic research has revealed that multiple genetic alterations, including oncogene activation and tumor suppressor gene inactivation, are necessary in colorectal carcinogenesis (1). Mutations of the p53 tumor suppressor gene are frequently detected in colorectal carcinomas, and are considered to play an important role in colorectal carcinogenesis (2-6). The *p53* mutation in colorectal carcinomas has been studied using two main approaches: analysis of DNA sequence with polymerase chain reaction amplification (4-6) and immunohistochemical analysis (3, 5). However, the circulating p53 protein or immune response against the mutant p53 protein, which is produced by the mutant *p53* gene, is not well understood.

Wild type p53 protein is present in minute amounts in normal cells (7), mutant p53 proteins are observed as a complex with a 70 kDa heat shock protein. They have a longer half life than wild type p53 proteins, and they accumulate to high levels within cells (8, 9). The accumulated mutant p53 protein may act as an immunogen that could produce anti-p53 antibodies. Several studies have successfully detected circulating anti-p53 antibodies in patients with cancer including colorectal cancer (10-18). However, there has been little study of detection of the circulating p53 mutant protein in patients with cancer. The mechanism of production of anti-p53 antibodies has not yet been explained, neither has the mechanism of production of mutant p53.

In this study, we investigated the presence of the circulating p53 protein in sera from patients with colorectal carcinoma. The relationship between the presence or concentration of the circulating p53 protein and clinical factors, such as staging, serum carcinoembryonic antigen (CEA) level and carbohydrate antigen (CA) 19-9 etc, was also investigated to clarify whether serologic testing for the mutant p53 protein is useful in preoperative management of colorectal carcinoma and postoperative follow up after curative surgical resection.

MATERIALS AND METHODS

Fifty consecutive patients with colorectal cancer diagnosed by histological examination, twenty-six patients after surgical resection (part of the 50 pre-op patients) and thirteen patients with adenomatous polyp of the

colo-rectum were included in this study. Informed consent was obtained. Colon cancer was in 18 patients and rectal cancer in 32 patients. The male:female ratio of the patients with colorectal cancer was 1.2:1(28:22). The mean age was 61 years old. Dukes classification (Modified Astler Coller classification in this study) revealed stage A in two patients who underwent local therapy, stage B in 14 patients (who all underwent curative surgical resection), stage C in 23 patients (19 patients underwent curative surgical resection and four patients underwent preoperative radiotherpy) and stage D in 11 patients (five patients underwent palliative resection of tumor). Histologic examination showed well differentiated adenocarcinoma in four patients, moderately differentiated in 29 patients, poorly differentiated in 8 patients, mucinous in five patients and undetermined in four patients.

Blood samples had been collected from the colorectal cancer patients by routine venipuncture technique upon admission and from twenty-six patients on the 7th day after surgical resection. The serum was separated and stored frozen at -70℃ until the time of analysis. Duplicates were used for final analysis.

We used the mutant p53 ELISA (QIA03, CALBIOCHEM, USA) system. p53 assay was performed according to methods outlined in the package insert. In brief summary of the procedure, we reconstituted p53 standard with deionized water and kept it on ice. Dilution of reporter antibody in assay buffer was carried out. Samples and each of the standards were added to precoated wells by pipetting 100 μl. Incubation at 37℃ was performed for 3 h, then the wells were washed. Reporter antibody was added and incubated for 2h at room temperature, then the wells were washed. Peroxidase conjugate was added and incubated for 1h at room temperature, then the wells were washed. After another 30 minutes of incubation upon addition of the substrate, absorbances in each well were measured using a spectrophotometric plate reader at a wavelength of 405 nm. To determine p53 concentration in each sample, we first calculated the average absorbance value in each set of duplicates. We used Softmax® software to determine the p53 concentration. Serum CEA levels were determined with CEA EIA Kit (Abbott, Tokyo Japan).

For statistical analysis, t-test (unpaired and paired), analysis of variance, correlation coefficient with SAS were used. Significance was defined as $p<0.05$.

RESULTS

Serum levels of the mutant p53 protein in the patients with colorectal cancer (mean=0.97±0.14 ng/ml, ranged from 0.7 ng/ml to 1.37 ng/ml, n=50) were significantly greater than those in the patients with adenomatous polyp (mean=0.73±0.06 ng/ml, ranged from 0.69 ng/ml to 0.83 ng/ml) ($p<0.001$). However, there was no difference in serum levels of the mutant p53 protein between the patients with colorectal cancer after curative surgical resection (mean=0.82±0.07 ng/ml, ranged from 0.55 ng/ml to 1.05 ng/ml) and the patients with adenomatous polyp (mean=0.73±0.06 ng/ml) (Fig. 1). It was very interesting that serum levels of the mutant p53 protein in the patients with adenomatous polyp showed very similar results.

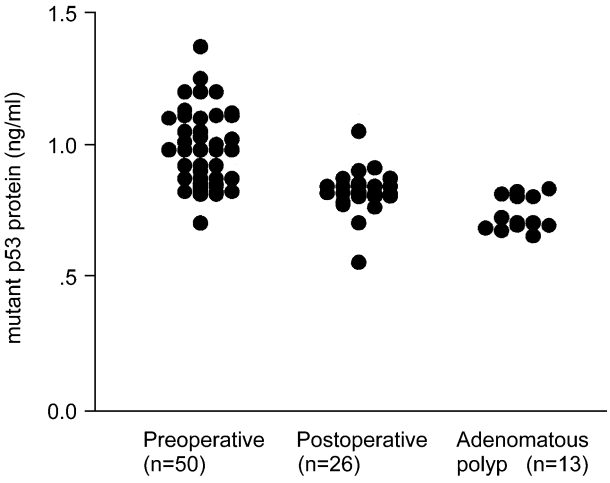

Fig. 1. Serum levels of mutant p53 protein in patients with colorectal cancer and adenomatous polyp. Serum p53 levels in patients with colorectal cancer were significantly higher than those in the patients with adenomatous polyp ($p<0.001$).

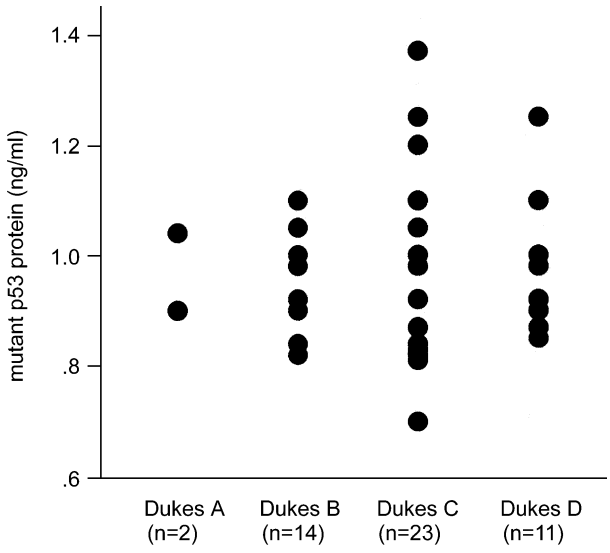

Fig. 2. Serum levels of mutant p53 protein according to modified Dukes' classification in patients with colorectal cancer. Some patients with stage C and D showed relatively high levels, but they are not statistically significant.

Table 1. Mean values of serum p53 protein levels according to variables of the patients

Variables		Mean of p53	p value
Age	<70 (n=34)	1.00 ± 0.15	0.009
	≥70 (n=16)	0.92 ± 0.08	
CEA	<5ng/ml (n=25)	1.02 ± 0.15	0.03
	≥5ng/ml (n=25)	0.94 ± 0.12	
Site	Colon (n=18)	0.97 ± 0.13	NS*
	Rectum (n=32)	0.97 ± 0.14	
Size	<5cm (n=14)	1.00 ± 0.16	NS
	≥5cm (n=29)	0.96 ± 0.14	
Sex	Male (n=28)	0.98 ± 0.11	NS
	Female (n=22)	0.97 ± 0.17	

* NS : not significant

Serum levels of the mutant p53 protein were 1.06±0.22 ng/ml (n=4) in the patients with well differentiated adenocarcinoma, 0.97±0.14 ng/ml (n=29) in those with moderately well differentiated, 0.96±0.13 (n=8) in those with poorly differentiated and 0.94±0.10 (n=5) in those with mucinous. They were not statistically significant. Serum levels of the mutant p53 protein were 1.02±0.24 ng/ml (n=2) in Dukes' A, 0.95±0.10 ng/ml (n=14) in Dukes' B, 0.98±0.16 ng/ml (n=23) in Dukes' C, and 0.98±0.12 ng/ml (n=11) in Dukes' D (Fig. 2). Relatively high levels of the mutant p53 protein were observed in the patients with advanced stage, but their mean values were not significant (ANOVA).

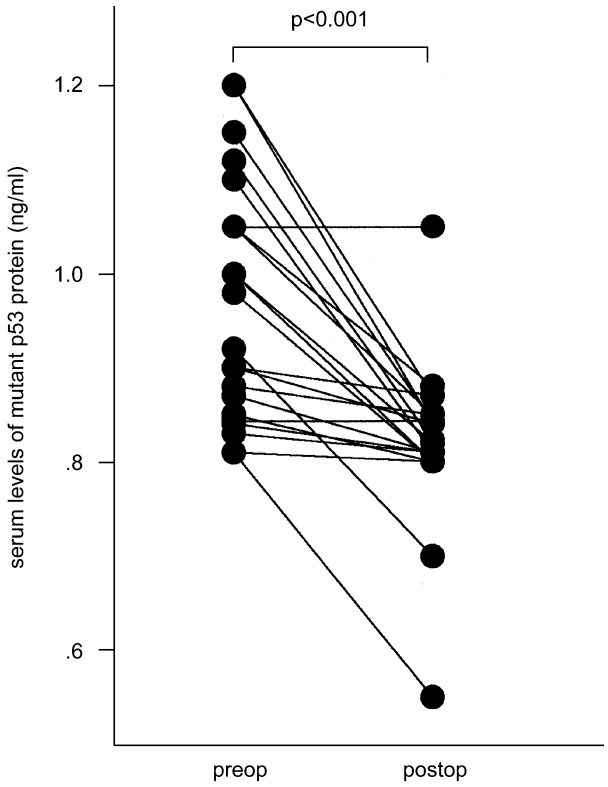

Fig. 3. Serum levels of mutant p53 protein in patients with colorectal cancer before and after surgical resection of the tumor (n=26). Serum levels of mutant p53 protein decreased significantly after surgical resection.

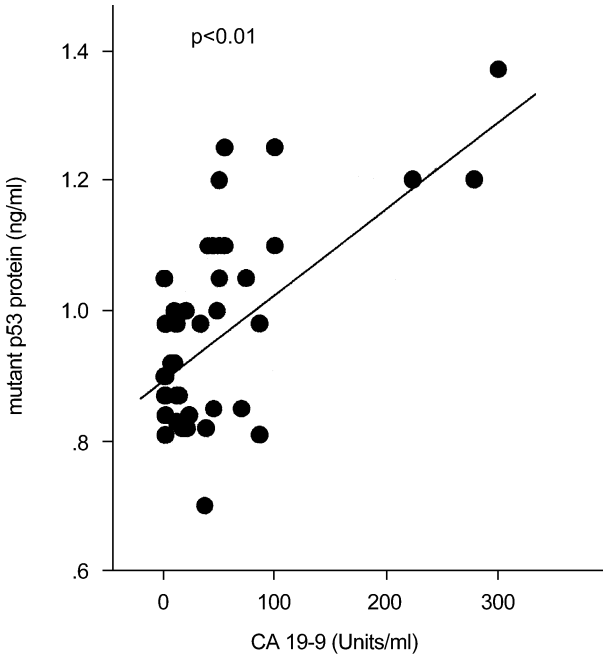

Fig. 4. The relationship between serum p53 protein levels and serum CA 19-9 levels in patients with colorectal cancer. Serum levels of mutant p53 protein increased with increasing CA 19-9 significantly (R=0.45).

Serum levels of the mutant p53 protein in younger patients with colorectal cancer (<70) were significantly higher than those in older patients with colorectal cancer (≥70). Serum levels of the mutant p53 protein in the patients with low CEA levels (<5 ng/ml) were significantly higher than those in the patients with high CEA levels (≥5 ng/ml). There were no significant differences in serum levels of the mutant p53 protein between male patients and female patients, and also between the patients with colon cancer and the patients with rectal cancer. Also, there were no differences in p53 protein levels according to tumor size (Table 1).

There was a significant correlation between serum levels of the mutant p53 protein and CA19-9 levels ($p<0.01$) (Fig. 3). Serum p53 levels increased with increasing CA 19-9. Serum levels of the mutant p53 protein prior to surgery (mean=0.97±0.13 ng/ml, n=26) significantly decreased after surgical resection of tumor (mean=0.82±0.07 ng/ml) ($p<0.001$, paired t-test) (Fig. 4).

DISCUSSION

p53 is the most commonly mutated gene in human

cancer with the majority of mutations being amino acid substitutions (19, 20). Mutations of the *p53* tumor suppressor gene are frequently detected in colorectal carcinoma and are considered to play an important role in colorectal carcinogenesis (2, 3, 4, 5). These mutations are associated with p53 overexpression caused by the decreased breakdown of the tetrameric form with mutant components and mutant *p53* blocks the function of wild type *p53* gene (21, 22, 23, 24). p53 mutation in primary tumor is associated with prognosis (25). In colorectal cancer, p53 is expressed in 50-70% of tumors by immunohistochemical analysis (5, 20, 26, 27).

The normal level of p53 protein is extremely low, but the aberrant accumulation of p53 in the tumor cell is detectable. Recently, antibodies against p53 protein in the serum of patients with colorectal cancer were successfully detected (18, 28). These results suggest that p53 status as a molecular marker might be used as a basis for the stratification of patients and eventually might play a role in the standard care of patients with colorectal cancer like CEA. However, it is unknown how p53 protein or antibodies against p53 protein appear in circulation.

We found that mutant p53 protein was detected in the sera of patients with colorectal cancer and in the patients with adenomatous polyp. However, we do not know why mutant p53 protein was detected in the sera of those patients and even in the sera of patients after curative surgical resection of colorectal cancer. One possible reason is the denaturation of p53 protein. The mutant p53 ELISA used in this study detects an epitope in p53 that is not exposed in wild type p53. Presumably, it is folded up inside the p53 molecule where the antibody cannot get to it. Many *p53* mutations alter the conformation and function of p53. When the conformation is altered, the epitope is exposed and the antibody can then detect the p53 molecule. If p53 is denatured, such as in a western blot, the epitope is also exposed, so the antibody will detect both mutant and wild-type p53 under denaturing conditions. The ELISA will detect any p53 molecule with a mutation that alters its conformation to expose the antibody binding site. We also studied using the same ELISA methods in the fresh sera from 41 healthy volunteers. Serum levels of the mutant p53 levels in all of them except 4 volunteers showed less than 0.05 ng/ml (0.09, 0.1, 0.23, 0.425 ng/ml). Thus, further study with fresh serum without stored serum in patients with colorectal cancer is needed to discriminate the real differences between fresh serum and stored serum.

General molecular biology techniques involving the study of DNA using Southern blotting, polymerase chain reaction and direct DNA sequencing are not easily performed in clinical laboratories. An alternative method for detecting genetic abnormalities is the study of the expression of the protein. While antibodies are available which can demonstrate the presence of the p53 protein in routine archival blocks, these techniques are generally restricted to use in pathology laboratories. Moreover, even when the same antibody is used there have been considerable differences in the interpretation of staining, in particular the degree of staining and the assessment of positivity. Although some studies have shown that the ELISA assay is not quite as sensitive as immunohistochemical technique, we think the ELISA assay might be an alternative method for the detection of the mutant p53 protein.

Measurements of anti p53 antibodies were used for the identification of the serum p53 protein and its significance was documented (10-18, 28). However, it is not clear how circulating p53 antibody is made because free p53 protein has to be presented to the immune system to make antibodies. In the same way, we can not explain how mutant p53 protein was detected in this study. Furthermore, mutant p53 protein in all patients with adenomatous polyp was detected in this study. We thought that this may result from denaturation of wild type p53 protein during serum storing or manipulation.

Currently, there are numerous studies on potential prognostic markers. An essential requirement for which is that the procedure for the assessment of a marker should be easy to perform and reproducible in a variety of laboratories. The development of the ELISA assay described in this paper fulfils this requirement and could facilitate more studies on the role of the mutant p53 protein in the management of colorectal cancer.

Serum levels of the mutant p53 protein before surgery decreased significantly after surgical resection. This result suggests that elevation of the circulating mutant p53 protein in the patients with colorectal cancer might have been produced in their colorectal tissue. Also this result indicates the possibility that mutant p53 protein might be used as a potential biomarker for follow up after curative surgical resection of colorectal cancer as in CEA. However, although relatively high levels of the mutant p53 protein were observed in some patients with advanced stage, further study with a large number of patients is warranted to determine its clinical usefulness because there is no close correlation between p53 protein levels and tumor stage.

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