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Immunohistochemical Study of p53 Overexpression in Radiation-Induced Colon Cancers

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The expressions of p53 and proliferating cell nuclear antigen (PCNA) were studied immunohistochemically from paraffin sections of 7 cases (9 lesions) of radiation-induced colon cancer and 42 cases of spontaneous colon cancer. Age distribution of radiation-induced and spontaneous colon cancer were 68.1 years (range, 56 to 77 years) and 67.4 years (range, 31 to 85 years), respectively. Among the radiation-induced colon cancers, there were 3 lesions of mucinous carcinoma (33%), a much higher than found for spontaneous mucinous cancer. Immunohistochemically, p53 protein expression was detected in 7/9 (78%) of radiation-induced cancers and in 23/42 (55%) of spontaneous colon cancers. χ^2 analysis found no significant differences between radiation-induced and spontaneous colon cancers in age distribution or p53-positive staining for frequency, histopathology, or Dukes' classification. In radiation colitis around the cancers including aberrant crypts, spotted p53 staining and abnormal and scattered PCNA-positive staining were observed. In histologically normal cells, p53 staining was almost absent and PCNA-positive staining was regularly observed in the lower half of the crypt. In radiation colitis including aberrant glands, cellular proliferation increased and spotted p53 expression was observed. This study suggests that radiation colitis and aberrant glands might possess malignant potential and deeply associate with carcinogenesis of radiation-induced colon cancer.

INTRODUCTION

Radiotherapy plays an important role in the management of cancer. For example at stage I–II of uterine cervical cancer, there is almost no difference in efficacy between operation and radiotherapy, both have a 5-year survival rate of about 80% for stage I and 70% for stage II.

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Improvements in radiotherapy methodology (e.g., brachytherapy) may further augment survival rates. However, acute and late complications in pelvic organs following radiotherapy are significant. One well-known late complication of pelvic irradiation is the development of colon cancers[1–5]. However, there have been few immunohistochemical studies to examine tumorigenesis of radiation-induced colon cancer. The carcinogenesis of colon cancer is a multi-step process entailing a series of genetic alterations that involve both oncogenes (e.g., *src, myc*) and tumor suppressor genes (e.g., *apc, mcc, dcc, p53* and possibly genes on chromosomes 8p, 1p and 22q) occurring in an adenoma-carcinoma sequence[6–8]. Some of these changes tend to occur in earlier stage and some in later stages[9]. One of the genes related to colon carcinogenesis is the *p53* tumor suppressor gene. Wild type *p53* is expressed in normal epithelial cells, but its half-life is too short to be detectable immunohistochemically. Most of *p53* mutations in human cancers are missense changes and the mutant type *p53* produced from such missense mutations has a long half-life and contributes to increased cell proliferation, genetic instability, and increased susceptibility to tumor formation[10–12]. The prolonged half-life and accumulation of this mutant p53 protein in the nucleus allows it to be immunohistochemically detectable. In radiation-induced human cancers, there have been two studies describing *p53* mutations in radon-associated lung cancer that have reported point mutations and deletions[13,14]. Although there have been some reports on radiation-induced colon cancers, few studies have examined gene mutations in radiation-induced colon cancer. Using a recently developed antigen retrieval immunohistochemical technique, we screened for mutant p53 protein and PCNA in radiation-induced colon cancer, radiation colitis and spontaneous colon cancers. In radiation colitis' cases, we examined mucosal cellularity and the expression of p53 and PCNA and compared them with spontaneous colon cancers. The mechanism of radiation-induced colon cancer is also discussed.

MATERIALS AND METHODS

Specimens

Among the 200,000 pathological reports at Nagasaki University School of Medicine we found 7 typical cases (9 lesions) of colon cancer occurring after pelvic irradiation for uterine cervical cancer. Radiation proctitis or enterocolitis around colon cancer were pathologically evident in these patients. The irradiation method, dosage, and interval between irradiation and subsequent development of colon cancer were recorded. For comparison with radiation-induced colon cancer, 42 spontaneous colon cancers (42 adenocarcinomas including 10 adenomas, at all resected specimens) were also examined.

Staining Procedures

After deparaffinization and rehydration, the specimens were soaked in phosphate-buffered saline (PBS, 0.01M, pH 7.4) for p53 detection or in 0.01M citrate buffer (pH 6.0) for PCNA detection. The specimens were then irradiated 3 times with microwaves for about 5 minutes each time[14]. After quenching endogenous peroxidase activity in methanol containing 0.3% hydrogen peroxidase for 10 minutes, sections were preincubated with 1% normal bovine serum antigen

(BSA) for 15 minutes to block non-specific binding. Primary anti-p53 antibody (monoclonal mouse anti-human p53 protein Clone DO-7, DAKO A/S, Glostrup, Denmark) diluted to 1:100, or anti-PCNA antibody (monoclonal mouse anti-PCNA. Clone PC10: DAKO A/S,) diluted to 1:50 were applied to the sections which were then incubated in a moist chamber overnight at 4°C. The p53-DO-7 antibody detects the wild and mutant forms of p53 protein in formalin-fixed paraffin-embedded archival materials. After the sections were washed with PBS, biotinylated goat anti-rabbit immunoglobulin G (IgG) was applied at a dilution of 1:200, and the sections incubated for 1 hour at room temperature. After washing again with PBS, immunoperoxidase staining was carried out by the avidin/biotinylated enzyme complex (ABC) method using a Vectastain kit (Elite, Vector Laboratories, Burlingame, CA). The excess complex was then washed out, and the localizations of p53 and PCNA were visualized by incubating the sections with diaminobenzidine. After being washed in distilled water, sections were counterstained with methyl green and dehydrated through an ethanol to xylene gradient.

Staining evaluation-p53

We assessed the staining intensity of definite nuclear p53 protein immunoreactivity among tumor cells and colitic mucosa, and the patients were classified into three groups as follows: (–) negative type containing no positive cells, (+) positive focal type containing aggregates of positive cells in focal areas, and (++) diffuse type containing homogeneously distributed positive cells. We were unable to analyze the specific mutations in *p53*.

Staining evaluation-PCNA

The staining pattern of PCNA was classified into 3 types: diffused with homogeneously distributed positive cells, abnormal and scattered with spottedly aggregated positive cells, or normal.

To ensure p53 and PCNA staining consistency between batches, a control colon cancer sample was included in each round. The omission of the primary antibody served as a negative control.

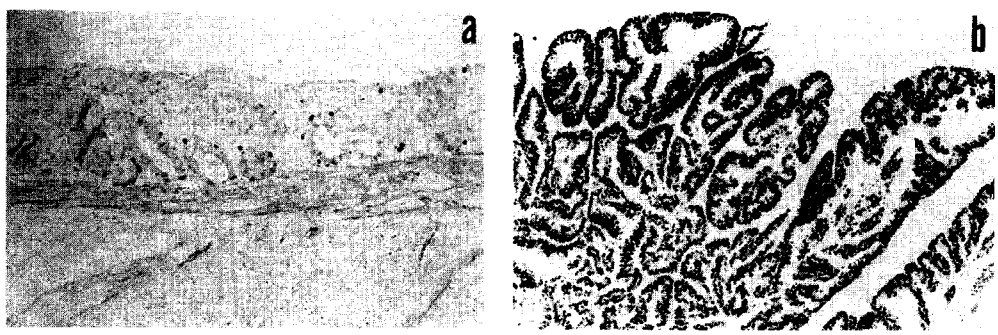

Figure 1. (a). p53 immunostaining: focal positive (= spotted) (+) in radiation colitis showing p53 expression.
(b). p53 immunostaining: strongly positive (++) in radiation-induced colon cancer showing p53 overexpression in most cells.
(Original magnification: a, × 25; b, × 25)

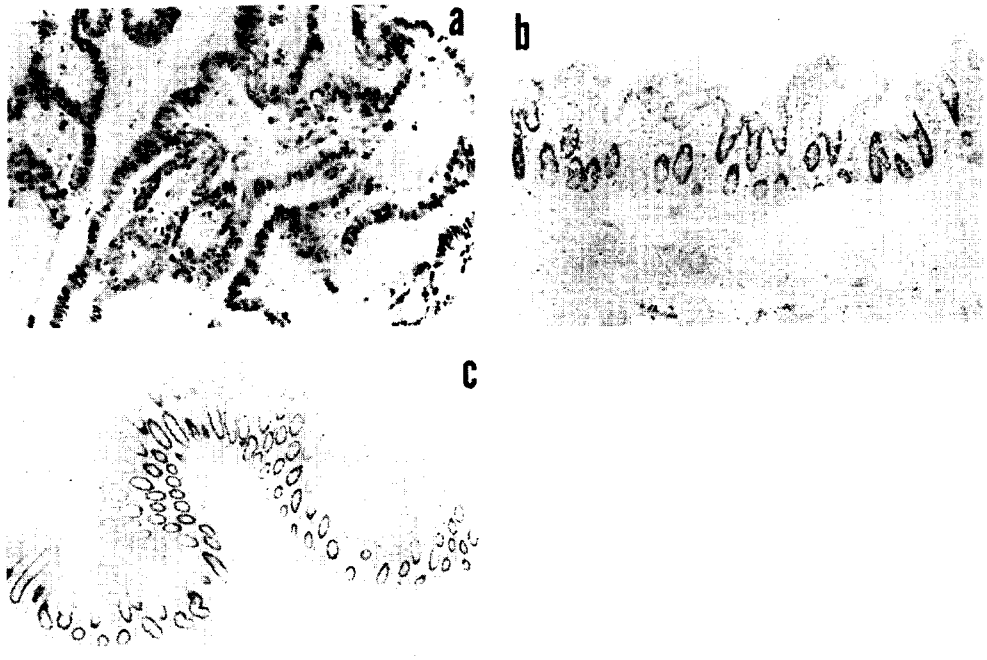

Figure 2. PCNA immunostaining in radiation-induced colon cancer and radiation colitis showed 3 patterns.
(a). Diffuse PCNA staining of cells was seen in colon cancer.
(b). Abnormal and scattered PCNA staining was seen in radiation-colitic mucosa.
(c). Normal PCNA staining was seen in normal colonic mucosa.
(Original magnification :a, × 25; b, × 25; c, × 10)

Statistical analysis

The frequency of p53 positive expression was compared for each variable using χ^2 statistics with the Stat View J-4.5 computer software program(Abacus Concepts Inc.,Berkeley, CA, USA).

RESULTS

There were 7 patients with 9 lesions of radiation-induced colon cancers. These patients and the spontaneous colon cancer patients studied were all female. Radiation-induced and spontaneous colon cancer patients had mean ages of 68.1 years (range, 56 to 77 years) and 67.4 years (range, 31 to 85 years), respectively. No positive association between the ages of the two groups was found. Cancer developed at a mean of 20.3 years (range, 10 to 27 years) after irradiation for the radiation-induced group.

The cancer site was the rectum in 25 cases and sigmoid colon in 17 cases in the group of 42 spontaneous colon cancers. Histologically, there were 4 lesions (44%) of well differentiated adenocarcinoma, 2 lesions (22%) of moderately differentiated adenocarcinoma, and 3 lesions (33%) of mucinous carcinoma in radiation-induced cancer group. Among the spontaneous colon can-

Table 1. Radiation-induced colon cancers

Case	Age (Years)	Site	Histology	Latency (Years)	Dosage classification	Dukes'
1	65	Rectum Sigmoid	Mucinous Well	25	4075mCih + 60Gy	B
2	77	Rectum	Mucinous	23	4573mCih + 60Gy	B
3	75	Rectum	Moderate	10	2299mCih + 50Gy	C
4	56	Rectum	Well Mucinous	19	6900mCih + 70Gy	A
5	63	Sigmoid	Well	18	3850mCih + 50Gy	A
6	71	Rectum	Moderate	20	unknown	A
7	70	Sigmoid	Well	27	unknown	B
mean	68.1			20.3		

Well: well-differentiated; Moderate: moderately differentiated; Mucinous: mucinous carcinoma

cers, there were 25 lesions of well differentiated adenocarcinoma, 12 lesions of moderately differentiated adenocarcinoma, and 5 lesions of mucinous carcinoma. Among the patients with radiation-induced cancer, no adenomatous remnant nor dysplasia was detected, while of the patients with spontaneous cancer, adenomatous remnants were seen in 10 patients. Pathologically, in radiation-induced cancers, various degrees of radiation injuries around colon cancers including mucosal atrophy with distorted atypical glands, irregularity of mucosal glands, expression of aberrant glands, fissuring formation, edematous submucosa and hyalinization of blood vessels were seen in all cases.

The overexpression of p53 was detected in 7 lesions (78%) of radiation-induced colon cancer, and in 23 cases (55%) of spontaneous cancer. (Table 2) χ^2 analysis did not detect a positive association between radiation-induced and spontaneous colon cancers with age distribution and

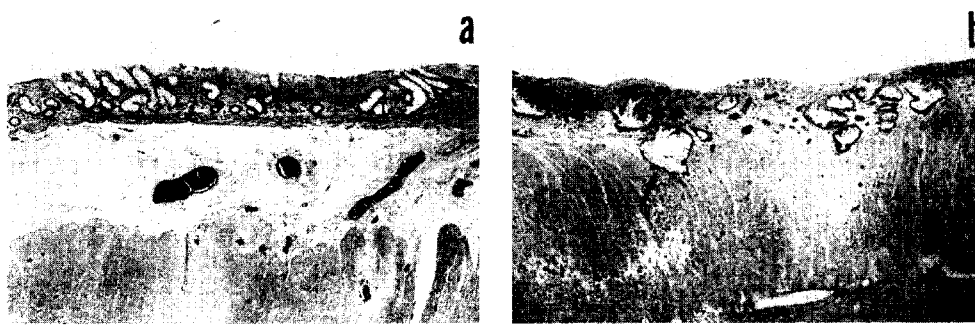

Figure 3. Radiation colitis, Hematoxylin-eosin stain. (a, b). Mucosal atrophy and irregularity, expression of aberrant gland, submucosal edema, fibrosis and hyalinization of blood vessels was observed. Aberrant glands (closed arrow) were sporadically encountered in the atrophic mucosa.
(Original magnification: a, × 10; b, × 10)

Table 2. p53 stainability of radiation-induced and spontaneous colon cancers

Variable	p53-positive cases (positive rate %)	
	Radiation (n = 9)	Spontaneous (n = 42)
Age	68.1 years	67.4 years
p53-positivity		
(++)	5	15
(+)	2	8
(–)	2	19
Histology	No. of positive / No. of tested (positive %)	
Well	3/4 (75%)	11/25 (44%)
Moderately	2/2 (100%)	9/12 (75%)
Mucinous	3/3 (100%)	3/ 5 (60%)
Dukes' classification		
A	2/4 (50%)	7/12 (58%)
B	4/4 (100%)	1/ 5 (20%)
C	1/1 (100%)	15/25 (60%)
Rectal cancer	5/6 (83%)	12/25 (48%)
Sigmoid colon cancer	2/3 (66%)	11/17 (65%)

Table 3. Immunohistochemical staining patterns of p53 and PCNA in patients with radiation colitic mucosa

Case	p53	PCNA
1	++	Abnormal and scattered
2	–	Abnormal and scattered
3	–	Abnormal and scattered
4	–	Abnormal and scattered
5	+	Diffuse
6	–	Normal
7	+	Abnormal and scattered

p53 staining. No significant correlations were observed between histological type and Dukes' classification. The degrees of p53-positive expression in radiation-induced cancers were (++) in 5 lesions, (+) in 2 lesions, and (–) in 2 lesions; in spontaneous colon cancers, the degrees were (++) in 15 lesions, (+) in 8 lesions, and (–) in 19 lesions. In radiation colitic mucosa, an overexpression of mutant-type *p53* was found in the nuclei of mucosal glands in 3 lesions (43%). The pattern of positive-PCNA cell distribution was scattered in 2 cases (67%) and diffuse in 1 case (33%).

Diffuse, granular and nuclear PCNA staining patterns were observed in all radiation-induced and spontaneous colon cancer cases. In 4 spontaneous colon cancer cases, abnormal and

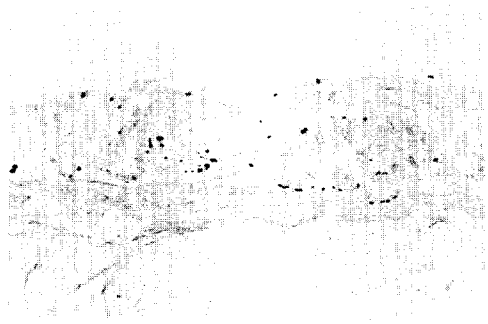

Figure 4. Spotted p53 staining in aberrant glands. (Original magnification × 25)

scattered PCNA staining was found. In general, the PCNA staining pattern was heterogeneous, being especially strong at the edge of cancers. In normal colon epithelium, PCNA was expressed mostly at the lower half of the crypt, with a variable number of stained nuclei. However, in spontaneous colon cancers, normal PCNA staining was observed around the lesions, whereas in radiation colitis, abnormal and scattered PCNA staining was seen in all lesions. The PCNA-staining pattern suggest activated irregular proliferation of radiated colitic mucosa. In addition, spotted mutant-type p53 and abnormal and scattered PCNA staining was detected in the nuclei of aberrant glands in radiation colitis.

DISCUSSION

Since Slaughter and Southwick reported radiation-induced colon cancer in 1957, there have been several studies of this type of cancer[1]. The criteria for radiation-induced colon cancer have not yet been established. Black, Ackerman and Castro *et al.* used the following criteria to identify radiation-induced colon cancer: 1) a period of at least ten years has passed from the time of radiotherapy to the diagnosis of cancer; 2) a relatively large dose of irradiation had been administered to the large intestine, and the colon cancer appeared at the site of irradiation; 3) severe radiation damage is demonstrable in and around the cancer; and 4) chronic proctocolitis is present symptomatically and clinically[2,3]. In a present series of patients, the colon cancers developed within the irradiated field at a mean of 20.3 years after irradiation. The radiotherapy for primary cancer consisted of combined external and intravaginal irradiation at variable dosages. In all cases, chronic proctocolitis was detected near the cancer and met all of the above-mentioned criteria for radiation-induced colon cancer. However, even if a cancer has met all these criteria, a cancer in an irradiated area may still be an incidental occurrence. Therefore, it is necessary to establish some objective indicators like molecular or histochemical profiles to the criteria for radiation-induced colon cancer. Castro *et al.* reported that 58% of the radiation-induced colon cancers examined were mucinous carcinoma, and 33% (3/9) of our cases were mucinous carcinomas[3]. So including our cases, the incidence of mucinous carcinoma occurring in radiation-in-

duced colon cancers was higher than in spontaneous cancer in the present series (approx. 10%). Denman *et al.* found that all of the colon cancers induced by radiation in rats were mucinous carcinoma[16]. These findings suggest that an abnormally high occurrence of mucinous carcinoma associated with radiation colitis may be related to radiation oncogenesis.

The association of mutant-type p53 accumulation with various cancer cells has been reported in many studies[17–19]. *p53* is a tumor suppressor gene which regulates the cell cycles and apoptosis. Genotoxic stress, such as ionizing irradiation, may activated *p53* function and cause G1 arrest or apoptosis[12]. Ootsuyama found *p53* mutations in radiation-induced skin and bone tumors in mice[20]. However, we found no definite correlation between the overexpression of p53 in radiation-induced and in spontaneous colon cancers. Therefore, the occurrence of *p53* gene mutations was not specific to the carcinogenesis of radiation-induced colon cancers compared with spontaneous colon cancers.

Expression of PCNA indicates proliferative cell activity, PCNA progressively accumulates in late G1 and early S phase and disappears at the end of mitosis[21,22]. Abnormal PCNA expression in colonic mucosa indicates abnormal mucosal proliferation of mucosal cells. In our study, abnormal and scattered PCNA-positive staining was seen in 6 cases (86%) of radiation colitis. Diffuse PCNA positive staining was seen in both spontaneous and radiation-induced colon cancers. In the dissemination regions of colon cancer, diffuse PCNA positive staining were seen in all cancer cells. Therefore in radiation colitic mucosa, cellular proliferative activity might increase and cellular movement stagnation might occur.

Various numbers of aberrant glands were seen in the radiation colitic mucosa. They were located in the lower level of the mucosa and did not open to the surface, inhibiting normal cryptic function. In many previous reports of colon cancer studies, aberrant crypts were identified putative precursor lesions and had many gene mutations[23,24]. The aberrant crypts in radiation colitis (showing abnormal and scattered PCNA-positive staining and spotted p53-positive staining) also potentiated colon cancer in the radiation colitic mucosa. The high frequency of mucinous carcinoma in radiation-induced colon cancers suggests a close association between the carcinogenesis of aberrant glands and the occurrence of mucinous carcinoma.

In spontaneous colon cancer, a multi-step carcinogenesis, similar to the adenoma-carcinoma sequence has been established, *p53* gene mutation occurs with this process. In contrast, in radiation-induced colon cancers, such a process has not been established, in part, because radiation exposure randomly induces various degrees of DNA damage, gene mutations, and loss of DNA repair systems[25–27]. Gene mutations in oncogenes and tumor suppressor genes may be responsible for radiation-induced colon cancers in radiation colitis. Therefore, in the radiation-induced and spontaneous colon cancers studied, similar rates of mutant-type p53 protein expression were detected. However, other differences in p53 expression might exist between the two cancer types. However, since radiation-induced colorectal cancers are very rare, it is difficult to collect enough information on these cancers from one institute. Further, in this study specimen conditions prevented mutant *p53* gene sequence analysis. To clarify the mechanism of carcinogenesis in radiation-induced colon cancer, pathological, biochemical and molecular examinations of a large number of cases of radiation-induced colon cancer are necessary.

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