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Correlation of p53 protein expression with apoptotic incidence in colorectal neoplasia

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Abstract The wild-type p53 gene suppresses cell proliferation and induces apoptosis when it is transfected into human colon cancer cell lines. Therefore, mutation of the p53 gene, which correlates closely with p53 protein overexpression, would be predicted to activate cell proliferation and limit apoptosis. We tested this hypothesis by correlating p53 protein expression with cell proliferation and apoptosis in 70 neoplasms (29 adenomas and 41 carcinomas) using p53 and Ki-67 immunohistochemical staining and DNA nick end labelling. The p53 immunoreactivity was independent of the Ki-67 positivity. The apoptotic incidence was less frequent ($P<0.005$) in tumours with diffuse p53 protein overexpression than in those with the sporadic overexpression, defined as p53 staining of isolated or scattered expression. In addition, apoptotic incidence only correlated directly ($P<0.05$) with Ki-67 positivity in tumours with sporadic p53-protein expression. These results indicate that p53 protein that is expressed sporadically in colorectal neoplasms is probably wild-type protein and induces apoptosis in response to active cell proliferation. In contrast, diffusely overexpressed p53 protein in colorectal neoplasms is probably mutant and correlates with a reduction in apoptotic cell death independently of cell proliferation.

Key words Apoptosis · p53 Expression · Colorectal cancer · Ki-67

Introduction

Mutation of the p53 gene is the most common abnormality in various human tumours, including colorectal cancer [12]. In addition, some authors have reported that p53 mutations are associated with a shorter survival of patients with colorectal cancer [10, 24, 29]. Therefore, it is hypothesized that p53 mutation plays an important role in the malignant behaviour of colorectal cancers, but the mechanism by which it causes the transformation has not been fully clarified. Previous experiments have demonstrated that introduction of the wild-type p53 gene inhibited cell growth [1] and mediated apoptosis [23] in human colon cancer cell lines. It has been reported that p53 immunoreactivity correlates with cell proliferative activity in human colorectal cancer specimens [29]. However, similar studies [4, 24] did not corroborate these findings. Moreover, the correlation between p53 protein overexpression or p53 gene mutation and apoptosis has not been evaluated in human colorectal cancer specimens. In this study, we assessed the level of p53 protein expression with cell proliferation and apoptosis in surgically resected colorectal neoplasms, using p53 and Ki-67 immunohistochemical staining and DNA nick end labelling [8].

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Materials and methods

Specimens and preparation

Seventy colorectal neoplasms were tested: 29 adenomas (5 with severe dysplasia and 24 with moderate dysplasia) and 41 invasive carcinomas (17 invading the submucosa and 24 invading into or beyond the muscularis propria). By Japanese criteria the 29 adenomas were categorized as intramucosal cancers, including 10 de novo cancers and 19 accompanied by adenoma [20, 27]. Fifty-two of the 70 tumours were resected endoscopically and the remainder surgically. None of the patients were treated prior to resection with chemotherapy or irradiation. The specimens were fixed in 10% formalin within 3 days and paraffin embedded. Four serial 3-μm-thick sections were prepared for haematoxylin and eosin (HE) staining, p53 or Ki-67 immunostaining, or DNA nick end labelling.

Histological examination

Colorectal adenocarcinomas were classified into well, moderately, and poorly differentiated types according to the World Health Organization's (WHO) histological typing of intestinal tumours [28].

Immunohistochemistry

Immunohistochemical staining of p53 and Ki-67 was performed as previously described [22]. Paraffin-embedded sections were placed on poly-L-lysine-coated glass slides and air-dried at room temperature. Deparaffinized and rehydrated sections were heated by microwave oven in 0.01 mol/l sodium citrate buffer (pH 5.9) for seven 3-min cycles, and then cooled for 60 min at room temperature. Endogenous peroxidase activity was inhibited by incubation with 0.3% hydrogen peroxidase in methanol for 20 min. After blocking non-specific reactions with 10% normal rabbit serum, the sections were incubated with anti-p53 antibody (PAb1801, 1:200; Oncogene Science, Manhasset, NY, USA) for 1 h or the Ki-67 antibody (MIB 1, 1:50; Immunotech, Marseille, France) for 2 h. The sections were then incubated with biotinylated rabbit anti-mouse immunoglobulin for 30 min followed by streptavidin-peroxidase complex (Histofine SAB-PO kit; Nichirei Corporation, Tokyo, Japan) for 30 min. The sections were rinsed with several changes of phosphate-buffered saline (PBS) between steps. The colour was developed with diaminobenzidine solution. The sections were lightly counter-stained with haematoxylin and mounted. Negative controls were treated identically, except that PBS was used instead of the primary antibody. The proliferative zone cells of the normal crypts and the germinal centre cells of the lymphoid follicles were used as internal positive controls for Ki-67 immunostaining. As for p53 immunostaining, positive control sections were included in each experiment and consisted of tissue from a colon cancer with known diffuse positivity and a p53 gene mutation detected by polymerase chain reaction and single-strand conformation polymorphism (PCR-SSCP) analysis.

DNA nick end labelling

Apoptotic cells were identified by terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated deoxyuridine-triphosphate nick end labelling (TUNEL) staining as described by Gavrieli et al. [8]. After deparaffinization and rehydration, paraffin-embedded sections adhered to poly-L-lysine-coated slides were incubated with 20 µg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) for 15 min at 37°C and then washed with distilled water. Endogenous peroxidase was inactivated by incubation with 3% hydrogen peroxidase in methanol for 10 min. The sections were rinsed with distilled water and immersed in TdT buffer (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride). TdT (0.3 eu/µl; Boehringer Mannheim GmbH) and biotinylated deoxyuridine-triphosphate (dUTP) (0.01 nmol/µl, Boehringer Mannheim, Germany) in TdT buffer was added to cover the sections and then they were incubated at 37°C for 60 min. The sections were rinsed in distilled water, immersed in PBS, and covered with 10% normal rabbit serum for 20 min. Then, they were incubated with streptavidin-peroxidase complex for 30 min and immersed in PBS. Finally, they were developed with diaminobenzidine solution, lightly counter-stained with haematoxylin and mounted. Positive controls were obtained by DNase (Boehringer Mannheim) treatment of the sections after peroxidase inactivation.

p53 Immunostaining assessment

p53-positive cells were defined as cells with brown staining on the nucleus, regardless of staining intensity. The patterns of p53 immunostaining were classified as follows: (-): negative type, no positive cells (Fig. 1A); (1+): sporadic type, one or a few scattered positive cells without any clusters (Fig. 2A); (2+): focal type, positive cells aggregated in focal area(s); and (3+): diffuse type, positive cells distributed homogeneously (Fig. 3A).

Counting procedure

We estimated the degrees of cell proliferation and apoptosis in the mucosal areas of 29 adenomas and 7 invasive (6 submucosal and 1 proper muscular) cancers. Submucosal areas of 34 other invasive cancers were evaluated. In each case, the areas selected were equivalent to a 100× total magnification lens field with the most homogeneous and frequent p53 immunostaining. p53, Ki-67 and TUNEL positivity were compared at the same selected field in serial sections. However, it is possible that intramucosal tumours showing focal p53 staining [p53(2+), n=3] were composed of two neoplastic clones with differing p53 status: aggregated positive cells and surrounding negative cells. Therefore, we excluded tumours with focal type p53 staining from this study because they could not be evaluated overall. Ki-67 positive cells were defined as cells with brown staining on the nucleus, regardless of staining

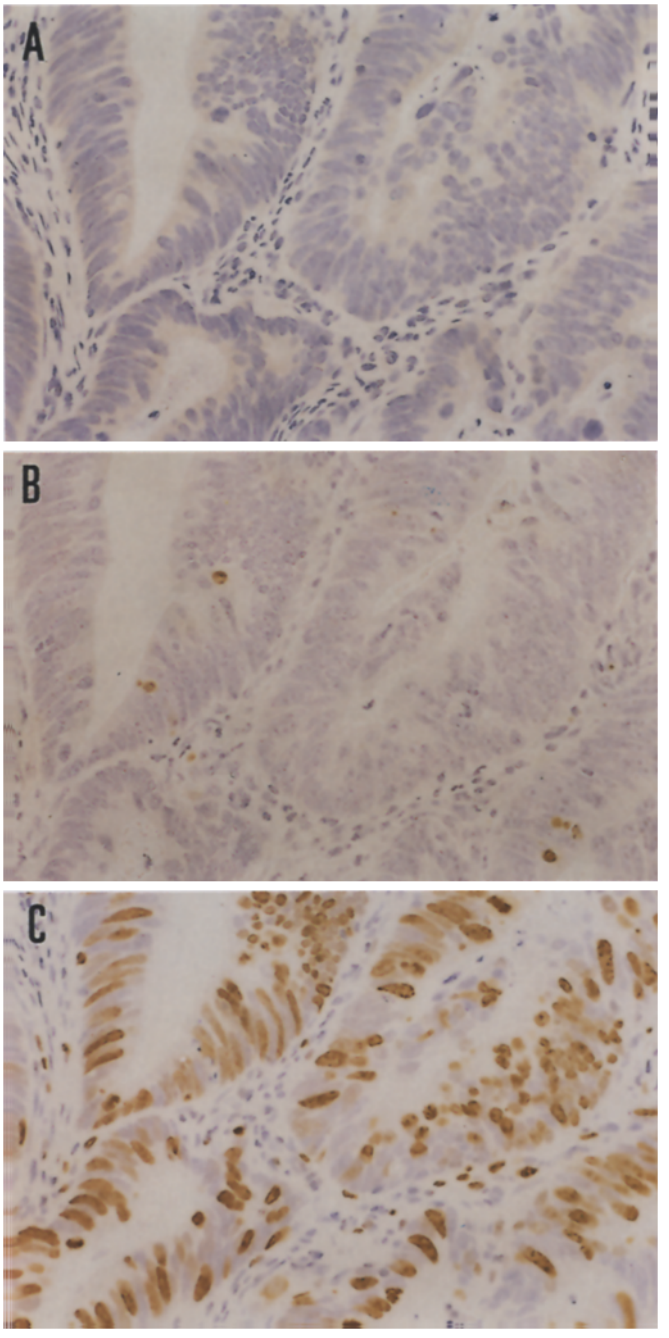

Fig. 1A–C An invasive cancer classified as p53-negative [p53(-)]. **A** p53 immunostaining; **B** DNA nick end labelling; **C** Ki-67 immunostaining. ×100

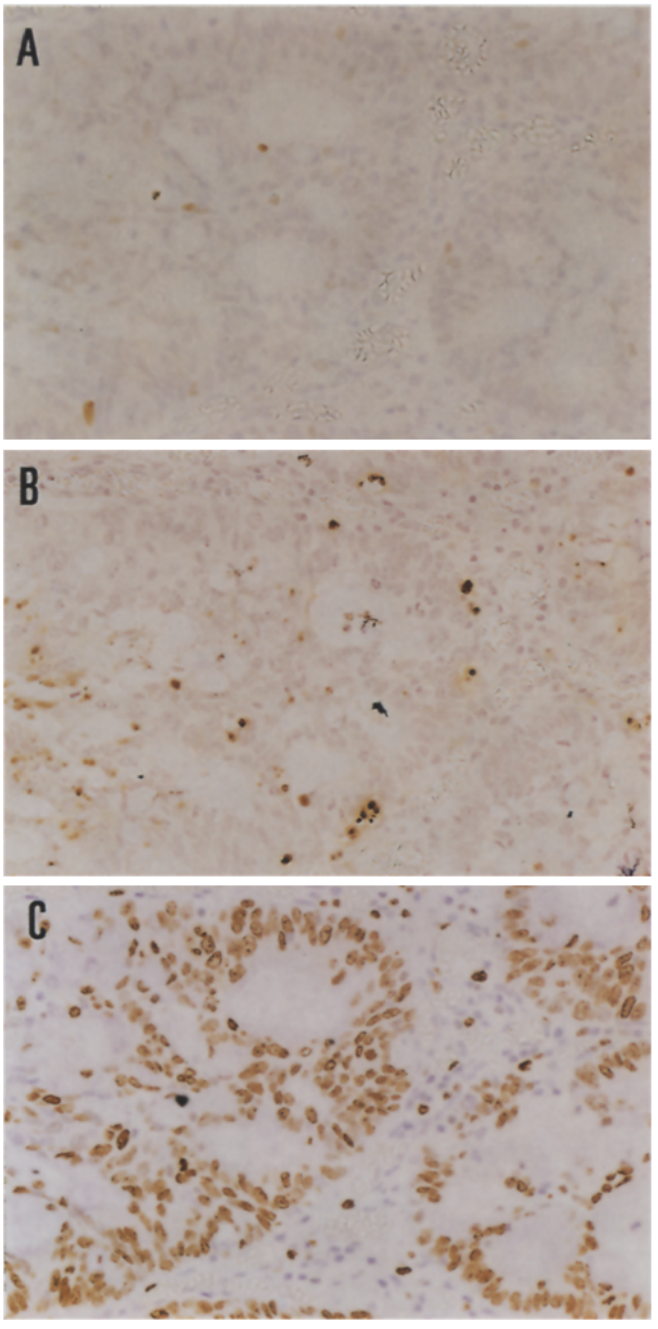

Fig. 2A–C An invasive cancer with sporadic p53-immunoreactive cells [p53(1+)]. **A** p53 immunostaining; **B** DNA nick end labelling; **C** Ki-67 immunostaining. ×100

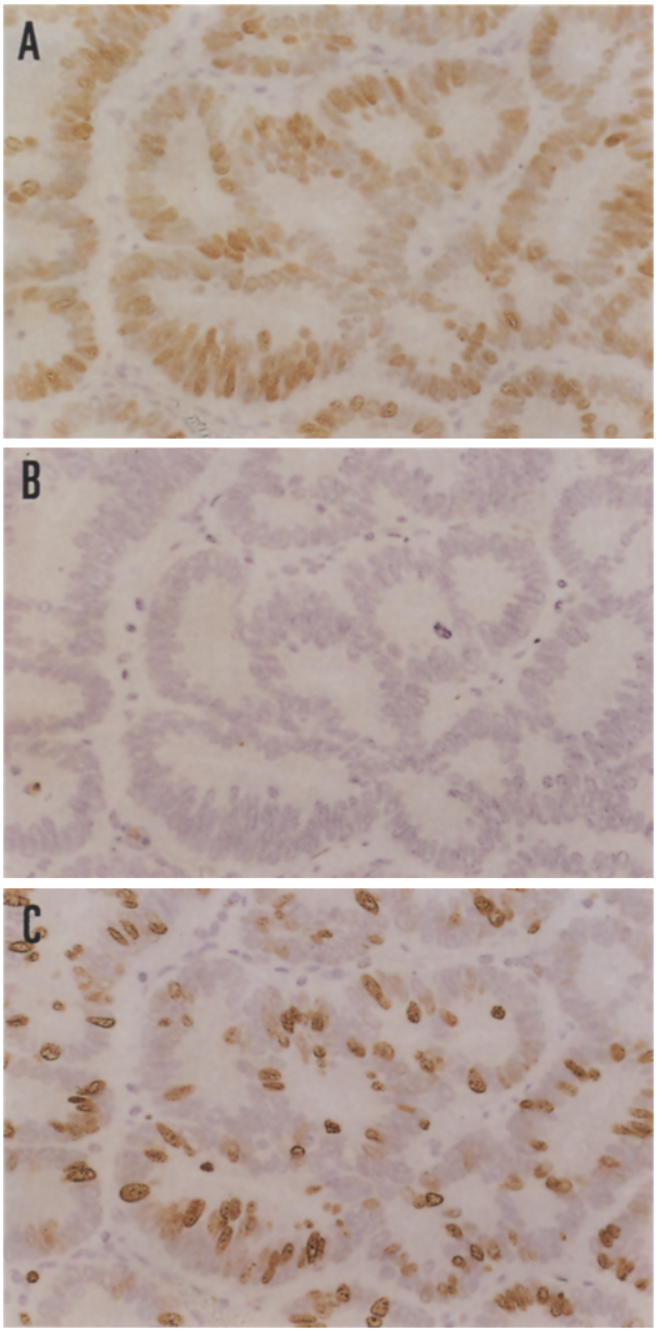

Fig. 3A–C An invasive cancer with diffuse, overexpressed p53-immunoreactive cells [p53(3+)]. **A** p53 immunostaining; **B** DNA nick end labelling; **C** Ki-67 immunostaining. ×100

intensity (Figs. 1–3C). TUNEL-positivity was identified as homogeneous brown or granular brown nuclear staining (Figs. 1–3B). The Ki-67-positive cell index (KI) and apoptotic cell index (AI) were expressed as the percentage of Ki-67- and TUNEL-positive cells, respectively, versus the total number of cancer cells. KI and AI were measured by randomly counting 1012–4828 (mean 3395) tumour cells per area using a standard light microscope at a magnification of ×400. TUNEL-positive apoptotic cells present inside a basement membrane were counted, whereas those located in the adjacent stroma or those extruded into the luminal space were excluded from the count. In addition to TUNEL-positive cells with normal appearance, TUNEL-positive nuclear fragments appearing as clusters, sometimes surrounded by halos (i.e. an apoptotic body), and single positive nuclear fragments larger than 2.5 μm in size were recorded as one apoptotic cell because they represented the remains of a single cell [11, 13, 14, 21].

Statistical analysis

KIs and AIs in groups of tumours based on p53 immunostaining pattern or invasion were compared using the nonparametric Mann-Whitney (two-sided) and Kruskal-Wallis (one-sided) tests. AIs and KIs of p53-sporadic tumours were compared by Pearson's correla-

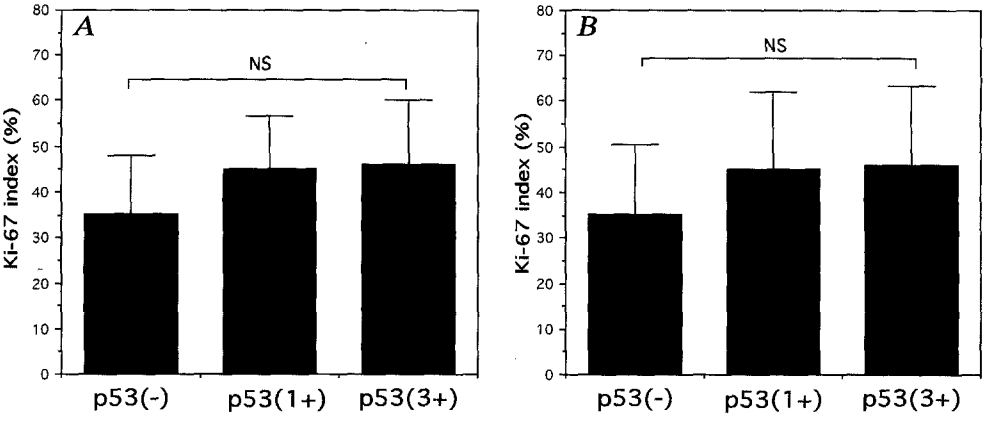

Fig. 4 Correlation between Ki-67 index and p53 immunoreactivity in **A** intramucosal and **B** invasive neoplasms. *Columns* and *bars* represent the mean±SD. There was no difference in Ki-67 index between tumours with different p53 immunostaining patterns

Table 1 Correlation between p53 immunoreactivity and histological types of colorectal tumours (*Well, Mod, Poor*, well, moderately, poorly differentiated adenocarcinoma)

Histological type	p53-immunoreactivity			
	p53(−)	p53(1+)	p53(2+)	p53(3+)
Adenomas (n=29)	2 (6.9%)	16 (55.2%)	3 (10.3%)	8 (27.6%)
Invasive cancers				
Well (n=26)	5 (19.2%)	6 (23.1%)	–	15 (57.7%)
Mod (n=11)	3 (27.3%)	4 (36.4%)	–	4 (36.4%)
Poor (n= 4)	–	2 (50.0%)	–	2 (50.0%)
Total (n=70)	10 (14.2%)	28 (40.0%)	3 (4.3%)	29 (41.4%)

tion analysis. Probability values smaller than 0.05 were considered statistically significant.

Results

p53 expression and histological type

p53 Immunoreactivity and histological types of colorectal neoplasms are presented in Table 1. The p53 immunostaining patterns were independent of histological type of cancer, but adenomas showed frequently (55.2%) the sporadic type.

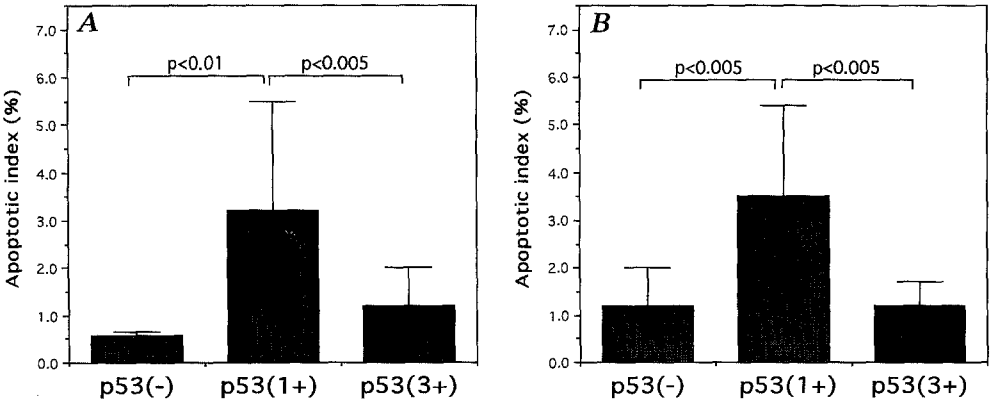

Fig. 5 Correlation between apoptotic index and p53 immunoreactivity in **A** intramucosal and **B** invasive neoplasms. *Columns* and *bars* represent the mean±SD. The apoptotic index was significantly higher in p53(1+) tumours than in p53(-) or p53(3+) tumours, regardless of depth of invasion (Kruskal-Wallis and Mann-Whitney test)

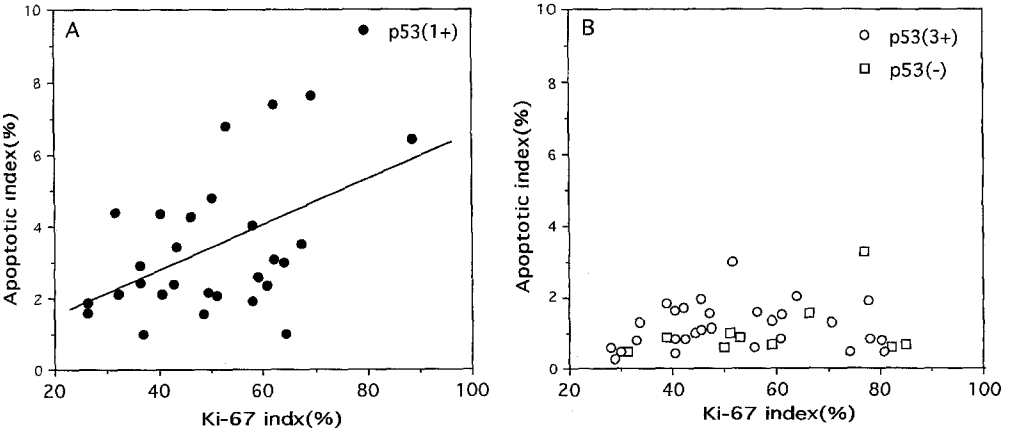

Fig. 6 Correlation between apoptotic index and Ki-67 index. There was a significant correlation (r=0.43, P=0.02, Pearson's test) in p53(1+) tumours (**A**), but not in p53(-) and p53(3+) tumours (**B**)

p53 expression and Ki-67 index

Ki-67-positive cells were observed mainly in the luminal portion of crypts in 23.1% (6/26) of adenomas. KIs were independent of invasion (mucosal areas 47.1±12.1%; submucosal areas 57.6±17.2%). There was no correlation between p53 immunostaining patterns and Ki-67 positivity in either intramucosal or invasive tumours (Fig. 4).

p53 expression and apoptotic index

There was no significant difference between the AIs of mucosal and submucosal areas (2.2±1.8% vs 2.0±1.7%) in the neoplasms tested. In both intramucosal and invasive tumours, AIs were higher in p53-sporadic type cancers [p53(1+)] than in p53-negative [p53(-)] or p53-diffuse types [p53(3+)] (Fig. 5).

Apoptotic index and Ki-67 index

There was a positive correlation (P=0.02) between KIs and AIs in p53-sporadic type tumours [p53(1+)] (Fig. 6A). The AIs and KIs of p53(-) and p53(3+) tumours did not correlate (Fig. 6B).

Discussion

It has been reported that mutation of the p53 gene is coincident (58–86%) with p53 overexpression in tumours of various organs [2, 7, 16, 25, 26]. Therefore, the p53 gene would be expected to be mutant in most of the tumours with a diffuse p53 immunostaining pattern. However, we believe that the sporadic p53 immunoreactivity seen in the neoplasms assessed in this study represents a transient expression of wild-type p53 protein, as previously discussed [2, 9]. This is supported by the observation that the anti-p53 antibodies PAb1801 and CM1, which recognize both wild-type and mutant p53 proteins, show a sporadic p53 staining pattern in non-neoplastic tissues derived from acute and chronic cholecystitis [22], normal bronchial mucosa [3] and normal larynx [6]. Furthermore, recent studies have shown that the stability of wild-type p53 protein increases temporarily in response to DNA damage by irradiation, mediating an arrest in G1 of the cell cycle [15, 17]. DNA damage could be caused by abnormal DNA replication. Therefore, it is possible that the temporal increase of wild-type p53 protein may be induced by DNA damage in p53-sporadic positive tumours. In contrast, the lack of p53 immunoreactivity in other tumours may reflect a lesion without chromosomal rearrangements or genetic alterations involving p53 gene. However, no p53 immunostaining cells were detected in bone and soft tissue sarcomas [26] and breast cancers [25] with p53 nonsense mutations or frameshift deletions/insertions. These gene alterations, which created stop codons, accounted for 39% (18/46) [10] or 16% (6/37) [16] of p53 gene alterations in colorectal cancers. These alterations corresponded with negative immunostaining in 100% (2/2) [16], 93% (14/15) [25] and 86% (6/7) [26] of cases. Furthermore, we observed p53 gene (exon 5 or 6) alterations in 3 of the 10 cancers without p53 immunoreactivity by PCR-SSCP analysis (data not shown). Therefore, although more detailed molecular analysis is needed, some of the tumours that were negative for p53 immunoreactivity could contain p53 nonsense mutations or frameshift deletions/insertions, while others might have wild-type p53 genes but lack DNA damage.

The present study showed a low incidence of apoptosis in p53-diffuse immunoreactive tumours. This is consistent with the recent proposal that cells with DNA damage cannot undergo apoptosis when the p53 gene is inactivated [18]. Apoptotic cell death mediated by wild-type p53 is considered an efficient method for removal of cells with genetic abnormalities. Therefore, p53 gene alteration accompanied with reduced apoptosis leads to the accumulations of mutation and aneuploidy [19, 30]. However, since we found a small number of apoptotic cells in the neoplasms with diffuse p53 immunoreactivity, some forms of apoptosis could be induced by other pathways not involving wild-type p53. In addition, we showed that apoptotic cell incidence correlates directly with cell proliferative activity in the tumours with sporadic p53 immunostaining. The correlation is probably the result of increased genetic mutation or abnormal replication in actively proliferating cells compared with quiescent cells. The result is an apparent increase in apoptotic cell death. This supports our speculation that sporadic p53 immunoreactivity represents transient expression of wild-type p53 protein.

In contrast, p53-negative tumours had a low apoptotic index. These data are compatible with the possibility that this group is composed of two kinds of neoplasms, those with p53 nonsense mutations or frameshift deletions/insertions and those with wild-type p53 but little DNA damage, because neither population of cells would exhibit any apoptotic response.

p53 mutation may not be the direct cause of cell proliferation, because the present study failed to observe a relationship between p53 protein expression and cell proliferative activity. The discrepancy with a previous study which corroborated the relationship [29] might be explained by the different antibody used for immunohistochemical detection of proliferating cells. In contrast to the anti-DNA polymerase α-antibody used in the previous research [29], Ki-67 (MIB 1) is applicable to formalin-fixed, paraffin-embedded samples [5]. Thus, in our study, p53 and Ki-67 immunoreactivity could be examined in the same tumour area using serial sections.

In conclusion, our findings indicated that the sporadic expression of p53 protein, which is probably wild-type p53 protein, mediated apoptotic cell death in colorectal neoplasms, especially in those with active cell prolifera-

tion. However, the overexpressed p53 protein observed in cells with diffuse p53 immunoreactivity was probably of a mutant type and could not mediate apoptosis. It may be that mutation of the p53 gene contributes to the progression of colorectal tumours by reducing apoptosis and allowing the subsequent rapid selection of malignant clones.

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