J Korean Med Sci 2000; 15: 667-74
ISSN 1011-8934

Topographic Expression of p21$^{WAF1/SDI1/CIP1}$, *bcl2*, and p53 is Altered at the Early Stage of Colorectal Carcinogenesis

We analyzed the expression of p21, bcl2, and p53 in normal and different pathologic mucosa of the human colorectum using immunohistochemistry and cold polymerase chain reaction-single strand conformation polymorphism. The topography of normal mucosa showed; bcl2 and p53 expression restricted to basal epithelial cells and p21 expressed only in superficial epithelial cells. This topographic expression was altered in hyperplastic polyps and adenomas. Hyperplastic polyps revealed absence of or weak bcl2 expression and strong p21 expression without topography. In adenomas, whereas bcl2 expression increased and extended to parabasal and superficial dysplastic epithelium, the increase of p21 expression was limited to surface dysplastic epithelium. p53 was weakly expressed throughout the full thickness of dysplastic epithelium. Bcl2 expression in adenomas was stronger than in carcinomas; p53 expression was converse and p21 expression was variable. In carcinomas, this topographic expression was largely abrogated but *p53* mutation (36%) was more frequent than in adenomas (2%). In carcinomas, p21 and p53 expression correlated inversely, but there was no relationship with bcl2. These results suggest that there is precisely ordered topographic pattern of p21, bcl2, and wild p53 expression in normal colorectal cells, but this becomes disordered during the early stage of colorectal carcinogenesis.

Key Words: *p21$^{WAF1/SDI1/CIP1}$; bcl2; p53; Colorectal Carcinogenesis; Topographic Expression; Immunohistochemistry; PCR-SSCP*

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Received: 7 August 2000
Accepted: 14 September 2000

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*This work was supported by the research fund of Korean Health Technology Planning & Evaluation Board (1996-1998). This abstract was presented in 89th Annual Meeting of American Association of Cancer Research.

INTRODUCTION

In the normal colonic mucosa, the equilibrium between cell proliferation and cell loss is essential for mucosal integrity, which is controlled by cell cycle and apoptotic kinetics. The disequilibrium between cell proliferation and loss is the basic pathologic event of morphologic and functional abnormalities of the colonic mucosa as well as other gastrointestinal mucosa. In this regards, colorectal cancer is believed to result from a series of genetic alterations that disorder normal mechanisms that control cell growth and cell death (1-3). *p53*, a tumor suppressor gene, is thought to play a critical role in the multistep process of colorectal carcinogenesis (4). It has been established that the accumulation of wild-type p53 protein results in two events, cell cycle arrest and programmed cell death, which carry out the tumor suppressor function (5, 6). Mutation of *p53* leads to disruption of this function, resulting in a selective growth advantage for tumor cells.

p53-induced cell growth arrest is through the regulation of one or more cell cycle checkpoint-related genes such as mdm-2 (7), GADD45 (8), and p21 (9). Of these three, the p21(WAF1/CIP1/SDI1/MDA6/CAP20) gene is thought to directly regulate the cell cycle. The product of p21 inhibits DNA replication by blocking cyclin-dependent kinase (CDK) (10, 11) and inhibits PCNA-dependent DNA repair (12). p21, therefore, can disrupt the progression of DNA-damaged eukaryotic cells through the cell cycle. These findings provide evidences for a direct link between p53 tumor suppressor protein and cell cycle control.

Bcl2 expression is one of the potential mechanisms by which tumor cells escape *p53*-mediated apoptosis. p53 down-regulates the expression of the *bcl2* gene and up-regulates the expression of the *bax* gene, which promotes *p53*-induced apoptosis. Bcl2 is expressed in a variety of human cancers (13, 14) and the apoptosis induced by wild-type p53 can be blocked by bcl2 in cultured cancer cells (15). The *bcl2* gene is obviously involved in the oncogenesis of human follicular lymphoma via a chro-

mosomal translocation t(14;18)(q32;q21) (16, 17). It is also expressed in the epithelial regenerative compartment or basal crypts of normal colonic and small intestinal mucosa (18, 19) as well as in the mucous neck region of gastric mucosa (20). Bcl2 appears to be important in many continuously proliferating epithelia through prevention of cell death in the regenerative compartments.

Genetic alterations of *p53* are most frequently observed in human colorectal cancers (2). Given the tumor suppressor role of p53 through either induction of G1 arrest or signaling apoptosis, we hypothesized that p21 and bcl2, mediating p53 function, might play a role in colorectal carcinogenesis. As a first investigative step, we performed an immunohistochemical analyses of normal mucosa, chronic inflammation, hyperplastic polyps, adenomas, and carcinomas of the colorectum together with cold polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP).

MATERIAL AND METHODS

Tissue samples

Endoscopic polypectomy, biopsy, and surgically resected specimens of 11 hyperplastic polyps from 9 patients and 41 adenomas from 33 patients (including 3 cancer patients) were examined. Within the same patient, 2 hyperplastic polyps in 2 patients, 2 adenomas in 8 patients were noted. Synchronous presence of a colon cancer within the adenomas was noted in 7 of 33 patients. One hyperplastic polyp and 12 of 41 adenomas were larger than 1.0 cm. Adenomas were divided into two groups (1): low-grade (10 cases) and high-grade dysplasia (31 cases). The histology were tubular (36 cases), villotubular (4 cases), or villous (1 case). Thirty-one surgically resected and untreated colorectal cancers were also examined, and most of tumors (26 cases) were well differentiated carcinomas. These included Modified Duke's stage B2 (15cases), C2 (13 cases), B1 (2 cases), and C1 (1 case) (21). Tumors were detected in the proximal colon (15 cases), distal colon (5 cases), and rectum (11 cases). Three carcinomas showed concurrent adenomas.

Normal mucosa and chronic inflammation contiguous to the carcinoma or adenoma were simultaneously analyzed. The diagnosis of carcinomas and assessment of their histologic grade were determined by examination of hematoxylin and eosin stained sections from the same block of each case.

Immunohistochemistry

The immunoperoxidase method, using the avidin-biotinylated horseradish peroxidase complex (Dako LSAB kit, Los Angeles, CA, U.S.A.), was carried out with formalin-fixed paraffin-embedded tissue sections. The microwave antigen retrieval procedure in 0.01 M sodium citrate buffer (pH 6.0) was performed. Primary p21 monoclonal antibody (mab) (Transduction Laboratories, Lexington, KT, U.S.A.) recognizes amino acid residues 1-150 of p21 protein, while primary anti-bcl2 mab (Dako, Carpinteria, CA, U.S.A.) recognizes a cytoplasmic epitope in amino acid residues 41-54 of bcl2 protein. Anti-p53 mab (DO-7, Novocastra, Inc, Manhasset, NY, U.S.A.) recognizes an amino-terminal residues 19-26 of p53 protein. Staining was developed by immersing slides in diaminobenzidine as chromogen and was counterstained in hematoxylin.

Controls

Fibroblasts in colorectal tissues served as a positive control for p21(++). Normal human tonsils and lymphoid cells infiltrated in tumors served as a positive control for bcl2 in all slides (+++). A gastric cancer with a high level of nuclear p53 immunoreactivity was used as a positive control for this protein (+++). A negative control was processed with each slide; it excluded the primary antibody but included all other steps of the procedure.

Scoring methods

Positive tumor cells were quantified twice, expressed as a percentage of the total number of tumor cells, and assigned to one of 6 categories: 0, <5%; 1, 5<<30%; 2, 30<<50%; 3, 50<<70%; 4, 70<<90%; 5, >90%. For all three proteins, less than 5% positive cells was regarded as negative. The intensity of p21, bcl2, and p53 immunostaining was scored as follows: 1+, weak; 2+, moderate; 3+, intense. The percentage of positive cells and staining intensity were multiplied to produce a weighted score for each tumor specimen. A score of less than 8 was designated as low expression (+), and one of more than 9, as high expression (++).

Cold PCR-SSCP of *p53* gene

Genomic DNA was isolated from 4 slices of 20 μm-thick sections from 11 formalin-fixed paraffin-embedded hyperplastic polyps, and 41 adenomas. Hyperplastic and dysplastic areas were microdissected from adjacent normal mucosa. After H&E examination of the first section, fresh frozen tissues from 29 carcinomas, which were retrieved from the tissue bank were trimmed from areas showing carcinomas and adjacent normal mucosa. Proteinase K digestion and phenol/chloroform extraction of

a microdissected tissues or detergent solution extraction of a biopsy tissue were performed as described (22, 23). Exons 5-8 of *p53* gene were amplified by PCR according to procedures described previously (22).

The method for nonradioactive SSCP was followed as described (24). A mixture consisting of 5 μL PCR product (equivalent to 20-200 ng of DNA), 0.4 μL 1 M methyl mercury hydroxide (Johnson Mathey Electronics, Ward Hill, MA, U.S.A.), 2 μL of loading buffer (15% Ficoll of M_r 40,000, 0.25% bromphenol blue, and 0.25% xylene cyanol), and 12.6 μL of DW were prepared to yield a total volume of 20 μL. This mixture was heated at 95℃ for 4 min to denature double-stranded DNA and then plunged into ice prior to loading the entire 20 μL on the gel. Precast 8×7 cm, 0.1 cm thick, 20% (for exon 6 and 7) or 4-20% (for exon 5 and 8) gradient polyacrylamide TBE gels were used with the matching commercial gel apparatus (Novex, SanDiego, CA, U.S.A.). The buffer chamber was filled with 1.25× TBE buffer for 20% gels or 1× TBE buffer for 4-20% gradient gels. A constant (10℃) temperature of internal buffer was maintained during the gel running using a thermostatically controlled chilled circulator (MULTI TEMP2209; LKB). The pumped-in buffer was allowed to overflow the outer chamber and to return to the circulator tank by gravity flow. The inner chamber was drained by siphon at a rate to equal the in-flow. The empirically determined optimal buffer temperature for each exon 5-8 was 10℃. Gels were run at 300 V (38 V/cm) for 1 hr 30 min (4-20% gel) to 3 hr (20% gel). The gels were stained with ethidium bromide (0.5 μL/mL in 1× TBE buffer) for 20 min at room temperature, destained in H_2O for 20 min, and finally photographed with Polaroid 57 film. Samples were considered SSCP-positive if one or more bands migrated apart from the wild-type bands by visual inspection.

Statistics

The associations between p21, bcl2, p53, and clinical findings were analyzed using Pearson's correlation coefficient. The relationship between p21, bcl2 or p53 staining and the dysplastic grade of adenomas or the presence of concurrent or synchronous carcinoma in adenomas, was analyzed using Kruskal-Wallis one-way ANOVA. Comparison of expression of these three proteins between adenomas and carcinomas was analyzed by t-test. Statistical significance was defined as $p<0.05$.

RESULTS

Topography of p53, p21, and bcl2 expression

In the normal mucosa, a few cells at the very base of the crypt exhibited nuclear p53 and cytoplasmic bcl2 expression, while nuclear p21 expression was restricted to surface epithelial cells. Cells in the mid-zone of the crypts were negative for all three proteins. Chronic inflammatory colorectal mucosa showed lymphoid follicles and diffuse plasma cell infiltration. Bcl2 and p53 expressions

Table 1. p21, bcl2, and p53 expression in colorectal hyperplastic polyps, adenomas, and carcinomas

	Hyperplastic polyp (%)	Tubular adenoma (%)		Carcinoma (%)
		Low D	High D	
Sca				
−	11 (100)	10 (24)	24 (59)	
+	0 (0)	0 (0)	7 (17)	
p21				
−	0 (0)	0 (0)	0 (0)	7 (23)
+	1 (9)	10 (24)	31 (76)	20 (65)
++	10 (91)	0 (0)	0 (0)	4 (13)
bcl2				
−	8 (73)	2 (5)	2 (5)	13 (42)
+	3 (27)	5 (12)	14 (34)	13 (42)
++	0 (0)	3 (7)	15 (37)	5 (16)
p53(DO7)				
−	5 (45)	0 (0)	0 (0)	0 (0)
+	6 (27)	9 (22)	18 (44)	8 (26)
++	0 (0)	1 (2)	13 (32)	23 (74)
SSCP				
Normal	11 (100)	9 (22)	31 (76)	18 (58)
Abnormal	0 (0)	1 (2)	0 (0)	11 (36)
ND	0 (0)	0 (0)	0 (0)	2 (6)
Total	11	10	31	31

Number of cases. D, dysplasia; Sca, synchronous carcinoma; SSCP, single strand conformation polymorphism; ND, not done

increased with the severity of chronic inflammation and especially in the epithelial cells above the lymphoid follicles.

All hyperplastic epithelial cells expressed p21 (Table 1, Fig. 1). All such polyps were negative or weakly positive for bcl2 and p53 immunostaining. p21, bcl2, and p53 expressions were diffusely distributed without topographic pattern in these polyps.

p21, bcl2, and p53 expressions showed characteristic topographic pattern in dysplastic crypts (Table 1 and Fig. 1, 2). All adenomas expressed p53. The full thickness of the dysplastic epithelium expressed p53 with weak staining intensity. p53 expression increased in severe dysplasia ($p<0.06$) and in the presence of concurrent carcinoma ($p<0.0002$). p53 expression was weaker in adenomas than in carcinomas ($p<0.0002$) (Fig. 1A). Bcl2 expression was detected in cells near the base of the crypt and extended upward to about half to two thirds of the dysplastic epithelium, where it abruptly ended, and p21 expression began, continuing up to the lumen (Fig. 2). A few dysplastic cells coexpressed bcl2, p21 and p53 with staining of serial sections. Bcl2 expression in adenomas was stronger than in carcinomas ($p<0.0001$) (Fig. 1B). p21 expression decreased with the presence of synchronous carcinoma ($p<0.002$). p21 expression increased in adenomas, but was variable in carcinomas (Fig. 1C). There was no relationship between bcl2 or p21 expression and grade of dysplasia and adenoma size. Multiple adenomas from the same patients revealed either variable or similar pattern of expression of these three proteins.

In carcinomas, topographic expression of these three proteins was abrogated (Fig. 1, 3). All carcinomas were positive for p53 antibody (Table 1). Twenty-three cases (74%) were strongly positive, whereas 8 cases (26%) were weakly positive (Fig. 4). Twenty-four cases (77%) expressed p21. Although a significant inverse correlation ($r=-0.36$, $p<0.04$) was found between p21 and p53 immunostaining, 24 of 31 carcinomas (77%) expressing p21 showed coexpression of p53, and the distribution of cells expressing either p53 or p21 was different. Seven cases of p21 negative carcinomas (23%) revealed strong p53 immunostaining; bcl2 expression was found in 18 cases (58%), but did not correlate with p53 or p21 expression (Fig. 4). No relationship was observed between the p21-, bcl2- or p53-weighted score and age, sex or clinical stage.

Relationship between p21, bcl2, and p53 expression and *p53* gene mutation

In normal mucosa and hyperplastic polyps, *p53* mutation was not detected by PCR-SSCP (Table 1, Fig. 1). In one adenoma (2%), which showed low grade dysplasia,

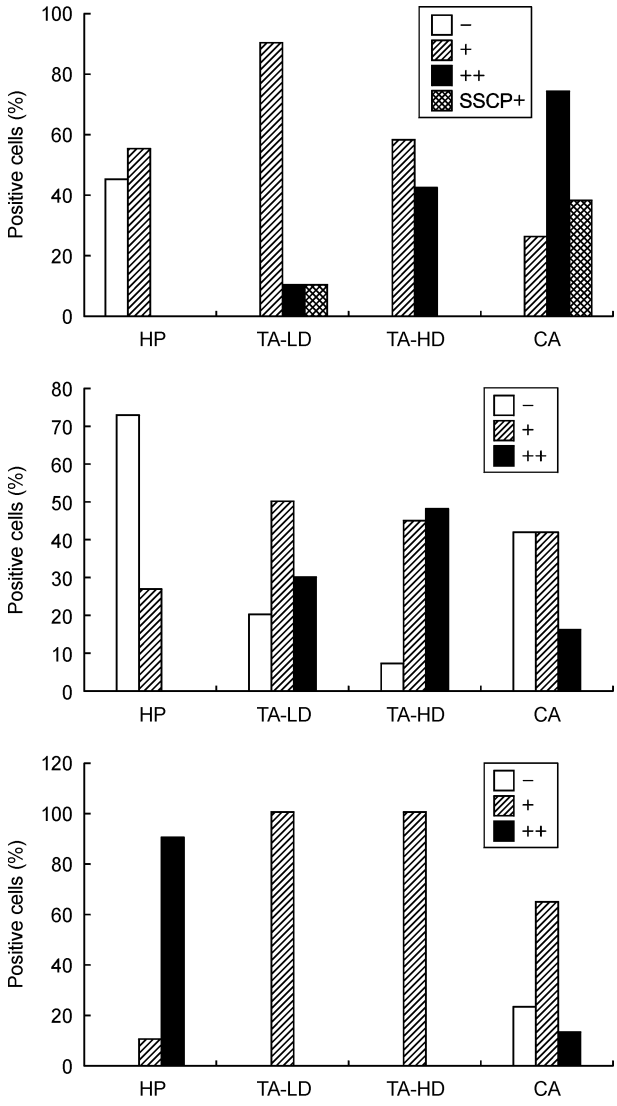

Fig. 1. Comparison of p53 (A), bcl2 (B) and p21 (C) expression in colorectal hyperplastic polyps, adenomas, and carcinomas. p53 expression increased with high-grade adenomas and was the strongest in carcinoma. *p53* gene mutation (SSCP+) was more frequent in carcinoma than in adenoma. Bcl2 expression increased with high-grade adenomas, but decreased in carcinomas. p21 expression increased in both hyperplstic polyps and adenomas, but was variable in carcinomas. HP: hyperplastic polyp, TA-LD: tubular adenoma with low-grade dysplasia, TA-HD: tubular adenoma with high-grade dysplasia, CA: carcinoma, SSCP+: presence of abnormally shifted bands in single strand conformation polymorphism.

weak p53 and p21 expression and strong bcl2 expression, *p53* mutation was detected in exon 8. Eleven of 29 carcinomas (38%) examined by PCR-SSCP showed shifted bands in exon 5 (6 cases), exon 6 (3 cases), and exon 7 (2 cases) (Table 1 and Fig. 1, 3); 1) seven cases (64%) with strong and 4 cases (36%) with weak p53 expression; 2) coincident p53 and p21 expression was observed in 8 cases with p53 mutation; 3) 6 cases showed no bcl2

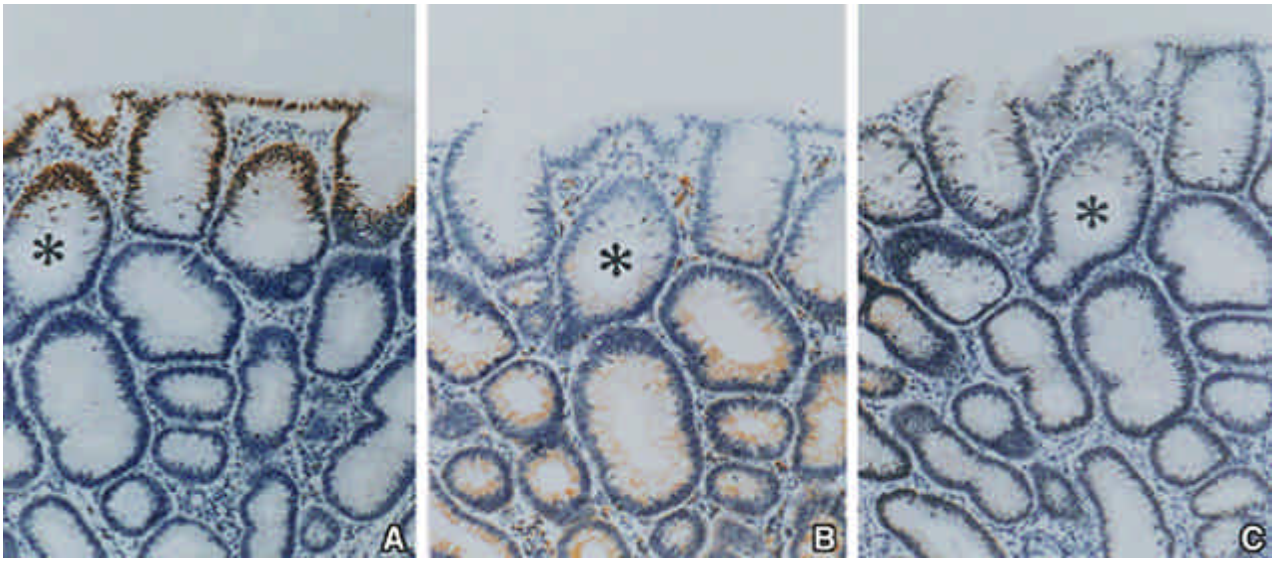

Fig. 2. Altered topographic expression of p21, bcl2 and p53 in one representative adenoma. Whereas bcl2 expression increased and extended to parabasal and superficial dysplastic epithelium, p21 expression limited to surface dysplastic epithelium. There was distinct separation between the p21- and bcl2-expressing compartments in a serial section. The full thickness of dysplastic epithelium showed weak p53 expression (ABC staining, ×400). A gland, marked by asterisk, represents the same gland in serial sections. **A:** p21, **B:** bcl2, **C:** p53 (DO7).

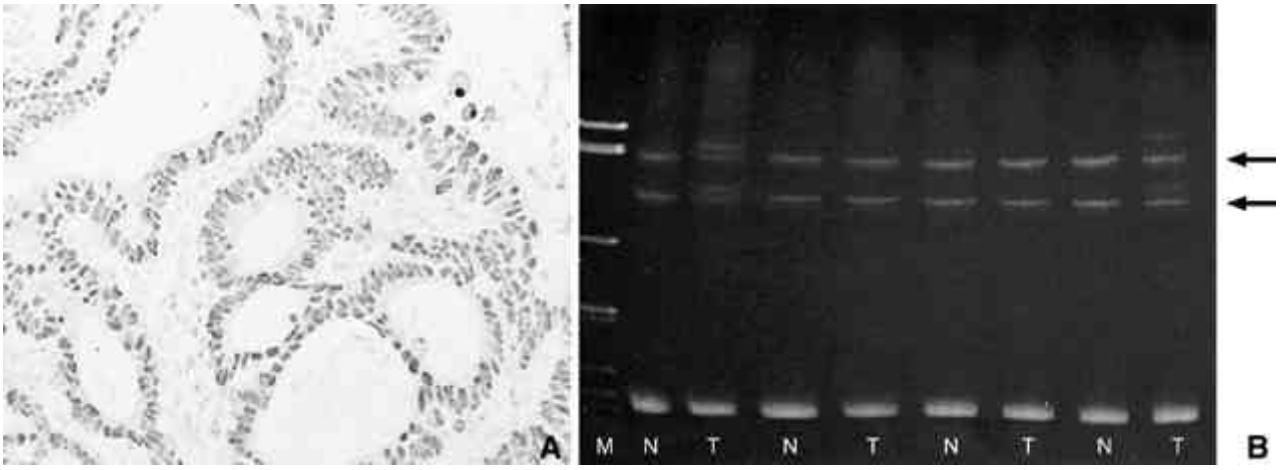

Fig. 3. Topographic expression was abrogated in carcinomas. Cold PCR-SSCP of exon 6 of p53 gene showed the abnormally shifted bands (arrow heads) in lane 2 (case 7) and 8 (case 10). The immunohistochemical result **(A)** of a case 7 (T*) showed strong p53 (DO7) expression in carcinoma cells (ABC staining, ×400). N: normal tissue, T: tumor tissue, M: marker.

expression (Fig. 4). Although there was no statistical correlation between p53 mutation and p21, bcl2, or p53 expression, p53 expression increased in carcinomas with p53 gene mutation (Fig. 4).

DISCUSSION

Our results indicate that whereas basal epithelial cells of the normal colonic crypts expressed bcl2 and wild-type p53 proteins, surface epithelial cells, which are post-replicative compartment of the crypt, did not express bcl2 or p53 but expressed p21. Given this observation along with the fact that the crypt cell population arises from basally located stem cells, it is suggested that bcl2 expression protects stem cells from apoptosis. However, during proliferation of regenerative cells, DNA alterations may occur frequently in these basal cells. Protection from apoptosis in basal cells may therefore be a consequence of bcl2 expression, but in some cells, further uncontrolled proliferation may be checked by the accumulation of wild-type p53 protein. The observation that

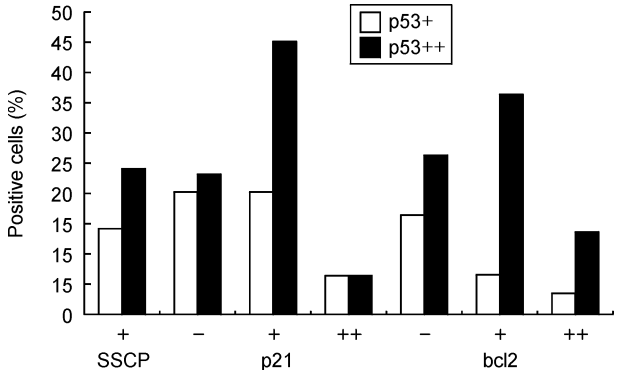

Fig. 4. Relationship between p53, p21, and bcl2 Expression in Colorectal Carcinomas. p53 expression increased in carcinomas showing p53 gene mutation (SSCP+). A significant inverse correlation was present between p21 and p53 expression. There was no positive correlation between bcl2 and p53 expression. SSCP+: presence of abnormally shifted bands in single stand conformation polymorphism.

expression of both bcl2 and wild-type p53 increased in severe chronic inflammation support these possibilities. A previous report demonstrated that p21 plays a critical role in negative control of the proliferating compartment in gastrointestinal mucosa (25). Expression of p21 alone in surface epithelial cells suggests that this topological control of p21 expression was probably independent of p53.

Colonic adenoma is believed to be the precursor lesion to cancer of the colon (26). Our study showed altered topographic expression of the three proteins in dysplastic crypts. Bcl2 expression in adenomas increased in high-grade dysplasia and was stronger than in carcinomas; p53 expression in adenomas also increased in high-grade dysplasia, but was weaker than in carcinoma. Therefore, these findings suggest that bcl2 overexpression promotes the survival of dysplastic cells, and exposes cells to possible genetic damage. Furthermore, absence of or weak bcl2 and strong p21 expression in hyperplastic polyps support the concept that bcl2 overexpression is not simply a characteristic of hyperproliferating colorectal epithelial lesions. A previous study (14) reported that overexpression of bcl2 did not affect the expression of the *p53* gene, but p53-dependent *p21* gene transcription was suppressed in breast epithelial cells. However, in the upper half of dysplastic crypts, cellular proliferation is controlled by loss of bcl2 expression and p21 overexpression. According to our data, in the colorectum, abnormal alteration of bcl2 gene may play a major role in very early phase of transformation of hyperplastic cells to dysplasia, which is in line with the results of our previous report with gastric adenoma (27).

p53 expression in adenomas increased with high-grade dysplasia and was weaker than in carcinomas. As 98% of adenomas examined in this study showed no *p53* mutation, the weak expression of p53 was interpreted as the accumulation of wild-type p53. According to these results, differences of the p53 expression pattern could be used for the differential diagnosis between high-grade dysplasia and early or microscopic carcinoma. In terms of the tumor suppressor role of wild-type p53, the role of the accumulation of wild-type p53 in dysplastic crypts remains to be elucidated in detail.

Topographic expression of bcl2, p21, and p53 was abrogated in carcinomas, which showed increased p53 expression with or without *p53* mutation. Previous studies (13, 28) reported an inverse correlation between bcl2 and p53 expression in human colorectal adenomas and breast cancers. A potential molecular basis for this effect may involve a p53-dependent negative response by the bcl2 gene through which p53 can directly or indirectly down-regulate bcl2 expression. However, our results showed no inverse correlation between bcl2 and p53 expression.

p53 mutation has been reported in over 50% of colorectal cancers (1, 3) and is thought to be an early event in both colorectal and gastric carcinogenesis. Our results revealed p53 mutation in 2% of adenomas and 38% of carcinomas and the latter frequency is lower than those in previous reports (1, 3). In this study, however, we focused on the relationship between p53 overexpression detected by immunohistochemical staining and the occurrence of p53 mutation by molecular method. The presence of wild-type p53 protein was confirmed by positive DO7 ab with no mutation in cold SSCP. Findings that normal mucosa and 98% of adenomas were weakly stained with DO7 ab with no mutation in cold SSCP reflect the presence of wild-type p53 protein. The staining intensity of wild-type p53 with DO7 ab was weaker than that of mutant p53. However, the results were controversial. One adenoma (2%), which were weakly stained with DO7 ab revealed p53 mutation; 37% of carcinomas stained strongly with DO7 ab revealed p53 mutation; 50% of carcinomas stained weakly with DO7 ab showed p53 mutation. The immunoreactivity of p53 paralleled the degree of mutation in one or both alleles, and weak staining of p53 indicated interaction of wild-type and mutant p53 proteins. We therefore propose that the detection of *p53* mutation should be done by both immunohistochemial staining (DO7 ab) and PCR-SSCP analysis of *p53* gene.

In our study, 77% of carcinomas expressed p21 with an inverse relationship with p53 staining. Previous studies on this have reported that at the cellular level, the relationship between p21 and p53 staining in colorectal tumors was inverse (13, 29). In this and other studies, however, tumor cells positive for p21 were identified in

about 70% of colorectal cancers; the actual proportion varied from case by case. Furthermore, tumor cells expressing both proteins were detected in 24 of 31 carcinomas (77%). In such cases, p21 expression cannot be explained by p53-dependent pathway, and alternative explanations for the coexpression of p21 and p53 in cancer cells have been proposed as follows: (a) although p21 expression may be basically regulated by p53-dependent pathway, it is potentially induced through the p53-independent pathway in cells with a mutation or a loss of *p53* gene (30); (b) p21 expression is largely unaffected in cells with a mutation in one allele of p53 and the other wild-type allele, and for the inhibition of p21 transcription, loss of both functional copies of p53 is necessary.

In summary, there is an ordered topographic pattern of p21, bcl2 and wild-type p53 expression in normal colorectal cells, but this pattern becomes disordered during the early stage of colorectal carcinogenesis. These results suggest a possibility that altered cell cycle and/or apoptosis control by wild-type p53 and its mediator appears to be an early event in colorectal carcinogenesis that may facilitate tumor progression.

ACKNOWLEDGEMENTS

We sincerely appreciate Dr. Mi-Na Ha, Department of Epidemiology, College of Medicine, Dankook University, for statistical analysis and Mr. Soon Chul Jang and Mr. Jae Hwan Lee for their excellent technical assistance.

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