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Immunohistochemical detection of truncated APC protein in sporadic human colorectal adenomas and adenocarcinomas

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Abstract Mutations of the *APC* gene frequently occur in sporadic forms of colorectal adenomas and adenocarcinomas. Phenotypically, the vast majority of these mutations result in the truncation of the APC protein. To demonstrate the defective *APC* gene product in human colorectal tumors, rabbit region-specific antisera raised against the APC protein of amino acid sequences between 371 and 390 (SP1) and between 1821 and 1840 (SP3) were used to exhibit the truncated APC protein. In all, 86 lesions from 67 cases of sporadic adenoma and adenocarcinoma were examined; abnormal staining patterns were distinguished in 43 lesions (50%); the incidence of abnormalities was not significantly different between adenomas and carcinomas. The majority, 75% exhibited epitopic change with the SP1-positive and SP3-negative phenotype (type P1), and 25% exhibited neither of these phenotypes (type P2). The staining pattern in all lesions was uniform, and studies of carcinomas arising in adenomas showed the same pattern of staining. These findings supported the view that the APC lesion is a very early event in colorectal carcinogenesis. Furthermore, this simple immunohistochemical approach demonstrated that different adenomas from the same patient showed different staining patterns.

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Introduction

The study of genes in tumorigenesis has been a central theme in recent years. The tumor suppressor gene *APC* has been identified as a causative factor in familial adenomatous polyposis (FAP), but its involvement in sporadic forms of colorectal tumors has been controversial [2, 4, 5, 8] and its functional role is still unclear. Evidence has given rise to the concept that alterations in the *APC* gene occur in more than 50% of sporadic tumors and that these changes consequently result in loss of gene function leading to the development of tumors [9].

Alterations of the *APC* gene detected in sporadic colorectal tumors are frameshift mutations, such as point mutations, small deletions or insertions [1, 8, 9]. All these mutations generate stop codons, and phenotypically they result in various lengths of truncated proteins [8]. Based on this evidence, we visualized the truncated APC protein expressed in colorectal adenomas and carcinomas immunohistochemically in paraffin-embedded sections. Rabbit antisera were obtained by immunizing synthetic peptides from various portions of the APC protein. In colorectal tumors with the defective APC protein, the cells would lose certain epitopes, and such phenotypic changes can be characterized by the region-specific antisera. To investigate these premises, we applied rabbit antisera against the APC protein to Western blot analysis and immunohistochemical staining, and the defective proteins were characterized in tumor cells from cultured human adenocarcinoma cells and in surgically resected or polypectomized specimens. We demonstrate and discuss the usefulness of this simple immunohistochemical technique.

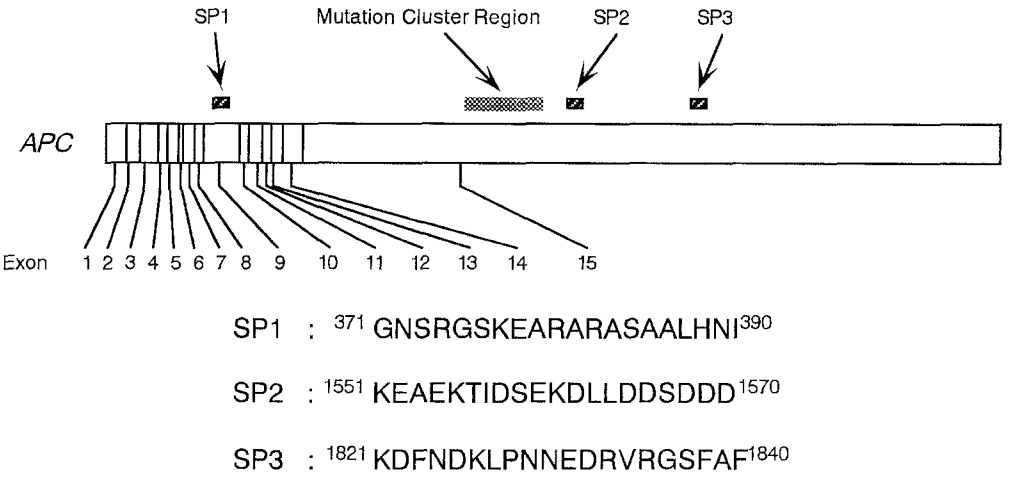


Fig. 1 Schematic map of synthetic peptides and amino acid sequences

Materials and methods

Preparation of cells and tissue samples

Human HT-29 colon cancer cells and MDA-MB-231 breast cancer cells were obtained from the American Type Culture Collection (Rockville, Md.). The cells were seeded (2×10^5 cells/plate) and cultured in 90-mm petri dishes (Greiner, Frickenhausen, Japan) in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum at 37° C in an atmosphere of 5% CO_2 in humidified chambers and harvested after 2 days. The defective 170-kDa APC protein and the intact 310-kDa one were detected in HT-29 and in MDA-MB-231 cells, respectively.

Tissue samples were obtained from the large intestine by polypectomy or by surgical resection at Ehime University Hospital from 1992 to 1994. Immediately after resection, all samples were routinely fixed in 10% formalin and embedded in paraffin. Sporadic colorectal tumors from 67 patients comprised 23 specimens from 23 cases of solitary adenoma, 29 specimens from 11 cases of multiple adenoma, and 34 specimens from 34 cases of adenocarcinoma, including 8 specimens of carcinoma in adenoma. One case of carcinoma overlapped with a case of multiple adenoma. None of the cases had any polyposis lesions of the FAP type or any family history of polyposis syndromes. In each resected specimen, mucosa 5 cm from the tumor edge without atypia was used as a normal control. In the polypectomized specimens, such controls were not available; therefore the stainability of the tumor cells was compared with that of adjacent mucosa with normal appearance; where such mucosa was not available, both negative and positive controls were prepared and stained together.

Histological diagnoses of the tumors were made according to the classification of Japanese Research Society for cancer of the colon and rectum [3].

Synthesis of peptides

Designation of the synthetic peptides is shown in Fig. 1. Three twenty mers from different portions, designated as synthetic peptide-1 (SP1) (^{371}GNSRGSKEARARASAALHNI^{390}), SP2 (^{1551}KEAEKTIDSEKDLLDDSDDD^{1570}), and SP3 (^{1821}KDFNDKLPNNEDRVRGSFAF^{1840}) [5], were synthesized with a peptide synthesizer (Model 431A, Applied Biosystems, Foster City, Calif.). The synthetic peptides were then removed from the solid face, purified with HPLC, and dried. Their amino acid sequences were confirmed with a protein sequencer (Model 477A, Applied Biosystems)

Preparation of antisera against synthetic peptide and Western blot analysis

The antiserum to the synthetic peptide was prepared by immunizing an adult female Japanese white rabbit (Inoue Laboratory Animal Center, Kumamoto, Japan) three times with ovoalbumin-peptide conjugate complex as an antigen. The IgG fraction was purified with protein A Sepharose column. The specific antibody to SP1 was further purified using an affinity column conjugated with SP1 as ligand (5 mg peptide bound to 1 ml of Sepharose CL-6B, Pharmacia Biotech, Uppsala, Sweden). Before electrophoretic separation of the cellular proteins, subcellular components were fractionated and immunoblot analysis were done on membranous, cytoplasmic, and nuclear fractions as described elsewhere [15]. Lysate from each fraction or marker protein was electrophoresed on 6% SDS-PAGE or 3% agarose gel and blotted onto Hybond-C-nitrocellulose membrane (Amersham-Japan, Tokyo, Japan). The nonspecific binding of antibodies was blocked with 5% skim milk, then the membranes were incubated with rabbit antiserum (3 μg/ml of IgG fraction of 0.2 μg/ml of peptide-specific antibody in phosphate-buffered saline, and 1% bovine serum albumin) for 2 h, with goat anti-rabbit IgG (0.2 μg/ml) for 1.5 h, and with peroxidase and rabbit antiperoxidase complex (0.1 μg/ml) for 30 min and then washed four times with buffer (PBS and 0.02% Tween-20). Peroxidase activity was visualized with the enhanced chemiluminescence detection kit (Amersham, UK) according to the manufacturer's instructions.

Immunocytochemical and immunohistochemical staining

Immunocytochemical staining was carried out as described previously [11]. In short, HT-29 and MDA-MB-231 cells were fixed with 2% formalin for 10 min, and with cold acetone for 90 s, then incubated with rabbit antiserum to APC peptide at 37° C for 30 min, and with FITC-conjugated antirabbit IgG (Zymed, South San Francisco, Calif.) for 30 min. Immunocytochemical findings were visualized with a Zeiss confocal laser-beam microscope (LSM 410, Carl Zeiss, Oberkochen, Germany).

Immunohistochemical staining was done according to the staining manual from Nichirei (Histofine SAB-PO(R) Kit; Nichirei, Tokyo, Japan]. Briefly, the thin sections were deparaffinized, washed with PBS, treated with 3% hydrogen peroxide to block endogenous peroxidase activity, and incubated with region-specific antisera to the APC protein for 12 h at 4° C, with biotin-labeled antirabbit IgG for 10 min at room temperature, with horseradish-labeled streptavidin for 10 min at room temperature, and with 0.2 mg/ml 3,3′-diaminobenzidine, 4 HCl (Wako, Osaka, Japan) and 0.6% hydrogen peroxide for 5 min. Finally, the sections were counterstained with 1% Methyl Green (Sigma, St. Louis, Mo.) and mounted. To confirm the specificity of the rabbit antisera to the

APC protein, the antisera were treated with excess peptides to abrogate the binding activity of the APC protein to the target sites.

Results

Reactivity of antisera raised to the synthetic peptides

The antisera to SP1 peptide (anti-SP1), purified with the affinity column chromatography, specifically reacted with the SP1 peptide at concentrations of more than 5 ng/ml. They did not react with the carrier protein ovoalbumin at less than 500 ng/ml (Fig. 2). Antisera to SP3 (anti-SP3) also revealed higher titration and reacted with SP3 at more than 1:1000 the dilution of the original; antisera to SP2 were not obtained, partly because SP2 showed the lowest antigenic index of the three peptides analyzed by the MacVector system (International Biotechnologies, New Haven, Conn.).

Anti-SP1 and anti-SP3 reacted with the 310-kDa APC protein in the membrane fraction of the MDA-MB-231 cells (Fig. 3, lanes 1 and 3). The anti-SP1 successfully reacted with the truncated 170-kDa protein in the cytoplasmic fraction of the HT-29 human colon cancer cells but not with the membrane fraction (Fig. 3, lane 6), This 170-kDa band, however, was not detected in any fractions of the HT-29 cells by anti-SP3 (Fig. 3, lanes 7 and 8), indicating that the antigenic epitope recognized by Anti-SP3 was absent in the truncated APC protein expressed in HT-29 cells.

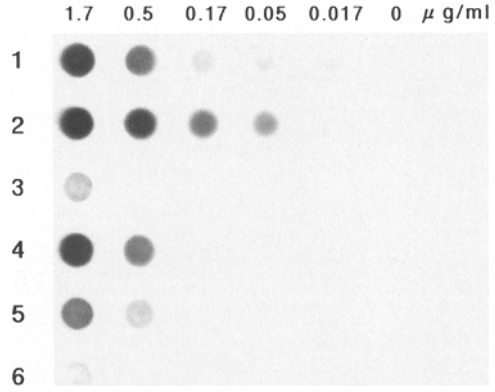


Fig. 2 Titration of anti-SP1 using dot blot. The reactivity of anti-SP1 was assayed with SP-1 peptide (*lanes 1, 2,* and *3*) or ovoalbumin (*lanes 4, 5,* and *6*). Each antibody was tested at concentrations of 17 ng/ml to 1.7 μg/ml. *Lanes 1, 4* crude anti-SP1; *lanes 2, 5* affinity-purified anti-SP1; *lanes 3, 6* preimmune serum

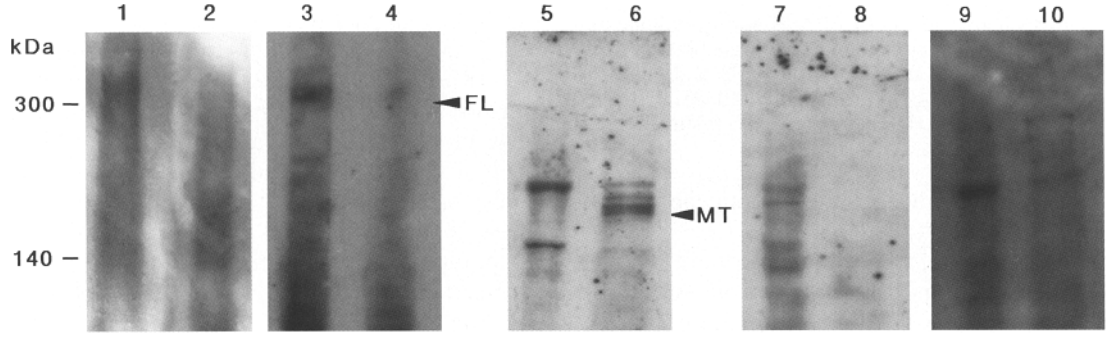
Fig. 3 Demonstration of the full-length and truncated APC proteins. Full-length 310-kDa APC protein (*FL*) was detected by both anti-SP1 and anti-SP3 in membrane fraction from MDA-MB-231 cells (*lanes 1* and *3*), while truncated 170-kDa mutant protein (MT) was detected by only anti-SP1 in the cytosolic fraction from HT-29 cells (*lane 6*). Several nonspecific bands appearing in *lanes 5–10* reacted with the preimmune and anti-APC sera. *Lanes 1–4* MDA-MB-231 cells, *lanes 5–10* HT-29 cells. *Lanes 1, 3, 5, 7, 9* membrane fraction; *lanes 2, 4, 6, 8, 10* cytosolic fraction. *Lanes 1, 2, 5, 6* anti-SP1; *lanes 3, 4, 7, 8* anti-SP3; *lanes 9, 10* preimmune serum

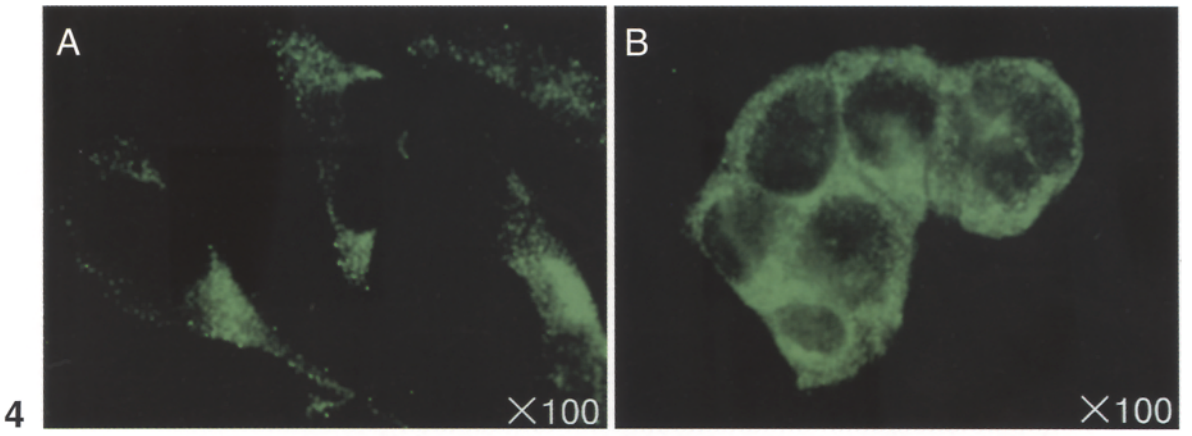
Immunocytochemical detection of truncated APC protein

The immunofluorescent staining of the APC protein is shown in Fig. 4. The intact 310-kDa protein expressed in MDA-MB-231 cells and the truncated one in HT-29 cells were visualized. In MDA-MB-231 cells, anti-SP1 and anti-SP3 manifested a patchy and, in places, filamentous pattern near the cytoplasmic and nuclear membranes (Fig. 4A). The two staining patterns were almost identical. In HT-29 cells, however, anti-SP1 manifested fluorescence-positive areas, without any foci or structural features, scattered in the (Fig. 4B). Anti-SP3 stained negative in HP-29 cells as demonstrated by Western blot analysis (data not shown). The fluorescence-positive features were not generated by either the preimmune serum of the antisera (anti-SP1 or anti-SP3) treated with excess synthetic peptides (antigen SP-1 or SP-3, respectively).

Immunohistochemical analysis of colorectal tumors

In the normal mucosa from different portions of the colon no staining differences were detected. Immunohistochemical staining with anti-SP1 and anti-SP3 are shown in Fig. 5A and B, respectively, Their staining patterns were the same. Mature goblet cells and columnar epithelia were stained more strongly than less mature cells near the base of crypts, where both SP1 and SP3 epitopes were observed mainly near the basement membrane and around the perinuclear area of colonocytes. Cytoplasmic





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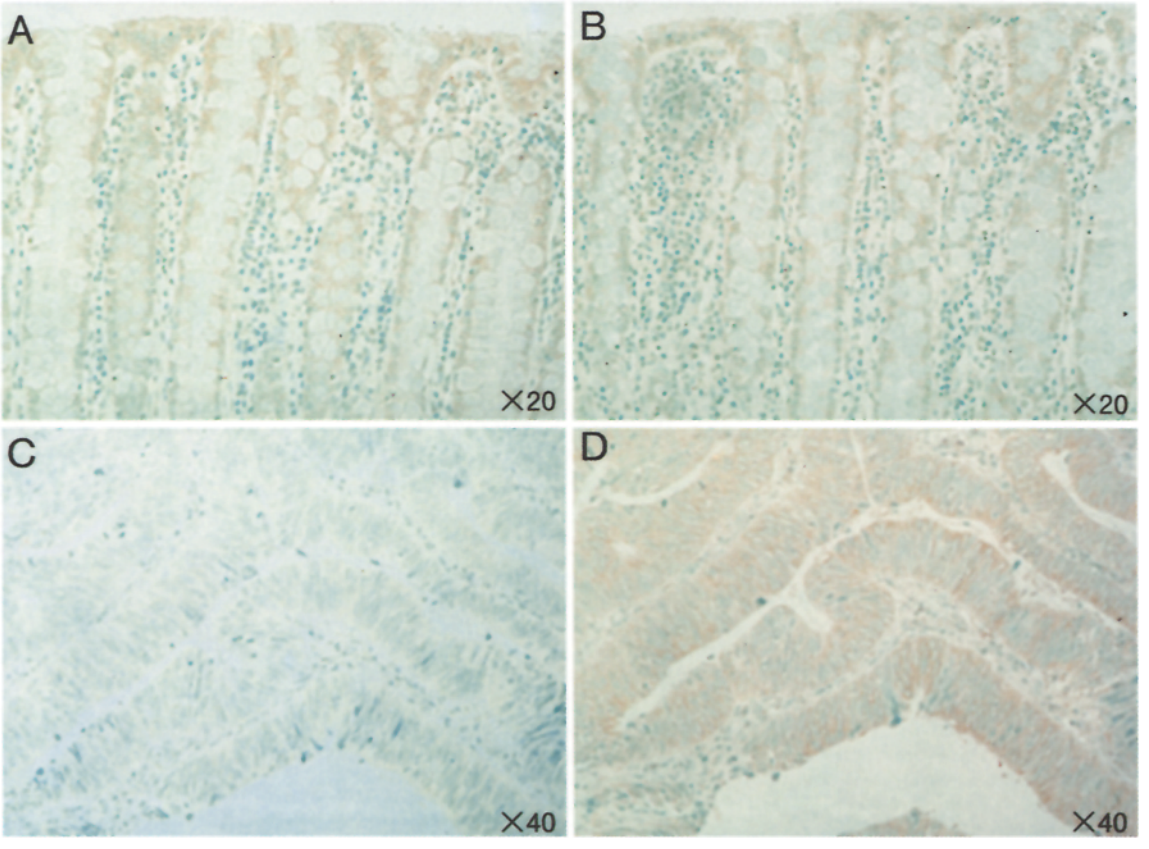

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Fig. 4A, B Analysis of the APC protein by confocal laser scanning microscopy. The APC protein was localized in the cytoplasm. The staining of the full length protein by anti-SP1 showed a patchy and partly filamentous pattern in MDA-MB-231 cells (**A**). The truncated protein was more diffusely stained in HT-29 cells (**B**), ×100

Fig. 5 Immunohistochemical staining of **A**, **B** normal colonic mucosa (×20) and **C, D** carcinoma in adenoma of case 940388 (×40) with **A, C** 3.0 µg/ml of IgG fraction of anti-SP3 and **B, D** 0.2 µg/ml of peptide-specific anti-SP1. In case no. 940388, the lesion stained positive with anti-SP1 (**D**) and negative with anti-SP3 (**C**). Refer to type P1 pattern in text

staining was more homogeneously and noticeably manifest in the upper half of crypts.

Unlike normal mucosa, tumor cells belonging to adenomas and carcinomas generated homologous staining in each lesion (Fig. 5). Three different staining patterns were distinguished in the tumors, however: type N was similar to normal mucosa with SP1- and SP3-positive epitopes, type P1 manifested phenotypic change with SP1-positive and SP3-negative epitopes, and type P2 exhibited neither the SP1-nor the SP3-positive epitope. In the 86 lesions that were adenomas and carcinomas, types N, P1, and P2 were detected in 43 (50%), 32 (37%), and 11 (18%) of the lesions, respectively (Table 1). There were no significant differences in the incidence of type between adenoma and carcinoma lesions. The lesions in 11 cases of multiple adenoma were of the following types: mixed N and P1 (3 cases), mixed N and P2 (1 case), N (2 cases), P1 (4 cases), and P2 (1 case). Among the polypectomized specimens, positive controls (normal mucosa) were available in only 6 cases, and of the remaining 28, specimens consisting of tumor cells, we

Table 1 Summary of immunohistochemical staining

Lesion	APC staining[a]			
	Positive	Negative		Total
		P-1	P-2	
Adenoma	25	20	7	52
Solitary	14	9	0	23
Multiple	11	11	7	29
Carcinoma in adenoma	5	3	0	8
Carcinoma	13	9	4	26
All	43	32	11	86

[a] APC staining patterns recognized: *Positive* both APC-2 and APC-3 regions were positively stained, *P-1* only the APC-3 region was stained, *P-2* neither APC-2 nor APC-3 was stained

confirmed 14 lesions as being of epitope type P1 and one as being of epitope type P2 (no. 941650, multiple adenoma). Another lesion of epitope type N was utilized as a normal control. The former 14 lesions were easily recognized as having pattern type P1 from the differences among the stainability of the tumor cells themselves with anti-SP1 and anti-SP3. Three lesions from single adenomas with abnormality type 2 were not taken into account, because these cases did not have definitive positive controls (data not shown).

In all the lesions examined, the areas of tumor cells stained homogeneously. Even in cases with carcinoma in adenoma, both lesions stained similarly. No mosaic staining pattern was detected in any of the lesions. Different staining patterns were recognized in cases with multiple adenomas only, where each lesion had a simple pattern.

These results suggested the clonal growth of tumor cells in each lesion.

Discussion

Immunohistochemical studies were carried out to demonstrate the APC protein expressed in colorectal tumors. The antisera that reacted with the specific epitope of the protein were used for Western blot analysis and immunohistochemical staining of the protein. We distinguished two abnormal immunohistochemical patterns: type P1 (lesions consisting of tumor cells with the SP1 epitope only) and type P2 (lesions without either SP1 or SP3 epitope). Type P1 tumors were common in both adenomas and carcinomas. Our results are in agreement with the finding that mutations of *APC* in sporadic cases have been detected in a narrow area coding amino acids from 1286 to 1513, which exists inside exon 15 and is called the mutation cluster region (MCR) [9]. In our study the MCR was localized between epitopes SP1 and SP3.

In this study, the key was to obtained epitope-specific antisera. We examined three synthetic peptides, and antisera were consequently raised against the peptides with the two highest antigenic scores (SP1 and SP3), but not against that with the lowest score (SP2). Anti-SP1 and anti-SP3 reacted with the immunized peptide and ovoalbumin, a carrier protein. We also purified anti-SP1 by affinity chromatography. Immunochemically and immunohistochemically, however, there were no differences between purified anti-SP1 and crude anti-SP1 in examining the APC protein (Fig. 2). The stainability of anti-SP1 and anti-SP3 was somewhat different from previous findings described by Kinzler et al. [15], who showed membranous staining of the basolateral margins of colonocytes in the upper crypt. Later, they demonstrated the association of wild-type APC protein with the microtubule cytoskelton [6]. Their immunofluorescent staining, which was close to ours shown in Fig. 4, indicated cytoplasmic localization of the APC protein. Thus, our immunohistochemical staining of colonocytes, which is shown in Fig. 5, is conceivably consistent with their previous findings. It is also possible that because our data (Fig. 5) were obtained from formalin-fixed paraffin-embedded sections, the figures are less sharp than would be obtained from frozen samples [8].

FAP is one of the hereditary disorders in which Knudson's double knockout model is used. In affected cases, only the mutant allele is retained in tumour cells [6, 7]. Similar genetic changes are also demonstrated in sporadic colorectal tumors [1, 8, 9]. The dominant negative hypothesis of the *APC* gene previously proposed [17] was that tumor cells might retain both wild-type and mutant alleles and express both messages. If this were so, our immunohistochemical system would not be viable. This idea was, however, negated by Oshima et al. [12] using transgenic mice. Thus, immunohistochemical findings that colorectal tumors exhibit truncated APC protein clearly imply a double knockout of the *APC* gene in these lesions.

Involvement of the *APC* gene is known to be an earlier event in oncogenesis in the colorectal mocusa. Our immunohistochemical studies support this concept; each lesion was homogeneously stained. In cases of carcinoma in adenoma, both lesions carried the same phenotype. Although different staining patterns were demonstrated in cases with multiple adenomas, each lesion expressed only one phenotype. These results suggest that the clonality of the *APC* gene was determined before or just as the adenoma arose, and no additional mutations of the gene occurred in any lesion.

Functional roles of the APC protein might be exhibited by binding to certain proteins, including β-catenin or other putative binding proteins [10, 13, 14, 16, 18, 19]. Our results indicate that the defective APC protein might

lose the binding activity to membrane components (Fig. 2). How the loss of the APC function leads to colorectal tumors is, however, still a subject of controversy. Further functional studies would be necessary to reveal the involvement of the *APC* gene in colorectal carcinogenesis; for this, region-specific antisera to the APC protein would be useful. In this study, we have demonstrated that the epitope-specific antisera to the APC protein are reliable and reproducible for detecting the truncated APC protein in colorectal ademonas and carcinomas, and are also helpful in suggesting particular regions where truncation occurs.

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