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# Frequent Allelic Imbalance on Chromosome 18q21 in Early Superficial Colorectal Cancers

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**Genetic alterations in early superficial colorectal cancers have rarely been reported. In the present study, we searched for alterations in the *APC* and *p53* genes in 27 superficial (20 depressed and 7 elevated) and 21 protruding colorectal cancers with submucosal invasion by means of PCR-single strand conformation polymorphism. Allelic imbalance (AI) on five loci, i.e., 1p34-36, 8p21-22, 14q32, 18q21 and 22q12-13, was also analyzed. Since a high incidence of 18q21 AI was detected in the superficial depressed cases, we further screened for alterations in *Smad2*, *Smad4* and *DCC*. *APC* alterations were observed in three superficial depressed, one superficial elevated, and 11 protruding colorectal cancers, indicating that the frequency of *APC* alterations in superficial depressed cases was significantly lower than that in the protruding ones. There was no significant association between *p53* alterations and macroscopic types. AI on 18q21 (13/20, 65%) was much higher than those on the other four loci in the superficial depressed cases. Moreover, the frequency of 18q21 AI in the superficial depressed cases was significantly higher than that in the protruding ones. *Smad4* alterations were only detected in 1 of the 13 superficial depressed and 3 of the 17 protruding cases, while *Smad2* and *DCC* alterations were not detected in any case examined. These data suggest that the carcinogenetic pathways of protruding and superficial depressed colorectal cancers are different, and that alterations of tumor suppressor gene(s) located on 18q21 other than *Smad2*, *Smad4* and *DCC* might be associated with most superficial depressed colorectal cancers.**

Key words: Allelic imbalance — APC — Early superficial colorectal cancer — Smad4 — 18q21

Colorectal carcinogenesis is regarded as a multistep process involving both activation of proto-oncogenes and inactivation of tumor suppressor genes.[1)] Loss of heterozygosity (LOH) on several specific chromosomes is thought to be an indicator of the presence of tumor suppressor genes within the lost regions. According to previous reports on allelotype profiles at several stages of colorectal cancers (CRCs), LOH was mostly found on chromosomes 1p, 5q, 8p, 14q, 17p, 18q and 22q.[1–3)]

The *APC* gene, located on chromosome 5q21, has been identified as the causative gene for familial adenomatous polyposis (FAP).[4)] Moreover, frequent somatic mutation or LOH of *APC* has been reported not only in FAP tumors, but also in sporadic colorectal adenomas and cancers.[5, 6)] Since *APC* alterations have been detected even in very small adenomas and *cancer in adenomas* in the colorectum,[5–7)] *APC* inactivation may initiate adenoma formation in colorectal tumorigenesis. The *p53* gene is a tumor suppressor gene located on chromosome 17p13, and alterations of it have been commonly observed in colorectal cancers, but not in adenomas. Thus, mutations of *p53* have been considered to occur in the adenoma-to-carcinoma conversion in colorectal carcinogenesis.[8)]

LOH on chromosome 18q21 was found at several stages of CRCs,[1, 9, 10)] and three candidate tumor suppressor genes, *DCC*, *Smad2* (also known as *JV18-1* and *MADR2*) and *Smad4* (also known as *DPC4*), were identified in the locus.[11–13)] The *DCC* gene encodes a cell surface glycoprotein exhibiting homology to the neuronal cell adhesion molecule family. LOH on 18q21 including the *DCC* locus, as well as the absence of the DCC protein, has been reported to be a prognostic marker in patients with stage II or III CRCs, suggesting that *DCC* alterations might be related to lymph node and liver metastases in the progression of CRCs.[9, 14)] Both Smad2 and Smad4 are intracellular transducers in the transforming growth factor (TGF)-β signaling pathway that inhibits the growth of colon epithelial cells. Alterations of *Smad2* or *Smad4* have been found in some advanced CRCs with 18q LOH.[12, 13, 15–17)] These alterations might play some role at a late stage of colorectal carcinogenesis.[16, 17)]

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It is widely accepted that colorectal carcinogenesis involves a pathway called the adenoma-carcinoma sequence, in which cancer is thought to arise from a pre-existing adenoma.[18] Early flat or superficial CRCs have been reported in Japan, most of which had no adenomatous component.[19–21] Depressed-type CRCs frequently invade the submucosal layer, even though their sizes are under 10 mm.[20] These cancers are thought to be *de novo* CRCs that directly develop from normal colonic mucosa.

It has been reported that the K-*ras* proto-oncogene is more frequently mutated in early protruding CRCs than in superficial ones.[22, 23] We found that the mutation frequency of *APC* in protruding (I) and superficial elevated (IIa) mucosal CRCs is higher than that in other superficial type CRCs, such as the depressed type.[23] These data suggested that protruding as well as some superficial elevated CRCs are strongly associated with the adenoma-carcinoma sequence, which is different from the pathway in other superficial CRCs. We also reported low frequencies of microsatellite instability (MSI) and frameshift mutations of the TGF-β type II receptor gene, both of which are induced from the defective mismatch repair system, in superficial mucosal CRCs as well as protruding ones.[23] Therefore, MSI may not be associated with most mucosal CRCs.

To clarify the genetic alterations of early superficial CRCs, *APC* and *p53* alterations were examined in early superficial as well as protruding CRCs with submucosal invasion. Moreover, we searched for allelic imbalance (AI) in several chromosomal regions, in which LOH has been reported to be frequent in CRCs.[1–3] Since a high incidence of AI was detected on the 18q21 locus, we further screened for alterations of *DCC*, *Smad2* and *Smad4*, which are located on this locus.

## MATERIALS AND METHODS

**Tissue samples** A total of 48 early CRCs with submucosal invasion and corresponding normal tissues were collected at the International Medical Center of Japan. We excluded CRCs that occurred in patients with FAP or hereditary nonpolyposis colorectal cancer.[4] According to the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus,[24] non-protruding cancers were macroscopically classified as the superficial (II) type as opposed to protruding (I) type submucosal cancers. Twenty-seven were superficial type and 21 were protruding type, and none showed metastasis to the lymph node or liver. We had previously searched for LOH in some of these cases.[10] Superficial-type early CRCs include superficial elevated (IIa), predominantly raised with central depression (IIa+IIc), flat (IIb), depressed (IIc) and raised with predominance of central depression (IIc+IIa) ones. Based on our previous report on *APC* alterations,[23] we divided the superficial type into two groups. Of the 27 superficial CRCs, 7 were categorized as the superficial elevated and 20 as the superficial depressed type (14 as IIa+IIc, 4 as IIc and 2 as IIc+IIa). None were categorized as flat CRCs in this study.

Two or three 10 μm sections of paraffin-embedded tissues were prepared and then microdissected. Genomic DNA was extracted from the tissues as described previously.[10] Moreover, an adjacent 3 μm section was stained with hematoxylin-eosin for histological examination.

**Mutation analysis of *APC*** Somatic mutations of *APC* mostly occurred within a small region, i.e., codons 1286–1513 in exon 15, indicating a mutation cluster region (MCR).[5] To search for *APC* mutations in early CRCs, we used seven overlapping primer sets that amplify codons 1267–1530 as described previously.[23] The size of each polymerase chain reaction (PCR) product of *APC* was less than 200 bp, this being suitable for the amplification of DNA from paraffin-embedded tissues. PCR comprised 35 cycles of 94°C for 1 min, 51–60°C for 2 min, and 72°C for 1 min in standard solutions. Nonradioisotopic single strand conformation polymorphism (SSCP) analysis was performed according to the method described previously.[23]

Nonsense mutations at codon 1450 in the *APC* MCR have frequently been observed in colorectal adenomas and carcinomas.[25] We reported finding the same mutation on PCR-restriction fragment length polymorphism (RFLP) analysis.[23] After PCR involving modified primers, the amplified DNA was digested with *Taq*I and then run on 15% polyacrylamide gels, followed by ethidium bromide staining.

When abnormal patterns were observed on SSCP and *Taq*I digestion analyses, the PCR products were sequenced directly with a cycle sequencing kit (TaKaRa, Kyoto) using end-labeled primers.

**LOH analysis of *APC*** Since there were two highly polymorphic sites, codons 486 and 1493,[6, 7] in the *APC* coding region, we screened for *APC* LOH at these regions in the early CRCs by PCR-RFLP. The primers used for PCR were 5′-GGACTACAGGCCATTGCAGAA-3′ and 5′-GGCTACATCTCCAAAAGTCAA-3′ for codon 486 in exon 11, and 5′-CGAAGGGTCCAGGTTCTTCC-3′ and 5′-TCCTGAACTGGAGGCATTATTC-3′ for codon 1493 in exon 15. After amplification of exons 11 and 15, the PCR products were digested with *Rsa*I and *BsaJ*I, respectively, and then electrophoresed on 12.5% polyacrylamide gels.

**Detection of *p53* alterations** *p53* alterations can be detected by immunohistochemical staining, because inactivation of the p53 protein by mutations prolongs its half-life.[8] The DO-7 monoclonal antibody (DAKO A/S, Copenhagen, Denmark) recognizes an epitope at the N-terminus of both the wild type and mutant p53 proteins. Four micrometer sections of paraffin-embedded tissues were

deparaffinized in xylene, followed by dehydration. Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase in methanol. Microwave-based treatment for antigen retrieval was performed. Sections were then incubated in 30 min at room temperature with the p53 antibody at the dilution of 1:50. The bound antibody was detected with a peroxidase-labeled polymer-conjugated anti-mouse antibody (ENVISION System; DAKO Corp., Carpinteria, CA),[7] and a 0.05% 3,3′-diaminobenzidine tetrahydrochloride solution. As a negative control, phosphate-buffered saline was used in each system. Each slide was counterstained with hematoxylin and then mounted.

To confirm the abnormal p53 expression in the early CRCs, we screened for mutations in exons 5–8 by PCR-SSCP analysis,[8] in which somatic mutations were frequently detected in CRCs.

**AI analysis using microsatellite markers** To study allelic deletion in early CRCs, we examined DNA for AI at 10 microsatellites containing the $(CA)_n$ repeat localized to chromosome arms 1p34-36 (D1S199), 8p21-22 (NEFL, D8S1725, D8S1731), 14q32 (D14S267) 18q21 (D18S1143, D18S467, D18S474, D18S487), and 22q12-13 (D22S282).[10]

PCR was performed in 25 $\mu$l reaction mixtures comprising 20–100 ng of template DNA, 5 pmol of each oligonucleotide primer pair (one end-labeled with [$\gamma$-$^{32}$P]ATP), 0.5 units of *Taq* DNA polymerase (Biotech International, Bentley, Australia), 2.5 $\mu$l of 10× buffer, and 1 $\mu$l of 1.25 mmol dNTP (Pharmacia, Uppsala, Sweden). After denaturation for 2 min at 94°C, each PCR was carried out for 30 cycles consisting of denaturation for 1 min at 94°C, annealing for 2 min at 53–60°C, and extension for 1 min at 72°C, followed by final extension for 10 min at 72°C. The PCR products were denatured in 98% formamide for 2 min at 80°C and then electrophoresed on denaturing 6–8% polyacrylamide sequencing gels. After electrophoresis, the gels were exposed to X-ray film for 2–48 h. AI was defined as a >50% reduction in the band intensity on visual inspection for either of the two alleles in a carcinoma in comparison with that in the corresponding normal tissue. The cancers that showed MSI at 2 or more of the 10 microsatellite markers were excluded, due to the difficulty in determining AI.

**Detection of alterations in the tumor suppressor genes, *DCC*, *Smad2* and *Smad4*** The DCC monoclonal antibody (PHARMINGEN, San Diego, CA) recognizes an epitope in the cytoplasmic domain.[14] As for Smad4, the monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) recognizes the epitope at the C-terminus of Smad4.[26] Immunostaining of DCC (dilution, 1:200) and Smad4 (dilution, 1:100) was performed with the ENVISION System and by the avidin-biotin peroxidase complex method (Santa Cruz Biotechnology), respectively.

It has been reported that somatic mutations of *Smad2* and *Smad4* are mostly located in the MH2 domain in exons 8–11.[12, 13, 15–17] We used 17 overlapping primer sets of the MH2 domains in *Smad2* and *Smad4*, i.e., nine for *Smad2* and eight for *Smad4*, and then screened for mutations in the domains on PCR-SSCP analysis.

Table I. Clinicopathological Features of Colorectal Cancers with Submucosal Invasion

| | | Macroscopic classification[a] | | |
|---|---|---|---|---|
| | | Superficial depressed (IIa+IIc, IIc+IIa, IIc) | Superficial elevated (IIa) | Protruding (I) |
| Total number | | 20 | 7 | 21 |
| Age range (mean) | | 41–83 (64) | 57–82 (69.4) | 49–77 (63.0) |
| Gender | Male | 12 | 5 | 18 |
| | Female | 8 | 2 | 3 |
| Location[b] | Proximal | 7 | 2 | 6 |
| | Distal | 13 | 5 | 15 |
| Size range in mm (mean) | | 5–41 (15.0) | 13–27 (20.4) | 3–40 (20.6) |
| Histology[c] | Well | 15 | 6 | 18 |
| | Mod | 5 | 1 | 3 |

*a*) IIa+IIc, predominantly raised with central depression; IIc+IIa, raised with predominance of central depression; IIc, depressed-type; IIa, superficial-elevated type; I, protruding type. There were no significant associations of clinicopathological features among the superficial depressed, superficial elevated and protruding colorectal cancers.
*b*) Locations of the colorectal cancers. Proximal, cecum, and ascending and transverse colon; distal, descending and sigmoid colon, and rectum.
*c*) The pathological features of cancers were classified according to the Japanese General Rules.[24] Well, well differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma.

## RESULTS

***APC* alterations in early CRCs** A total of 48 early CRCs with submucosal invasion were divided into three types, i.e., protruding (I), superficial elevated (IIa) and superficial depressed (IIa+IIc, IIc+IIa, IIc) types (Table I). There were no significant associations of clinicopathologic features among these three types.

Abnormal SSCP or altered *Taq*I digestion patterns at MCR in *APC* exon 15 were detected in 3 (15%) of the 20 superficial depressed CRCs, none of the 7 superficial elevated ones and 9 (42.9%) of the 21 protruding ones (Fig. 1 and Table II). The mutant band for each of the cancers was not observed for the corresponding normal tissue, indicating somatic mutations. Sequencing revealed that all the mutations encoded truncated APC proteins through nonsense or frameshift mutations. When we searched for *APC* LOH at two intragenic polymorphic sites, i.e., codons 486 and 1493, by means of PCR-RFLP, 11/20 (55%) patients

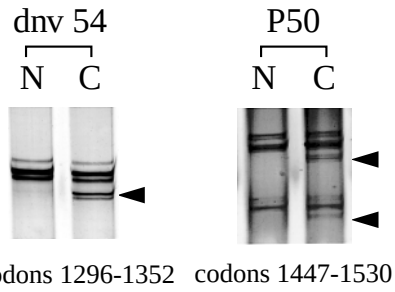


Fig. 1. PCR-SSCP analysis of the *APC* MCR in the early CRCs with submucosal invasion. The numbers at the top are the case numbers (left, superficial depressed type; right, protruding type). N, normal mucosa; C, cancer. The mobility shifts are indicated by arrowheads. After direct sequencing, a nonsense mutation (CAG to TAG) at codon 1328 and a frameshift mutation (CAT to C) at codon 1490 were detected in the superficial depressed and protruding colorectal cancers, respectively.

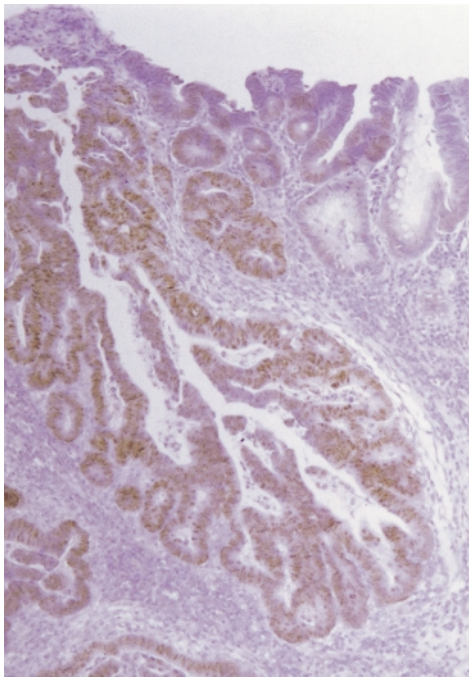

Fig. 2. Immunohistochemical staining of the p53 protein in a superficial depressed colon cancer. Positive staining of the p53 protein localized in the nuclei was detected in the cancerous region but not in the normal mucosa. Magnification ×80.

Table II. Frequencies of Genetic Alterations in Colorectal Cancers with Submucosal Invasion

| Macroscopic type | Frequencies of genetic alterations | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *APC* alterations | | | | | *p53* alterations | | | | 18q21 | AI (%) | | Smad4 | (%)[a] |
| | Mutation | LOH | Total | (%) | | Mutation | Staining | Total | (%) | | | | | |
| I | 9/21[b] | 3/14 | 11/21 | (52.4) | | 7/21 | 8/21 | 8/21 | (38.1) | 6/21 | (28.6) | | 3/17 | (17.6) |
| IIa | 0/7 | 1/1 | 1/7 | (14.3) | [c] | 2/7 | 2/7 | 2/7 | (28.6) | 2/7 | (28.6) | [d] | 0/3 | (0) |
| Subtotal | 9/28 | 4/15 | 12/28 | (42.9) | | 9/28 | 10/28 | 10/28 | (35.7) | 8/28 | (28.6) | | 3/20 | (15) |
| IIa+IIc | 1/14 | 0/8 | 1/14 | (7.1) | | 3/14 | 7/14 | 7/14 | (50) | 7/14 | (50) | | 0/9 | (0) |
| IIc+IIa | 1/2 | 0/1 | 1/2 | (50) | [c] | 2/2 | 1/2 | 2/2 | (100) | 2/2 | (100) | [d] | 0/2 | (0) |
| IIc | 1/4 | 0/2 | 1/4 | (25) | | 1/4 | 2/4 | 2/4 | (50) | 4/4 | (100) | | 1/2 | (50) |
| Subtotal | 3/20 | 0/11 | 3/20 | (15) | | 6/20 | 10/20 | 11/20 | (55) | 13/20 | (65) | | 1/13 | (7.7) |

*a*) Loss of Smad4 expression.
*b*) Alteration-positive cases/total number of cases examined.
*c*) The frequency of *APC* alterations in the superficial depressed cases was significantly lower than that in the I type ($P<0.02$) or subtotal of I and IIa types ($P<0.04$), Fisher's exact test.
*d*) The frequency of 18q21 AI in the superficial depressed cases was significantly higher than that in the I type ($P<0.02$) or subtotal of I and IIa types ($P<0.02$), $\chi^2$ test.

with superficial depressed CRCs, 1/7 (14.3%) with superficial elevated ones and 14/21 (66.7%) with protruding ones were informative for at least one locus. LOH was found in none of the 11 superficial depressed, one superficial elevated and 3 (21.4%) of the 14 protruding CRCs (Table II).

Totally, somatic mutations in the *APC* MCR and/or *APC* LOH were observed in 15 (31.3%) of the 48 early CRCs, i.e., 3/20 (15%) superficial depressed ones, 1/7 (14.3%) superficial elevated ones and 11/21 (52.4%) protruding ones (Table II). The frequency of *APC* alterations in the superficial depressed type was significantly lower than that in the protruding one ($P<0.02$, Fisher's exact test). When we combined the data for protruding and superficial elevated CRCs (12/28, 42.9%), and compared

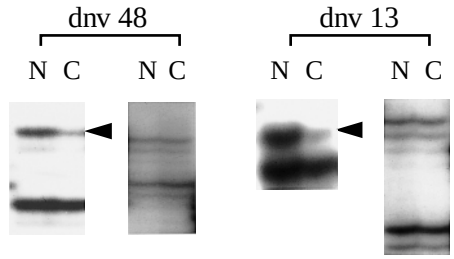


Fig. 3. Representative autoradiographs showing AI in the superficial depressed CRCs. Microsatellite DNA markers D1S199, D14S267, and D18S487, located on chromosomes 1p34-36, 14q32, and 18q21, respectively, were used to detect AI. The numbers at the top are the case numbers. N, normal mucosa; C, cancer. AI in the D18S487 region was found in the cancers, as indicated by arrowheads.

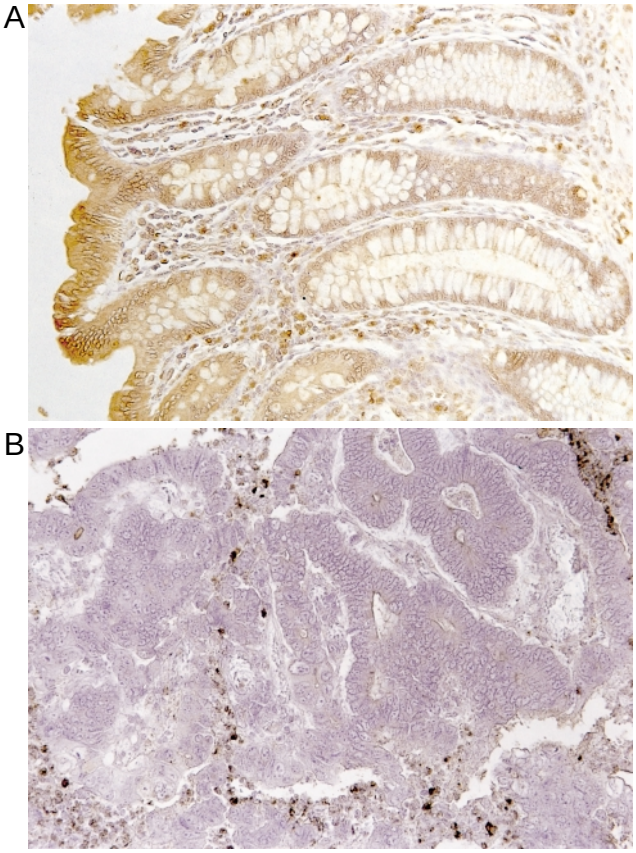


Fig. 4. Immunohistochemical staining of the Smad4 protein in normal (A) and cancer (B) cells of the colon. (A) Smad4 was strongly expressed in the cytoplasm of normal epithelial cells, particularly in the surface epithelium. (B) Smad4 expression was not seen in the cancer cells. Magnifications ×160.

Table III. Frequencies of AI in Superficial Depressed Colorectal Cancers with Submucosal Invasion

| Chromosomal locations | Microsatellite markers | Informative cases[a] (%) | | AI[b] (%) |
|---|---|---|---|---|
| 1p34-36 | D1S199 | 18/20 (90) | | 4/18 (22.2) |
| 8p21-22 | NEFL | 12/20 (60) | | |
| | D8S1725 | 18/20 (90) | 18/20 (90) | 3/18 (16.7) |
| | D8S1731 | 15/20 (75) | | |
| 14q32 | D14S267 | 17/20 (85) | | 1/17 (5.9) |
| 18q21 | D18S1143 | 13/20 (65) | | |
| | D18S467 | 13/20 (65) | 20/20 (100) | 13/20 (65)[c] |
| | D18S474 | 15/20 (75) | | |
| | D18S487 | 16/20 (80) | | |
| 22q12-13 | D22S284 | 14/20 (70) | | 0/14 (0) |

*a*) Informative cases/total number of cases examined.
*b*) AI/informative cases.
*c*) The frequency of 18q21 AI was significantly higher than those on other regions. Range, $P<0.0001-0.01$, Fisher's exact test.

them with those for superficial depressed CRCs, according to our previous report on mucosal CRCs,[23] we observed a statistically significant difference ($P<0.04$, Fisher's exact test, Table II).

**p53 overexpression and/or mutations** Positive nuclear staining of p53 (Fig. 2) and/or *p53* mutations in exons 5–8 were detected in 21 (43.8%) of the 48 early CRCs, i.e., 11/20 (55%) superficial depressed ones, 2/7 (28.6%) superficial elevated ones and 8/21 (38.1%) protruding ones (Table II). There was no significant association of *p53* alterations in the superficial depressed CRCs in comparison with in the protruding ones or in the combined protruding and superficial elevated CRC group (10/28, 35.7%).

**AI in early CRCs** When we screened for AI using one or more microsatellite markers at the five chromosomal loci in the 20 superficial depressed CRCs, more than 70% were informative for each chromosomal region. AI was detected in 4/18 (22.2%) for 1p34-36, 3/18 (16.7%) for 8p21-22, 1/17 (5.9%) for 14q32, 13/20 (65%) for 18q21, and 0/14 (0%) for 22q12-13 (Fig. 3 and Table III). The frequency of 18q21 AI is significantly higher than that on other regions (range; $P<0.0001-0.01$, Fisher's exact test, Table III). To determine whether or not allelic deletion on 18q21 is essential in these CRCs, we further examined AI on the 18q21 locus in the superficial elevated and protruding CRCs. AI was found in 2 (28.6%) of the 7 superficial elevated and 6 (28.6%) of the 21 protruding CRCs (Table II). In total, 18q21 AI was detected in 21 (43.8%) of the 48 early CRCs. The frequency of 18q21 AI in the superficial depressed CRCs was higher than that in the protruding ones or in the combined protruding and superficial elevated CRC group (8/28, 28.6%) ($P<0.02$, Table II).

**Alterations in the tumor suppressor genes, *DCC*, *Smad2* and *Smad4*** We examined DCC and Smad4 protein expression in the 13 superficial depressed CRCs, 8 of which exhibited 18q21 AI, by means of immunohistochemistry. The 3 superficial elevated and 17 protruding CRCs, none of the former and 4 of the latter showing 18q21 AI, were also analyzed. DCC was expressed in the cytoplasm of normal epithelial cells throughout the mucosal surface and crypts (data not shown), this being consistent with previous data.[14] DCC expression was observed in all the cancerous regions as well as corresponding normal cells, indicating no DCC alterations. As for Smad4 in normal mucosa, strong stainability was observed in the cytoplasm of the surface epithelium, while the crypt epithelium was weakly stained (Fig. 4A). Smad4 was also expressed in the cytoplasm in most cancer cells. These staining patterns were similar to those described previously.[27] Loss of Smad4 expression (Fig. 4B) was only detected in 1 (7.7%) of the 13 superficial depressed, none of the 3 superficial elevated and 3 (17.6%) of the 17 protruding CRCs (Table II). Among them, one superficial depressed and two of the three protruding CRCs also showed 18q21 AI.

It has been reported that most mutations of *Smad2* and *Smad4* are located in the MH2 domain in exons 8–11, indicating a mutation hot-spot region.[12, 13, 15–17] We screened for mutations in the MH2 domains of *Smad2* and *Smad4* in the 23 early CRCs (17 superficial depressed, 3 protruding and 3 superficial elevated cases), 4 of which showed loss of the Smad4 protein. No mutation at either MH2 domain was detected in any case.

## DISCUSSION

*APC* inactivation appears to initiate colorectal tumorigenesis, thereby contributing to adenoma formation.[5–7] Previous reports demonstrated that over 60% of *APC* somatic mutations in colorectal tumors were located on MCR of the gene.[5, 25] When we searched for alterations in *APC* MCR in submucosal CRCs, significantly frequent *APC* alterations were found in the protruding CRCs in comparison with in the superficial depressed ones ($P<0.02$, Fisher's exact test). The incidence in the combined protruding and superficial elevated CRC group was also higher than that in the superficial depressed CRCs ($P<0.04$, Fisher's exact test). This significant association is consistent with our previous report on mucosal CRCs.[23] Our data suggest that *APC* alterations are related to protruding CRCs, and that the carcinogenetic pathways of protruding and superficial depressed CRCs are different.

*APC* mutation is known to be dominant for transcriptional activation via β-catenin/Tcf pathway in CRCs. Oncogenic mutations of *β-catenin* have been found in CRCs without *APC* mutations, and these mutations mainly occurred in exon 3 serine/threonine phosphorylation residues, leading to β-catenin accumulation.[28, 29] Interestingly, several reports demonstrated significant *β-catenin* mutations in CRCs with MSI-high.[30, 31] In this study, we examined for genetic alterations in early CRCs without MSI. Therefore, if *β-catenin* mutations are associated with CRCs showing a defective mismatch repair system, mutations of it might be rare in our superficial depressed CRCs. Nevertheless, it is valuable to analyze *β-catenin* mutations in order to study its role in superficial depressed CRCs.

We searched for *p53* alterations in the 48 early CRCs by means of immunohistochemistry and PCR-SSCP, and 21 (43.8%) cases showed alterations. There was no significant association between *p53* alterations and macroscopic types. Several studies revealed that the *p53* mutation frequencies for protruding and superficial CRCs with submucosal invasion were similar.[32] Our data further support a relationship of *p53* inactivation with both superficial and protruding CRCs.

The accumulation of genetic alterations, such as *APC* and *p53*, is associated with colorectal tumorigenesis based

on the adenoma-carcinoma sequence, and mutations of such genes are commonly found in early protruding as well as advanced CRCs.[1, 7, 23] In contrast, *APC* mutations were very rare in superficial CRCs, although *p53* alterations were often detected in this study. Our data indicate that *p53* may be responsible for superficial depressed CRCs, but it is not clear whether or not *p53* inactivation alone is sufficient for a malignant change in this type of colorectal carcinogenesis. It is likely that alterations of tumor suppressor gene(s) other than *APC* also occur in superficial depressed CRCs.

To identify tumor suppressor gene(s) other than *APC*, we examined AI on the five chromosomal loci, i.e., 1p34-36, 8p21-22, 14q32, 18q21 and 22q12-13, using microsatellite markers. The incidence of 18q21 AI was much higher than those on the other four chromosomal regions in superficial depressed CRCs, indicating an important role in these colorectal carcinogeneses. Previous cytogenetic findings have demonstrated high frequency of chromosomal deletions on 18q in CRCs,[33, 34] hence AI on the 18q21 locus can be interpreted as chromosomal deletions, but not as chromosomal gains. Moreover, 18q21 AI was significantly more frequent in the superficial depressed cases (13/20, 65%) in comparison with in the protruding ones (6/21, 28.6%), or the combined protruding and superficial elevated CRC group (8/28, 28.6%) ($P<0.02$). Therefore, the 18q21 deletion is likely to be an essential genetic change and tumor suppressor gene(s) located on this region may contribute to formation of superficial depressed CRCs.

We previously reported that frequent allelic deletions on 18q21 and 8p21-22 were detected in submucosal CRCs with lymph node metastasis,[10] suggesting the presence of metastasis-related tumor suppressor gene(s) in these regions. Since 18q21 AI was commonly found in superficial depressed CRCs without lymph node metastasis in this study, some tumor suppressor gene(s) may be located on this region. However, it is uncertain whether superficial depressed type- and metastasis-related tumor suppressor gene(s) are the same or not. The frequency of 8p21-22 AI as well as that of AI on the three other loci was found to be low in superficial depressed CRCs. AI on these four chromosomal loci might be a late event in colorectal carcinogenesis, because these chromosomal loci were often deleted in advanced CRCs.[1–3]

To identify a candidate tumor suppressor gene(s) located on 18q21, we searched for alterations of *DCC*, *Smad2* and *Smad4*. DCC protein expression was detected in all the 33 early CRCs and corresponding normal mucosa, suggesting that DCC alterations are not correlated to an early stage of colorectal carcinogenesis. Loss of Smad4 expression was found in both the superficial depressed (1/13, 7.7%) and protruding (3/17, 17.7%) CRCs, but their frequencies of alteration were low. *Smad4* may be a target on 18q21 LOH in some early CRCs, because three of the four cases with loss of Smad4 expression also exhibited 18q21 AI. When we screened for mutations in the MH2 domains of *Smad2* and *Smad4* in 23 early CRCs, 4 of which exhibited loss of Smad4 expression, no mutation was found in any case. It is possible that a mutation occurred in another region, such as the MH1 domain, in the four cases without Smad4 expression. Nevertheless, neither *Smad4* nor *Smad2* alteration seems to be specific to the development of most superficial depressed CRCs as an initial step. Since the frequencies of alterations of these three tumor suppressor genes were very low in comparison with that of 18q21 AI in superficial depressed CRCs, other tumor suppressor gene(s) located on 18q21, as yet unidentified, might be responsible for most superficial depressed CRCs.

The genetic alterations of the superficial depressed CRCs were different from those of the protruding ones. *APC* and *p53* inactivations may be involved in the protruding CRCs, while *p53* inactivation and 18q21 deletion might play important roles in the superficial depressed CRCs. Judging from the present findings taken together with previous reports of clinicopathologic and genetic studies on superficial depressed CRCs,[19–22, 32] these cancers are likely to be *de novo* type CRCs that directly develop from normal colonic mucosa. Alterations of tumor suppressor gene(s) located on 18q21 other than *DCC*, *Smad2* and *Smad4* might be essential for the development of *de novo* type CRCs.

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