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Analyses of the *APC* and *TGF-β Type II Receptor* Genes, and Microsatellite Instability in Mucosal Colorectal Carcinomas

Osmar Kenji Yagi,[1] Yoshimitsu Akiyama,[2] Yasuo Ohkura,[3] Shin-ichi Ban,[4] Mitsuo Endo,[1] Kiyoshi Saitoh[5] and Yasuhito Yuasa[2,6]

[1]*First Department of Surgery,* [2]*Department of Hygiene and Oncology, Tokyo Medical and Dental University School of Medicine, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113,* [3]*Department of Pathology, Tokyo Metropolitan Cancer Survey Center, 2-5 Surugadai, Kanda, Chiyoda-ku, Tokyo 101,* [4]*Second Department of Pathology, Saitama Medical School, 38 Moro-Honcho, Moroyama-cho, Iruma-gun, Saitama 350-4 and* [5]*Department of Pathology, International Medical Center of Japan, Toyama, Shinjuku-ku, Tokyo 162*

APC and _transforming growth factor-β type II receptor_ (_TGF-β RII_) gene mutations, and microsatellite instability have been found in sporadic colorectal carcinomas. To clarify further the early alterations in colorectal carcinogenesis, we investigated these genetic changes in 23 protruding- and 24 superficial-type mucosal colorectal carcinomas. _TGF-β RII_ gene mutations and microsatellite instability were rarely found in these lesions. Nevertheless, _APC_ was mutated in 16 of the 47 (34.0%) mucosal colorectal carcinomas and was significantly more frequently mutated in protruding- (I) and superficial elevated-type (IIa) (14/32, 43.8%) than in other superficial-type (IIa+IIc, IIb, IIc, and IIc+IIa) (2/15, 13.3%) mucosal colorectal carcinomas ($P<0.04$). These results indicate that the _APC_ gene may be involved from the beginning in the tumorigenesis of many early colorectal carcinomas, particularly of the protruding and superficial elevated types. However, there might be a distinct pathway for other superficial-type colorectal carcinomas, possibly not involving _APC_ as an initial step of tumorigenesis.

Key words: Early colorectal carcinoma — *APC* — Microsatellite instability — *Transforming growth factor-β type II receptor* gene — K-*ras*

The generally accepted "adenoma-carcinoma" sequence in the histogenesis of colorectal carcinomas (CRC) is based on the concept that carcinomas develop from preexisting adenomas.[1] A multistep genetic model for colorectal tumorigenesis, based on this "adenoma-carcinoma" sequence, has been proposed.[2] In this model, mutations in K-*ras* and various tumor suppressor genes are known to accumulate during the progression from normal to malignant tissue. The role of each mutant gene during development and/or progression has been suggested; *APC* for an initial step of adenoma formation, K-*ras* for making adenomas larger and more severely dysplastic, *p53* for malignant transformation, and *DCC* for late changes that cause progression of carcinomas and worsen the prognosis of patients with CRC.[3]

Genetic analysis of colorectal adenomas and carcinomas demonstrated that significantly more K-*ras* alterations were found in adenomas with severe atypia, when compared with sporadic CRC, leading to the speculation that many CRC do not arise from adenomas with severe atypia.[4] Furthermore, in early CRC,[5] less frequent alteration of the K-*ras* gene was found in the flat elevated type compared with the polyp-forming type carcinomas, and therefore it was suggested that flat elevated carcinomas may originate from a pathway different from the "adenoma-carcinoma" sequence.

Germline mutations of the *APC* gene cause familial adenomatous polyposis (FAP), and somatic mutations of *APC* also occur in about 60% of sporadic colorectal adenomas and carcinomas.[6,7] Most of these mutations cause loss of the carboxyl terminus of APC protein, thus deleting some of the binding sites for proteins that may be important for APC's growth-controlling function, such as EB1[8] and DLG,[9] or proteins implicated as signal transducers in the Wg/WNT signaling pathway.[10] Recently, it was suggested[3] that the genetic defect in FAP targets the gatekeeper function of the *APC* gene for colonic epithelial cell proliferation, altering the homeostatic balance of these populations, and finally affecting the rate of tumor initiation. Therefore, it is of great interest to know if *APC* mutations are equally involved in the genesis of different types of mucosal CRC.

According to the macroscopical classification for CRC,[11] mucosal carcinomas can be divided into two groups, i.e., the nonprotruding carcinomas classified as superficial-type, and the protruding-type early carcinomas. Previous clinicopathological studies[12,13] showed that these two groups present distinct degrees of invasiveness: the superficial-type tends to reach deeper layers at an earlier stage than the protruding-type carcinomas. A study of *APC* in six superficial-type early CRC[14] in-

[6] To whom all correspondence should be addressed.

dicated that it was mutated in some of these cases (2/6, 33.3%). However, because of the small number of samples and the lack of protruding-type samples for comparison, it remained uncertain whether or not the *APC* gene was equally involved in the tumorigenesis of both protruding-type and superficial-type CRC.

It was postulated that, in hereditary nonpolyposis colorectal cancer (HNPCC), mutations in the mismatch repair gene cause genetic defects in the DNA repair system, which are manifested as replication errors (RER) in tumors.[3] These DNA repair defects would target the short repeat sequence $(A)_{10}$ in the *transforming growth factor-β type II receptor* (*TGF-β RII*) gene, a crucial regulator of cell growth,[15] and inactivate it through frameshift mutations and the resultant protein truncation. Once sequential genetic changes have occurred, the cells will lose their responsiveness to TGF-β inhibition and progress to malignancy.[16–19] Our previous study[20] demonstrated high frequencies of RER and *TGF-β RII* mutations as early as in the adenoma stage of HNPCC patients. The same carcinogenetic mechanism as in HNPCC may operate in about 10% of sporadic CRC.[21] However, RER and the *TGF-β RII* gene mutation frequency in mucosal sporadic CRC have not been investigated yet, and it is also unclear in which stage of carcinogenesis these alterations occur.

To address the question of distinct pathways for development of CRC, we searched for alterations in *APC*, the *TGF-β RII* $(A)_{10}$ repeat site and K-*ras* codon 12, as well as for RER in mucosal colorectal lesions.

MATERIALS AND METHODS

Subjects A total of 47 tumors pathologically diagnosed as mucosal CRC, without polyposis or family history for HNPCC, and corresponding normal tissues were obtained by surgical or endoscopical resection. Twenty-three (48.9%) were protruding-type and 24 (51.1%) were superficial-type early CRC. They were collected at the International Medical Center of Japan, Tokyo, the Tokyo Metropolitan Cancer Survey Center, Tokyo, and the Saitama Medical School, Saitama, from 1989 through 1996. Each tumor was examined carefully by the same pathologist to confirm the diagnosis and classification. Genomic DNA was extracted from paraffin-embedded tissues as described previously.[22] The mean age of the patients was 63.5±10.3 years (mean±standard deviation). There were 38 (80.9%) males and 9 (19.1%) females. Eighteen (38.3%) carcinomas were located in the proximal colon (cecum, and ascending and transverse colon) and 29 (61.7%) were located distally (descending and sigmoid colon, and rectum). The mean maximum diameter of the superficial-type mucosal CRC was 12.7 mm (range 2–46 mm).

Macroscopically the nonprotruding mucosal lesions were classified as a superficial-type (II type) as opposed to the protruding-type (I type) mucosal lesions.[11] Superficial-type early CRC include superficial elevated (IIa type), predominantly raised with central depression (IIa +IIc), flat (IIb), depressed (IIc), and raised with predominance of central depression (IIc+IIa). Among the 24 superficial-type carcinomas, 9 (37.5%) were categorized as IIa, 10 (41.7%) as IIa+IIc, 2 (8.3%) as IIb, 1 (4.2%) as IIc, and 2 (8.3%) as IIc+IIa. With reference to the depth of invasion, the lesion is considered as mucosal when it is limited to within the mucosa and does not reach the submucosa.[11, 12] As to histological classification, all lesions were well differentiated tubular adenocarcinomas according to the classification for CRC.[11]

Analysis of microsatellite instability We determined the microsatellite instability at four microsatellite loci containing $(CA)_n$ repeats (D2S119, D2S123, D3S1029 and D10S197)[23, 24] and a locus containing an $(A)_n$ repeat (BAT26).[19] Polymerase chain reaction (PCR) amplification of these markers was performed as described previously.[23] The PCR products were denatured in 95% formamide for 2 min at 94°C and then electrophoresed on 6% polyacrylamide sequencing gels. Then the gels were exposed to X-ray film for 2–48 h. We classified the cancers as positive for RER, when the PCR product with tumor DNA gave extra bands that were not observed for the PCR product of the corresponding normal tissue DNA.

Mutation screening of *TGF-β RII* by PCR-single strand conformation polymorphism (SSCP) We designed a set of genomic DNA primers according to the *TGF-β RII* mRNA sequence (Genbank#M85079).[17] The primer sequences for the $(A)_{10}$ microsatellite sequence of nucleotides 709 to 718 were: RIIU1, 5′-AGATGCTGCTTCTCCAAAGTGC-3′, and RIID1, 5′-TTGCACTCATCAGAGCTACAGG-3′, which amplify the 90 bp target sequence from nucleotides 677 to 766. This region has already been reported to exhibit mutations in HNPCC and sporadic colorectal cancer cell lines with RER.[16–19]

PCR-SSCP analysis was performed as described previously.[25] Briefly, each PCR was run for 35 cycles consisting of denaturation for 1 min at 94°C, annealing for 2 min at 58°C, and extension for 1 min at 72°C, followed by final extension for 10 min at 72°C. The PCR products and denaturing stop solution (95% formamide, 10 m*M* EDTA, 0.25% bromophenol blue and xylene cyanol FF) were heated at 80°C for 5 min, cooled rapidly on ice, and then electrophoresed on non-denaturing 12.5% polyacrylamide gels containing 10% glycerol in Tris-glycine buffer (25 m*M* Tris-HCl, 200 m*M* glycine, pH 8.3), using a commercially available minislab gel (90×70×1.0 mm) apparatus (ATTO Co., Ltd., Tokyo). The running conditions were 300 V for 2.5 h at 15°C, and the gel was stained with silver (Dai-ichi Co., Ltd., Tokyo).

Mutation screening of *APC* by PCR-SSCP Overlapping primer sets numbered 1 to 7 (5′ to 3′) were designed for genomic DNA screening of *APC* exon 15 codons 1274 to 1523. This region has already been reported to be a mutation cluster region (MCR) in nonFAP colorectal tumors.[6] The sizes of the PCR products ranged from 126 to 176 bp. The primer sequences are 1U 5′-ACTCCAATATGTTTTCAAGATG-3′ and 1D 5′-GGAACTTCGCTCACAGGAT-3′; 2U 5′-GCAGATTCTGCTAATACCCT-3′ and 2D 5′-AACAGCTTTGTGCCTGGCT-3′; 3U 5′-CTGCAGGGTTCTAGTTTATC-3′ and 3D 5′-ATCAAGTGAACTGACAGAAG-3′; 4U 5′-GACCCCACTCATGTTTAGC-3′ and 4D 5′-TTACTTCTGCTTGGTGGCAT-3′; 5U 5′-GATCTTCCAGATAGCCCTGG-3′ and 5D 5′-TCTTTTCAGCAGTAGGTGCTTT-3′; 6U 5′-AAACAGCTCAAACCAAGCGA-3′ and 6D 5′-TCTGGAGTACTTTCCGTGG-3′; 7U 5′-CAGAGGGTCCAGGTTCTTCC-3′ and 7D 5′-TCCTGAACTGGAGGCATTATTC-3′.

PCR-SSCP was carried out as described above. The annealing temperature varied from 53 to 58°C. The electrophoresis was performed on non-denaturing 10–15% polyacrylamide gels containing 10% glycerol. The running conditions were 300 V for 1.5–3 h at 15°C. After electrophoresis, the gels were stained with silver.

Analysis of mutations at APC codon 1450 by *Taq I* digestion The database for *APC* mutations[26] indicates that there are some hot spots inside the exon 15 MCR, and the most somatically mutated place is at codon 1450. Therefore, we designed a primer set with an artificial *Taq I* restriction enzyme site in codon 1450 to allow PCR-SSCP mutation screening. The sequences of the primers are: APC1450U 5′-TCCTCAAACAGCTCAAACCAAT-3′ and 6D above. PCR was carried out as described above with annealing at 55°C. The amplified DNA was digested with *Taq I* (20 units; New England Biolabs, Beverly, MA) and then electrophoresed on 12.5% non-denaturing polyacrylamide gels in Tris-glycine buffer. The running conditions were 350 V for 40 min at 15°C, followed by ethidium bromide staining.

DNA sequencing When abnormal patterns were observed on SSCP analysis, the PCR products were purified using a QIA-quick spin PCR purification kit (QIAGEN Inc., Chatsworth, CA), and were sequenced directly with a cycle sequencing kit (TaKaRa, Kyoto) using end-labeled primers and the conditions specified by the manufacturer.

Detection of mutations in K-*ras* codon 12 To detect point mutations in codon 12, selected amplification of the K-*ras* gene sequence was performed using modified primers.[4,27] The 5′ 22mer was modified to contain GCC instead of GCT (codon 11) to introduce an artificial *Msp I* restriction site (CCGG) into codon 12. PCR was carried out as described above, with annealing at 50°C. The amplified DNA was digested with *Msp* I (20 units; New England Biolabs) and then electrophoresed on 15% non-denaturing polyacrylamide gels in Tris-glycine. The running conditions were 350 V for 1 h at 15°C, followed by ethidium bromide staining.

RESULTS

RER analysis Using five microsatellite markers, we examined 25 mucosal CRC for which we could obtain corresponding normal tissues. RER with one positive marker was observed in none among the 5 (0%) protruding-type carcinomas and in 2 among the 20 (10%) superficial-type, and none was positive for two or more markers (Table I).

Mutations of *TGF-β RII* at the small repeated sequence Twenty-three protruding- and 24 superficial-type CRC were screened for the *TGF-β RII* $(A)_{10}$ region. One among the 23 (4.3%) protruding-type (case 124T) and one among the 24 (4.2%) superficial-type (case 24T) lesions showed different SSCP variant patterns (Fig. 1 and Table I). The mobility shift was not observed for the

Table I. RER and *TGF-β RII* Alterations in Mucosal Colorectal Cancers

	RER ≥2 loci[a]	*TGF-β RII* (+)[b]
Protruding-type	0/5 (0%)	1/23 (4.3%)
Superficial-type	0/20 (0%)	1/24 (4.2%)
Total	0/25 (0%)	2/47 (4.3%)

a) RER ≥2 loci: positive for two or more loci.
b) *TGF-β RII* (+): mutations at the $(A)_{10}$ repeat of the *TGF-β RII*.

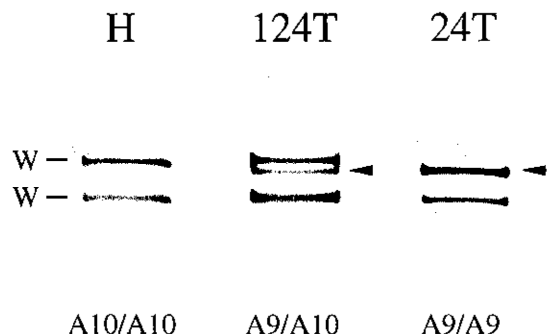

Fig. 1. PCR-SSCP analysis of the $(A)_{10}$ repeat in the *TGF-β RII* gene. H, healthy individual; 124T, A9/A10 heterozygous case; 24T, A9 homozygous case. The mobility shifts shown by arrowheads indicate one A deletion. W, wild type allele.

Table II. Survey on *APC* and *TGF-β RII* Mutational Status and Clinicopathological Data for Mucosal CRC

Case	Age	Sex[a]	Site[b]	Size (mm)	Macroscopic type[c]	*TGF-β RII*[d]	*APC*			
							Mutation[e]	Codon	Nucleotide change	Predicted effect
34	81	M	S	18	I		+	1398	T deletion	frameshift
97	58	M	S	8	I		+	1376	TA deletion	frameshift
110	52	M	D	10	I		+	1450	C to T	nonsense
111	52	M	S	11	I		+	1450	C to T	nonsense
115	76	M	T	5	I		+	1487–1488	T insertion	frameshift
116	72	M	S	9	I		+	1319	C deletion	frameshift
118	76	M	T	17	I		+	1450	C to T	nonsense
121	72	M	S	10	I		+	1307–1311	AAAAG deletion	frameshift
122	72	M	S	5	I		+	1322	G to T	nonsense
124	50	F	S	23	I	A9/A10				
127	65	M	S	5	I		+	1393	T deletion	frameshift
31	44	M	A	24	IIa		+	1437–1438	A deletion	frameshift
33	80	M	R	40	IIa		+	1302–1303	A deletion	frameshift
99	32	M	T	7	IIa		+	1490–1492	ATTTTGC deletion	frameshift
107	59	F	D	7	IIa		+	1307–1311	AAAAG deletion	frameshift
23	64	M	R	15	IIa+IIc		+	1388	A insertion	frameshift
24	60	M	A	12	IIa+IIc	A9/A9				
39	52	M	T	5	IIc+IIa		+	1307–1311	AAAAG deletion	frameshift

a) M, male; F, female.
b) A, ascending colon; T, transverse colon; D, descending colon; S, sigmoid colon; R, rectum.
c) I, protruding-type; IIa, superficial elevated-type; IIa+IIc, predominantly raised with central depression-type; IIc+IIa, raised with predominance of central depression-type.
d) Mutation in the *TGF-β RII* polyadenine repeat region. A9/A9, homozygous; A9/A10, heterozygous for one A deletion.
e) +, mutation detected in *APC* gene exon 15 codons 1274 to 1523.

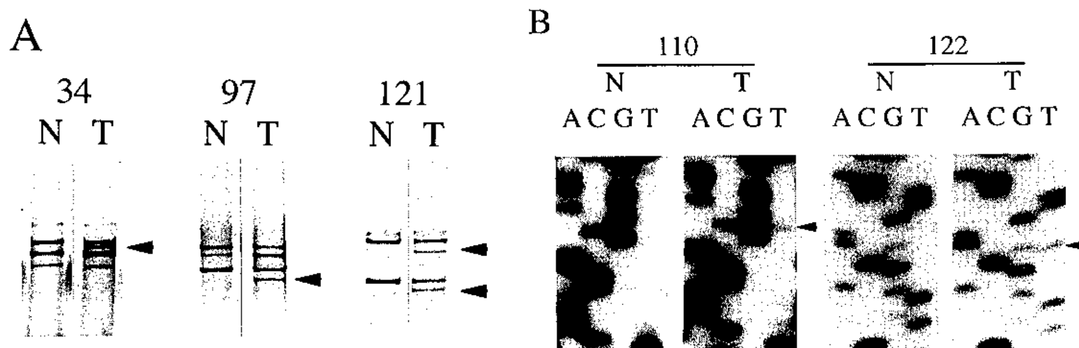

Fig. 2. *APC* gene analysis in mucosal CRC. A, PCR-SSCP analysis of the primer set numbers 4 (case 34), 3 (case 97) and 1 (case 121). Arrowheads indicate mobility shifts in tumors (T), in comparison with normal DNA (N). B, sequence analysis of tumors (T) in cases 110 and 122 compared to normal tissues (N). Arrowheads indicate the position of C-to-T transition in case 110 and G-to-T transversion in case 122, as well as the wild type allele.

corresponding normal DNA of case 24T, indicating that the mutation was somatic. Direct sequencing of the PCR products revealed that case 124T was heterozygous and 24T was homozygous for one A deletion. The clinicopathological data are shown in Table II. Case 24T did not show any RER of five microsatellite markers examined. Normal tissue was not available for case 124T. The superficial-type (case 24T) lesion with *TGF-β RII* mutation was an ascending colon carcinoma, whereas the protruding-type (case 124T) was a sigmoid colon lesion.

Mutations in *APC* exon 15 MCR Sixteen of the 47 (34.0%) mucosal cases showing different SSCP variant patterns or altered *Taq* I digestion patterns (codon 1450) for the *APC* PCR products were confirmed to present

Table III. *APC* and K-*ras* Mutations in Mucosal Colorectal Lesions

Macroscopic type[a]	*APC* (%)[b]		K-*ras* (%)[c]	
I	10/23 (43.5)		10/23 (43.5)	
IIa	4/9 (44.4)		3/9 (33.3)	
Subtotal	14/32 (43.8)	d)	13/32 (40.6)	e)
IIa+IIc	1/10 (10)		1/10 (10)	
IIb	0/2 (0)		0/2 (0)	
IIc+IIa	1/2 (50)		0/2 (0)	
IIc	0/1 (0)		0/1 (0)	
Subtotal	2/15 (13.3)		1/15 (6.7)	

a) I, protruding-type; IIa, superficial elevated-type; IIa+IIc, predominantly raised with central depression; IIb, flat; IIc+IIa, raised with predominance of central depression; IIc, depressed-type.
b) *APC* mutation frequencies.
c) K-*ras* mutation frequencies.
d) Comparing I and IIa to other lesions, Fisher's exact test, $P<0.04$.
e) Comparing I and IIa to other lesions, Fisher's exact test, $P<0.02$.

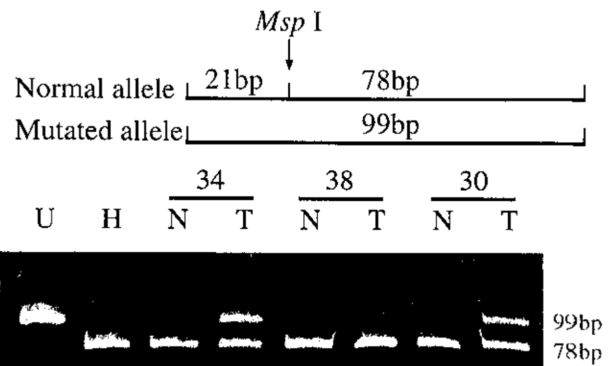

Fig. 3. Detection of point mutations in c-K-*ras* 2 codon 12 using PCR *Msp* I digestion. U, uncut; H, healthy individual; N, normal tissue; T, tumor tissue. The arrow in the schematic diagram indicates the *Msp* I restriction site in the normal allele, dividing the normal allele of 99 base pairs into two fragments of 21 and 78 base pairs.

mutations by means of direct sequencing. Representative data are shown in Fig. 2, A and B, and clinicopathological data for cases presenting mutations are shown in Table II. The alterations were either frameshift or nonsense mutations, and resulted in truncation of the APC protein. *APC* was mutated in 10 of the 23 (43.5%) protruding-type (I), 4 of the 9 (44.4%) superficial elevated-type (IIa), and in 2 of the 15 (13.3%) other superficial-type CRC (Table III). Comparing the data in the protruding-type (I) and superficial-type (II) CRC, we found no statistically significant difference. However, when we combined the data from protruding-type (I) and superficial elevated-type (IIa) CRC (14/32, 43.8%), and compared the combined group with the other superficial-type mucosal lesions (2/15, 13.3%), we observed a statistically significant difference ($P<0.04$) in the frequencies of the *APC* exon 15 MCR mutations.

Mutations in the K-*ras* gene K-*ras* codon 12 mutations were present in 14 of the 47 (29.8%) cases examined. K-*ras* was mutated in 10 of the 23 (43.5%) protruding-type (I), 3 of the 9 (33.3%) superficial elevated-type (IIa), and in 1 of the 15 (6.7%) other superficial-type CRC (Fig. 3 and Table III). Combining the data from protruding-type and superficial elevated-type CRC (13/32, 40.6%), and the combined group with the other superficial-type mucosal lesions (1/15, 6.7%), we observed a statistically significant difference ($P<0.02$) in the frequencies of the K-*ras* codon 12 mutations.

Correlation among genetic alterations Simultaneous mutations of the *APC* and K-*ras* genes frequently coexisted in our samples. Among the 16 *APC*-mutated cases, 8 (50%) presented alterations in K-*ras*. On the other hand, in the 31 *APC* negative cases, only 6 (19.4%) presented K-*ras* mutations ($P<0.04$). Statistically significant differences were also observed when we combined data from I- and IIa-type lesions, where among the 14 *APC*-mutated cases, 8 (57.1%) presented alterations in K-*ras*, but only 5 (27.8%) among the 18 *APC* negative cases did so ($P<0.03$). The other superficial-type cases did not present such an *APC*/K-*ras* mutation correlation (data not shown). As for *APC* and *TGF-β RII*, among the 16 *APC*-mutated cases, none presented *TGF-β RII* alterations (Table II), indicating that these mutations occurred independently.

DISCUSSION

In this study, we found RER for one marker in 2 of the 20 (10%) and *TGF-β RII* mutations in 1 of the 24 (4.2%) superficial-type mucosal CRC, and RER for one marker in none of the 5 and *TGF-β RII* mutation in 1 of the 23 (4.3%) protruding-type mucosal CRC. The superficial-type tumor 24T, carrying mutations in the $(A)_{10}$ sequence of *TGF-β RII* was homozygous for one A deletion, that is A9/A9, and the protruding-type (124T) was heterozygous for it. In case 24T, *TGF-β RII* was mutated, even though none of the five microsatellite markers tested showed RER. This may not be unusual, because the frequency of alterations in the $(A)_{10}$ sequence of *TGF-β RII* is very high among microsatellite markers[17, 19] and such alterations would be advantageous for cell growth. There was no significant association between the *TGF-β RII* mutation and clinicopathological data. As to the location in *TGF-β RII* -mutated cases, the

superficial-type case (24T) was on the proximal side and the protruding-type case (124T) was located in the sigmoid colon. These data suggest that the frequencies of RER and *TGF-β RII* mutations in mucosal colorectal cancers are very low, and hence these alterations may not be commonly involved in both protruding- and superficial-type mucosal CRC. However, it is of interest that mutations in *TGF-β RII* and *APC* were detected independently in mucosal CRC, indicating that these two genes may play independent roles in colorectal tumorigenesis.

Our results showed a similar frequency of K-*ras* mutations to the previous data on early CRC.[5] We also observed a close correlation between *APC* and K-*ras* mutations. The *APC* mutations might occur earlier than K-*ras* mutations according to the previous data.[3] We speculate that a mutation in one of these genes may facilitate or even lead to the mutation in the other one.

In mucosal lesions, *APC* mutation frequencies in MCR examined were significantly higher in the protruding-type (43.5%) and superficial elevated-type (44.4%) than in other superficial-type (13.3%) early CRC ($P<0.04$). Despite the macroscopical differentiation, the protruding- (I) and superficial elevated-type (IIa) of lesions may undergo similar pathways of tumorigenesis involving *APC*, different from other types of early superficial-type CRC. This hypothesis is consistent with the previous report that the superficial elevated-type (IIa) early CRC may develop into a protruding-type, then may grow into advanced carcinoma.[28] Comparing *APC* mutation sites and types of our cases with previously reported data for advanced carcinomas,[26] we observed similar site and type distribution rates throughout the hot spots.

According to the "adenoma-carcinoma" sequence, *APC* mutation has been considered one of the first steps in the tumorigenesis of CRC, followed by the remaining gene mutations.[3] In this study, we observed that such *APC* alterations are very frequent in protruding- and superficial elevated-type early CRC from the beginning. Therefore, lesions of these types may constitute the group of colorectal carcinomas that follows the "adenoma-carcinoma" sequence. However, the other superficial-type carcinomas rarely showed *APC* mutations as initial steps, supporting the hypothesis that there might be a distinct pathway in colorectal tumorigenesis, where possibly other gene mutations are involved.

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