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Mutational analysis of β-catenin and the RAS-RAF signalling pathway in early flat-type colorectal tumours

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ABSTRACT

Morphologically, early colorectal tumours can be divided into two groups, protruded-type and flat-type. However, little is known about genetic mechanisms of the latter. We investigated mutations of *β-catenin*, *KRAS*, *BRAF*, and *PIK3CA* in 310 early colorectal tumours. *β-catenin* mutation was detected in 7.1% of 310 tumours. *β-catenin* mutation was detected in a significantly higher percentage of flat-type tumours with depressed areas (4/17, 23.5%) than in other tumours (18/293, 6.1%; $p = 0.0246$). *KRAS*, *BRAF*, and *PIK3CA* mutations were detected in 21.6%, 5.4%, and 1.0% of 310 tumours, respectively. Concomitant mutations of *β-catenin* and *KRAS* or *BRAF* were detected in seven tumours. Mutation of at least one gene was detected in a significantly higher percentage of flat-type tumour tissues (75/193, 38.9%) than in protruded-type tumour tissues (25/117, 21.4%; $p = 0.0014$), and it was correlated significantly with size ($p = 0.0001$). In conclusion, *β-catenin* mutation seemed to play an important role in flat-type tumours, especially in those with depressed areas. The genetic abnormalities could arise and accumulate in the early stage of colorectal tumourigenesis, and seem to contribute to the development of flat-type tumour.

1. Introduction

Morphologically, early colorectal tumours can be divided into two groups, protruded-type tumours and flat-type tumours. Recently, flat-type tumours have been reported not only in Japan[1] but also in Western countries.[2] Since early detection of flat-type tumours is difficult compared with protruded-type tumours, they are sometimes overlooked and tend to be found at a late stage.[3–5] Moreover, it has been thought that some flat-type colorectal cancers correspond to *de novo* cancers, which contain no observable adenomatous component,[4,5] and may develop through a distinct genetic pathway.[6,7] Although some studies have shown genetic alterations in early colorectal tumours, including sporadic hyperplastic polyps, serrated adenomas, tubular or tubulovillous adenomas, and early invasive carcinomas, genetic alterations in early flat-type colorectal tumours have remained unknown.

The RAS-RAF signalling pathway mediates cellular responses to growth signals not only in advanced colorectal carcinomas but also in early colorectal tumours. Previous studies have shown that *KRAS* mutations are rare in flat-type colorectal tumours.[2] *BRAF* mutations have also been reported in

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hyperplastic polyps and serrated adenomas. Although the acquisition of *BRAF* mutation has been shown to be associated with the progression of hyperplastic polyp to serrated adenoma,[8] the frequency of *BRAF* mutations in early flat-type colorectal tumours has not been reported.

Recently, mutations in *PIK3CA* have been identified in various human tumours, such as colorectal cancer, gastric cancer, glioblastoma, breast cancer, and lung cancer. The *PIK3CA* gene encodes the catalytic subunit p110alpha of phosphatidylinositol 3-kinase (PI3K) belonging to class IA of PI3Ks. Since oncogenic RAS activates PI3Ks, activation of the PI3Ks signalling pathway via RAS-RAF signalling or *PIK3CA* mutations was considered one of the most common mechanisms involved in colorectal carcinogenesis.[9,10]

Flat-type colorectal tumours are not a homogeneous group and they are sometimes classified into three tumour types: small flat-type tumours, flat-type tumours with depressed areas, and laterally spreading tumours (LSTs). The latter are superficial neoplasms that spread laterally over the mucosa, and these have recently received significant attention from gastroenterologists. LSTs of the colon and rectum are defined as lesions greater than 10 mm in diameter with a low vertical axis that extend laterally along the luminal wall.[11–14] Recently, we reported a case of LST in which there was interstitial deletion, including exon 3 *β-catenin*.[15] The β-catenin protein has two major functions. First, it acts as a cell-cell adhesion regulatory protein which binds cadherin.[16] Second, it is thought to act as a downstream transcriptional activator in the Wnt signal pathway. It has been reported that cytoplasmic β-catenin is degraded after phosphorylation at serine/threonine residues in the NH2 terminus by glycogen synthase kinase-3 β (GSK-3β) through the formation of a complex with APC. Aberrant transactivation of T-cell factor (TCF)-4-regulated genes by β-catenin plays a key role in colorectal carcinogenesis. APC dysfunction or abnormalities of the *β-catenin* gene result in cytoplasmic accumulation of unphosphorylated β-catenin.[17,18] This stabilised β-catenin protein translocates into the nucleus, where it modulates gene transcription by interacting with TCF-4/ lymphoid enhancer factor (LEF)-1, resulting in transcriptional activation of target genes such as *c-myc*, *cyclin-D1*, and *matrix metalloproteinase (MMP)-7* (*matrilysin*).[19] However, the frequency of *β-catenin* mutations in sporadic flat-type colorectal tumours has not been reported.

Thus, it seems important to clarify the role of β-catenin and the RAS-RAF signalling pathway in human early colorectal carcinogenesis. We investigated mutations of exon 3 *β-catenin*, *KRAS*, *BRAF*, and *PIK3CA* by using direct DNA sequencing and expression of the β-catenin protein by immunohistochemistry in 310 human early colorectal tumour tissues. Twenty-two early colorectal tumours with exon 3 *β-catenin* mutations were also analysed for *APC* mutations and microsatellite instability (MSI).

2. Materials and methods

2.1. Patients and tissue samples

Formalin-fixed paraffin-embedded tumour specimens of 310 colorectal tumours were obtained from patients who had undergone polypectomy or surgical treatment. These tumour samples consisted of 34 hyperplastic polyps, eight serrated adenomas, 106 adenomas with low-grade dysplasia, 39 adenomas with high-grade dysplasia, 82 intramucosal carcinomas and carcinoma *in situ*, and 41 early invasive carcinomas (pT1 in the TNM classification of the Union International Contre Cancer). In all of the early invasive carcinomas, submucosal invasion was shallow. None of the patients reported a family history of colorectal carcinoma fulfilling a criterion for hereditary non-polyposis colorectal cancers (HNPCC) in interviews. The subjects were classified according to the most advanced lesion identified. Advanced neoplasia was defined as an adenoma of 10 mm or more in diameter, an adenoma with high-grade dysplasia, or invasive carcinoma.[20,21] Intramucosal carcinoma and carcinoma *in situ* were classified as adenoma with high-grade dysplasia. The criterion for diagnosing cancer was invasion of malignant cells beyond the muscularis mucosa. Tubular adenomas with low-grade dysplasia less than 10 mm in diameter were defined as minor neoplasias. Hyperplastic polyps were not included as neoplasias.[21] Locations of the colorectal tumours were divided into proximal colon (caecum, ascending and transverse colon) and distal colon (descending and sigmoid colon and rectum). Macroscopic types were divided into protruded-type (height of tumour $\geqq$3 mm) and flat-type (height of tumour <3 mm). The clinicopathological characteristics of colorectal tumours are shown in Table 1. Informed consent was obtained from each subject, and the institutional review committee approved this study.

2.2. Detection of exon 3β-catenin mutation

Exon 3 of *β-catenin* was amplified by PCR using primer pair: forward, 5′-GAACCAGACAGAAAAGCGGCTG-3′ and reverse, 5′-ACTCATACAGGACTTGGGAGG-3′. Products were purified and then sequenced in both directions using Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA). The sequence reactions were run and analysed on an ABI 3100 Genetic Analyzer (Applied Biosystems).

2.3. Detection of KRAS, BRAF and PIK3CA mutations

KRAS mutations at codon 12 and codon 13, *BRAF* mutations at codon 600, and *PIK3CA* mutations in exon 9 and exon 20 were detected by direct DNA sequencing as described previously.[8,10,22]

2.4. Immunohistochemistry

Immunohistochemistry with an anti-human β-catenin mouse monoclonal antibody (10 mg/ml, BD Biosciences, San Jose, CA) was done as described previously.[19] The sections were examined microscopically by two well-trained pathologists who were blinded to the clinicopathological characteristics. Nuclear and cytoplasmic expression of β-catenin was defined as positive when an immunoreactivity was observed in more than 10% of tumour cells.

2.5. MSI and APC mutation

Twenty-two tumour tissues with *β-catenin* mutations were analysed for MSI using five microsatellite markers (BAT-25,

Table 1 – Clinicopathological characteristics in 310 early colorectal tumour tissues

Clinicopathological characteristics	Morphology	
	Flat-type (n = 193)	Protruded-type (n = 117)
Age (year ± SD)	66.0 ± 10.4	62.7 ± 10.6
Mean size (mm ± SD)	17.1 ± 13.8	15.9 ± 11.5
Gender		
Male	116	82
Female	77	35
Location		
Proximal	123	40
Distal	70	77
Histopathology		
Hyperplastic polyp	32	2
Serrated adenoma	5	3
Adenoma with low-grade dysplasia	61	45
less than 10 mm (minor neoplasia)	14	26
10 mm or more (advanced neoplasia)	47	19
Adenoma with high-grade dysplasia	20	19
Intramucosal carcinoma and carcinoma in situ	54	28
Early invasive carcinoma (pT1)	21	20

BAT-26, D2S123, D5S346, and D17S250) as described previously.[23] These tumour tissues were also analysed for *APC* mutation (codon 680–1693) as described previously.[24]

2.6. Statistical analysis

Mutation of each gene was assessed for correlations with clinicopathological characteristics using the following statistical tests: Mann–Whitney U-test for age and size, and the chi-square two-tailed test or Fisher's exact test for the remaining parameters.

3. Results

3.1. Exon 3 β-catenin mutation

Exon 3 *β-catenin* mutation was detected in 22 (7.1%) of the 310 early colorectal tumours tissues (Table 2). These mutations were single-base substitutions that were located within the critical serine/threonine codons for GSK-3β phosphorylation of β-catenin (codons 29–48): TCT → TAT at codon 33; GGA → GAA, AGA at codon 34; TCT → TGT at codon 37; ACC → GCC, ATC at codon 41; TCT → TTT at codon 45. All sites of exon 3 *β-catenin* missense mutation have been reported previously.[22,25,26] Interstitial deletions of exon 3 *β-catenin* were detected in two early colorectal tumours tissues (Fig. 1). In both cases, short nucleotide sequences at both ends of the deletion were complementary, and the deletions included exon 3 containing critical serine/threonine codons.

β-catenin mutation was detected in 16 (8.2%) of the 193 flat-type tumour tissues and in six (5.1%) of the 117 protruded-type tumour tissues. The frequencies of *β-catenin* mutation were not significantly different. However, *β-catenin* mutation was detected in a significantly higher percentage (p = 0.0246) of flat-type tumours with depressed areas (23.5%; four of 17 tumours) than in other tumours without depressed areas (6.1%; 18 of 293 tumours). *β-catenin* mutation was detected

Table 2 – Details of β-catenin alterations detected in early colorectal tumour tissues

Case	Morphology	Age (Years)	Size (mm)	Gender	Location	Histopathology	Mutation & Amino acid	IHC	Other mutation
1	flat	58	10	M	proximal	advanced neoplasia	33TCT-TAT (S33Y)	cytoplasm	(–)
2	flat	63	30	M	distal	advanced neoplasia	33TCT-TAT (S33Y)	nuclei	(–)
3	flat	61	7	M	proximal	advanced neoplasia	33TCT-TAT (S33Y)	nuclei	(–)
4	flat	48	12	M	proximal	hyperplastic polyp	34GGA-GAA (G34E)	(–)	BRAF
5	flat	65	9	F	proximal	advanced neoplasia	34GGA-AGA (G34R)	cytoplasm	(–)
6	flat	67	35	F	proximal	advanced neoplasia	34GGA-AGA (G34R)	nuclei	KRAS
7	flat	64	20	M	proximal	advanced neoplasia	41ACC-GCC (T41A)	cytoplasm	(–)
8	flat	74	15	M	proximal	advanced neoplasia	41ACC-GCC (T41A)	cytoplasm	(–)
9	flat	59	15	F	distal	advanced neoplasia	41ACC-GCC (T41A)	cytoplasm	(–)
10	flat	81	40	F	distal	advanced neoplasia	41ACC-GCC (T41A)	cytoplasm	KRAS
11	flat	81	20	F	proximal	advanced neoplasia	41ACC-GCC (T41A)	cytoplasm	(–)
12	flat	63	75	F	proximal	advanced neoplasia	41ACC-GCC (T41A)	nuclei	(–)
13	flat	74	6	M	distal	hyperplastic polyp	41ACC-ATC (T41I)	(–)	(–)
14	flat	74	14	F	proximal	advanced neoplasia	45TCT-TTT (S45F)	cytoplasm	(–)
15	flat	62	35	F	proximal	advanced neoplasia	1-42 deletion (394bp)	nuclei	(–)
16	flat	73	15	M	distal	advanced neoplasia	25-36 deletion (33bp)	nuclei	KRAS
17	protruded	72	30	M	ditsal	advanced neoplasia	33TCT-TAT (S33Y)	nuclei	(–)
18	protruded	55	40	M	proximal	advanced neoplasia	33TCT-TAT (S33Y)	nuclei	KRAS
19	protruded	63	14	M	ditsal	advanced neoplasia	37TCT-TGT (S37C)	cytoplasm	BRAF
20	protruded	81	20	F	ditsal	advanced neoplasia	41ACC-GCC (T41A)	cytoplasm	KRAS
21	protruded	74	7	M	proximal	minor neoplasia	41ACC-GCC (T41A)	cytoplasm	(–)
22	protruded	57	12	M	proximal	advanced neoplasia	45TCT-TTT (S45F)	cytoplasm	(–)

Abbreviations: M, male; F, female; IHC, immunohistochemistry.

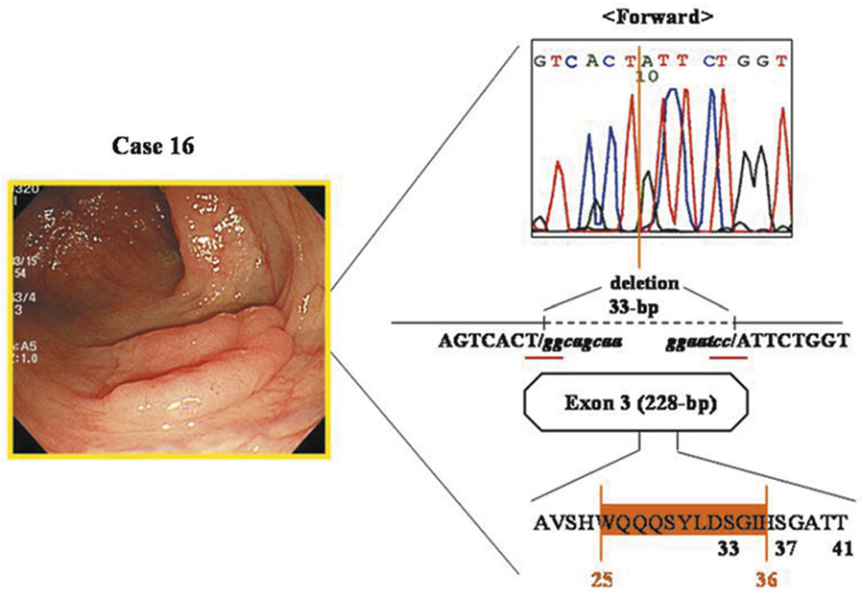

Fig. 1 – (A) Endoscopic picture showing a flat-type colorectal tumour (case 16 in Table 2). (B) Chromotogram showing interstitial deletion of a 33-bp region in the tumour tissues. Three-base inverted repeats, TGG and CCA, were found in the sequences flanking the interstitial deletion. The deletion included the part of exon 3 containing critical serine and threonine codons for GSK-3β phosphorylation.

in 19 (8.2%) of the 233 advanced neoplasia tissues and in three (3.9%) of the 77 hyperplastic polyp and minor neoplasia tissues. The frequencies of *β-catenin* mutation were not significantly different. Other clinicopathological characteristics were not correlated significantly with *β-catenin* mutation.

3.2. *KRAS codon 12 and codon 13 mutations*

KRAS mutation was detected in 67 (21.6%) of the 310 early colorectal tumours tissues. *KRAS* codon 12 mutation, codon 13 mutation, and both mutations were detected in 40, 24, and 3 of the 67 tumour tissues, respectively (Fig. 2A). Although *KRAS* mutation was detected in a higher percentage of flat-type tumour tissues (24.9%; 48 of 193 tumours) than in protruded-type tumour tissues (16.2%; 19 of 117 tumours), the difference was not significant. *KRAS* mutation was detected in a significantly higher percentage ($p = 0.0147$) of advanced neoplasia tissues (24.9%; 58 of 233 tumours) than in hyperplastic polyp and minor neoplasia tissues (11.7%; nine of 77 tumours).

In addition, *KRAS* mutation was correlated significantly with age ($p = 0.0016$), size ($p < 0.0001$), and gender ($p = 0.0049$; female > male). *KRAS* mutation was not detected in any flat-type tumours with depressed areas (0% of 17 tumours).

3.3. *BRAF codon 600 (V600E) mutation*

BRAF mutation was detected in 17 (5.4%) of the 310 early colorectal tumours tissues. All of the tumours demonstrated missense mutations at codon 600 (V600E) (Fig. 2B). *BRAF* mutation was detected in a significantly higher percentage ($p < 0.0001$) of hyperplastic polyps (20.6%; seven of 34 tumours) and serrated adenomas (87.5%; seven of eight tumours) than in other tumours. Although the adenoma with *BRAF* mutation was a serrated adenoma, no *BRAF* mutation was found in tubular or tubulovillous adenomas. The mutation was detected in 14 (7.3%) of the 193 flat-type tumour tissues and in three (2.6%) of the 117 protruded-type tumour tissues. The frequencies of mutation were not significantly different. Other clinicopathological characteristics were not correlated significantly with *BRAF* mutation.

3.4. *PIK3CA mutation in exon 9 and exon 20*

PIK3CA mutation was detected in three (1.0%) of the 310 early colorectal tumours tissues. It was not correlated significantly with any clinicopathological characteristics. These mutations occurred in exon 9 (codons 545) and exon 20 (codons 1047 and 1049) (Fig. 2C).

3.5. *Relationships between KRAS, BRAF, and PIK3CA mutations and β-catenin mutation*

Although there was a mutually exclusive relationship between *KRAS* and *BRAF* mutations, concomitant mutations of *β-catenin* and *KRAS* were detected in five tumours (Table 2). Concomitant mutations of *β-catenin* and *BRAF* were detected in two tumours. Concomitant mutations of *PIK3CA* and *KRAS* or *BRAF* were detected in two tumours. Although *BRAF* mutation was detected in a higher percentage of tumours with *β-catenin* mutation (9.1%; two of 22 tumours) than in tumours without *β-catenin* mutation (5.2%; 15 of 288 tumours), the difference was not significant. Likewise, no significant difference was found between frequencies of *KRAS* mutations in those two groups (22.7%, five of 22 tumours versus 21.5%, 62 of 288 tumours). Mutation of at least one gene was present in 100 (32.3%) of the 310 early colorectal tumour tissues (Table

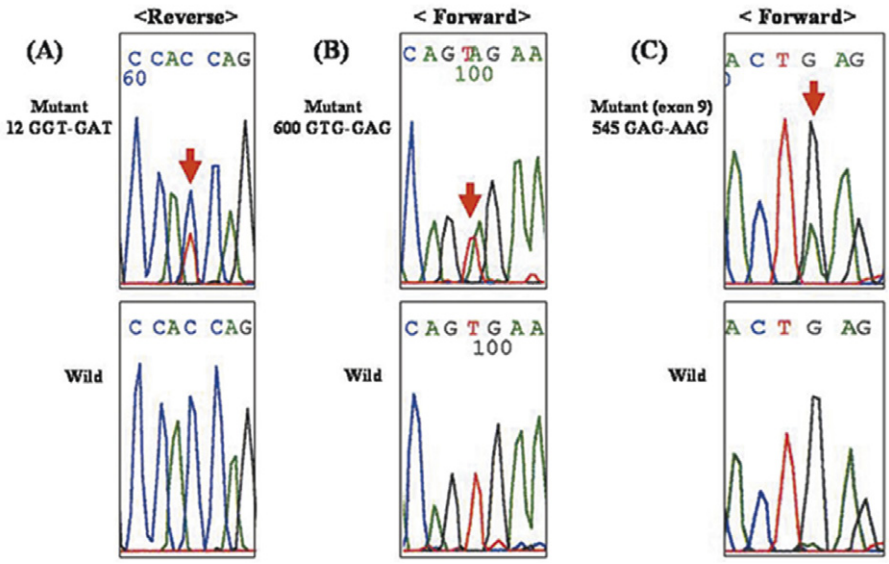

Fig. 2 – DNA direct sequencing of early colorectal tumour tissues with *KRAS*, *BRAF*, or *PIK3CA* mutation. Wild, sequence of a wild case; Mutant, sequence of a mutated case. (A) *KRAS* codon 12: There is a GGT-to-GAT change (G12D) at codon 12. (B) *BRAF* codon 600: There is a GTG-to-GAG change (V600E) at codon 600. (C) *PIK3CA* codon 545: There is a GAG-to-AAG change (E545K) at codon 545.

3). Mutation of at least one gene was detected in a significantly higher percentage ($p = 0.0014$) of flat-type tumour tissues (38.9%; 75 of 193 tumours) than in protruded-type tumour tissues (21.4%; 25 of 117 tumours), and it was correlated significantly with age ($p = 0.0133$), size ($p = 0.0001$), gender ($p = 0.0060$; female > male), and location ($p = 0.0405$; proximal > distal). Although mutation of at least one gene was detected in a higher percentage of advanced neoplasias (33.9%; 79 of 233 tumours) than in hyperplastic polyps and minor neoplasias (27.3%; 21 of 77 tumours), the difference was not significant.

Table 3 – Mutation of at least one gene in early colorectal tumour tissues

Clinicopathological characteristics	Mutation of at least one gene		P-value
	Yes ($n = 100$)	No ($n = 210$)	
Age (years ± SD)	66.9 ± 10.6	63.7 ± 10.5	0.0133
Mean size (mm ± SD)	20.7 ± 13.4	14.7 ± 12.3	0.0001
Gender			
Male	53	145	0.0060
Female	47	65	
Location			
Proximal	61	102	0.0405
Distal	39	108	
Morphology			
Flat-type	75	118	0.0014
Protruded-type	25	92	
Histopathology			
Hyperplastic polyp and Minor neoplasia	21	56	0.2804
Advanced neoplasia	79	154	

3.6. *Mutational analysis of advanced neoplasia*

We analysed 146 flat-type advanced neoplasia (FAN) tissues and 87 protruded-type advanced neoplasia (PAN) tissues. Mutations of *β-catenin*, *KRAS*, *BRAF*, and *PIK3CA* were detected in 14 (9.6%), 42 (28.8%), five (3.4%), and one (0.7%) of the 146 FAN tissues, respectively. On the other hand, mutations of *β-catenin*, *KRAS*, *BRAF*, and *PIK3CA* were detected in five (5.7%), 16 (18.4%), two (2.3%), and one (1.1%) of the 87 PAN tissues, respectively. No significant difference was found between frequencies of those mutations in FAN and PAN tissues. Mutation of at least one gene was detected in a significantly higher percentage ($p = 0.0066$) of FAN tissues (40.4%; 59 of 146 tumours) than in PAN tissues (23.0%; 20 of 87 tumours).

3.7. *Immunohistochemical staining of β-catenin in colorectal tumour*

Immunohistochemical staining of β-catenin was positive in 20 (90.9%) of the 22 tumours with *β-catenin* mutations (Table 2). Immunohistochemical staining of the tumours showed a high level of β-catenin protein in the cytoplasm (Fig. 3A) or nuclei (Fig. 3B). On the other hand, in normal epithelial cells of the colon, β-catenin protein was found in the membrane but not in the cytoplasm or nuclei. Intense cytoplasmic and nuclear expressions of β-catenin were seen in 15 and five of the 22 tumours, respectively.

Intense cytoplasmic and/or nuclear expression of β-catenin was detected in a higher percentage of flat-type tumours with depressed areas (64.7%; 11 of 17 tumours) than in other tumours without depressed areas (50.2%; 147 of 293 tumours),

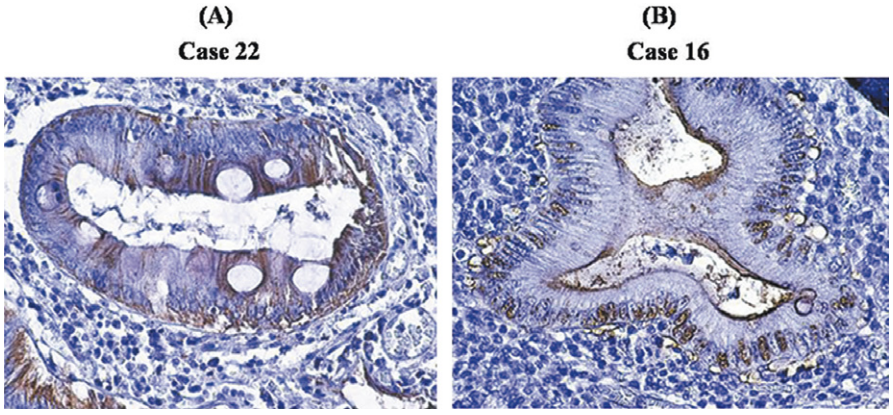

Fig. 3 – Immunohistochemical staining of β-catenin protein in colorectal tumour tissues with *β-catenin* mutation. (A) Staining of β-catenin protein is observed in the cytoplasm of tumour cells (case 22 in Table 2) (stain, hematoxylin and eosin; magnification, 400×). (B) Intense nuclear expression of β-catenin is immunohistochemically observed within the nuclei of tumour cells (case 16 in Table 2) (stain, hematoxylin and eosin; magnification, 400×).

but the difference was not significant. On the other hand, it was detected in a significantly higher percentage ($p < 0.0001$) of early invasive carcinomas (87.8%; 36 of 41 tumours) than in other tumour tissues (41.3%; 111 of 269 tumours).

3.8. *MSI and APC mutation*

We analysed the 22 tumour tissues with *β-catenin* mutations for MSI. No MSI-H was found in these tumour tissues, and these tumour tissues were classified as MSS or MSI-L according to the NCI guideline (data not shown). *APC* mutation was not detected in 22 tumour tissues with *β-catenin* mutations (data not shown).

4. Discussion

In the current study, *β-catenin* mutation was frequently detected in flat-type tumours with depressed areas. These tumours are thought to develop through a distinct genetic pathway and correspond to *de novo* cancers.[6,27] Akiyama and colleagues[28] reported that the frequency of 18q21 allelic imbalances in flat-type tumours with depressed areas was significantly higher than that in tumours without those areas and that the frequency of *APC* alterations was significantly lower in the former than in the latter. No significant difference was found between frequencies of *p*53 alterations in those two groups.[28] Therefore, not only these genetic abnormalities but also *β-catenin* mutation seems to contribute to the development and/or progression of flat-type tumours with depressed areas.

Johnson and colleagues[18] recently reported that exon 3 *β-catenin* mutations were rare in small (<10 mm) sporadic adenomas (1/83, 1.2%), HNPCC adenomas (1/37, 2.7%), and in both MSI-positive (0/34) and MSI-negative (0/78) sporadic colorectal cancers. In contrast, a significantly higher frequency (8/44, 18.2%) of mutations was found in HNPCC cancers.[18] Considering our results in the current study, it was thought that exon 3 *β-catenin* mutation could often occur not only in HNPCC cancers but also in sporadic colorectal adenomas and cancers.

MSI-H due to defective DNA mismatch repair occurs in the majority of HNPCC and in 10–15% of sporadic colorectal cancers. It has been reported that *β-catenin* mutations occur more often in MSI-H colorectal cancers.[18,25,26] Although we analysed 22 tumour tissues with *β-catenin* mutation for MSI, no tumour tissue was MSI-H. This result might be due to the fact that all tumour samples were early colorectal tumours.[6,29] Mutations in *β-catenin* and *APC* have been reported to be mutually exclusive, consistent with their almost equivalent effects on β-catenin stability and TCF-4 transactivation.[24] In the current study, *APC* mutation was not detected in any of the 22 tumour tissues with *β-catenin* mutations, compatible with results presented in that previous report.[24,26]

In the current study, with the exception of two hyperplastic polyps, immunohistochemical staining of tumours with *β-catenin* mutations showed a high level of β-catenin protein in the cytoplasm and nuclei.[30,31] Interestingly, all of the tumours with intense nuclear expression were intramucosal carcinoma or early invasive carcinoma. These findings suggest that cytoplasmic or nuclear expression of β-catenin correlates with the biological malignancy.[31] In the mechanism by which β-catenin proteins are translocated into the cytoplasm or nuclei, colorectal tumours seemed to require not only the missense mutations but also other alterations in co-activators of TCF-4/β-catenin or epigenetic alterations such as DNA promoter hypermethylation. These mechanisms and/or *β-catenin* mutation in focal area of tumours may underlie the lack of immunohistochemical overexpression of β-catenin in two hyperplastic polyps with *β-catenin* mutation. Further analysis is needed to clarify these issues.

Intense cytoplasmic and/or nuclear expression of β-catenin was detected in a higher percentage of flat-type tumours with depressed areas (64.7%) than in other tumours without depressed areas (50.2%), but the difference was not significant. Overexpression of β-catenin in tumours without *β-catenin* mutations could be achieved by APC deficiency and/or other alterations. The deregulation of the APC-β-catenin pathway is not necessarily accompanied by overexpression of β-catenin. Therefore, the frequency of alterations of the APC-β-catenin pathway could be higher than that of β-catenin

overexpression in early colorectal tumourigenesis. Although mutually exclusive, *APC* and *β-catenin* mutations are not functionally equivalent, and APC has other tumour suppressor functions besides degrading β-catenin. Further analysis is needed to clarify the roles played by the deregulation of the APC-β-catenin pathway in early colorectal tumourigenesis and by *β-catenin* mutations in flat-type tumours with depressed areas.

Although it has been reported that *KRAS* mutations are infrequently detected in flat-type colorectal tumours,[2,6] a significant difference was not found between frequencies of *KRAS* mutation in flat-type and protruded-type tumours in the current study. However, *KRAS* mutation was not detected in any flat-type tumours with depressed areas. Umetani and colleagues[7] reported that the frequency of *KRAS* mutations in flat-type tumours with depressed areas was significantly lower than that in flat-type tumours without depressed areas. Our findings are consistent with the results of that study.

BRAF mutation was detected in a significantly higher percentage of hyperplastic polyps and serrated adenomas than in other tumours. Since other clinicopathological characteristics, including morphology, were not correlated significantly with *BRAF* mutation, this mutation seems to be specific for a hyperplastic polyp-serrated adenoma-carcinoma pathway.[8]

In colorectal tumours, *PIK3CA* mutations preferentially occur in exons 9 and 20, affecting the two functionally important helical and kinase domains of the protein. *PIK3CA* mutations occurred more frequently in accumulation with *KRAS* or *BRAF* mutations than in isolation. Previous studies have shown that the prevalence of *PIK3CA* exon 9 and exon 20 mutations in advanced colorectal cancers range between 13.6% and 27%.[9,10] In the current study, the frequency of *PIK3CA* mutation was low not only in adenomas but also in early invasive carcinomas. Samuels and colleagues[9] reported that *PIK3CA* mutations were rare in colorectal adenomas (2/76, 2.6%) and that colorectal adenomas in which mutations were detected were very advanced tubulovillous adenomas greater than 5 cm in diameter. Therefore, these results suggest that *PIK3CA* mutation arises in the late stage of colorectal carcinogenesis.

It has been reported that *β-catenin* mutation does not coexist with *KRAS* mutation in colorectal tumours.[22] On the other hand, Li and colleagues[32] reported that oncogenic KRAS stimulates the Wnt signal pathway in colorectal cancer through inhibition of GSK-3β. In the current study, concomitant mutations of *β-catenin* and *KRAS* or *BRAF* were detected in seven tumours. That is to say, 31.8% (7/22) of the early colorectal tumours with *β-catenin* mutations have another genetic abnormality. Concomitant mutations of *PIK3CA* and *KRAS* or *BRAF* were detected in two (66.7%) of the three early colorectal tumours with *PIK3CA* mutations. These findings suggest that genetic abnormalities accumulate in the early stage of colorectal carcinogenesis.

Mutation of at least one gene was detected in a significantly higher percentage of flat-type tumour tissues than in protruded-type tumour tissues. When the samples were limited to advanced neoplasias to match the histopathology of flat-type and protruded-type tumours, the frequency in FAN was significantly higher than that in PAN. Regarding histopathology, a significant difference was not found between advanced neoplasia and minor neoplasia. Therefore, multiple genetic abnormalities could arise in the early stage of colorectal tumourigenesis. Indeed, multiple genetic alterations in early colorectal tumourigenesis have been previously reported. For example, Chan and colleagues[33] reported the concurrent alterations of *KRAS* mutation, loss of heterozygosity of 1p, and methylation of one or more genes in several aberrant crypt foci. Takayama and colleagues[34] also reported multiple genetic alterations in early colorectal tumourigenesis. Our results further suggest that flat-type tumours have a higher mutational rate than protruded-type tumours. Since flat-type tumours have been reported to exhibit relatively aggressive clinical behavior with a tendency to invade deep mucosal layers with metastatic invasion over a short time course,[3–5] their high mutational rate might contribute to the biological malignancy.

In conclusion, *β-catenin* mutation was detected in sporadic early flat-type colorectal tumours and seems to play an important role, especially in those with depressed areas. Since concomitant mutations of *β-catenin* and *KRAS*, of *β-catenin* and *BRAF*, and of *PIK3CA* and *KRAS* or *BRAF* were detected in early colorectal tumours, genetic abnormalities could accumulate in the early stage of colorectal tumourigenesis. In flat-type colorectal tumours, genetic abnormalities seem to play an important role in the development and progression.

Conflict of interest statement

None declared.

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