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Analysis of *SMAD4/DPC4* gene alterations in multiploid colorectal carcinomas

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Background. Although recent animal studies have shown that *SMAD4*/*DPC4* gene alterations are essential for late-stage intestinal tumorigenesis, the role of *SMAD4*/*DPC4* gene alterations in primary human colorectal carcinomas is not fully understood. Therefore, we attempted to clarify the role of the *SMAD4*/*DPC4* gene during tumor progression of colorectal carcinoma. ***Methods.*** Differences in allelic imbalance (AI) and mutations of the *SMAD4*/*DPC4* gene between diploid and aneuploid populations were analyzed for 30 sporadic DNA multiploid colorectal carcinomas (used as a tumor progression model and defined as the coexistence of diploid and aneuploid cells within the same tumor). The crypt isolation technique was coupled with DNA cytometric sorting and a polymerase chain reaction assay. In addition, hypermethylation of the promoter region was examined to clarify whether inactivation of gene expression occurred. ***Results.*** Although a *SMAD4*/*DPC4* gene AI was detected in only 5 of 27 informative diploid populations, 25 of 27 aneuploid populations had a *SMAD4*/*DPC4* gene AI. Mutation of the *SMAD4*/*DPC4* gene was detected in only one aneuploid population of multiploid colorectal carcinomas, but not in the corresponding diploid population. In total, 20 available multiploid carcinomas were selected for methylation analysis, and no evidence of hypermethylation of the promoter region was found. ***Conclusions.*** We suggest that, although mutation of the *SMAD4*/*DPC4* gene and hypermethylation of the promoter region are infrequent events in colorectal tumorigenesis, AI at the *SMAD4*/*DPC4* gene locus may play a key role in the progression of colorectal carcinomas.

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Introduction

The theory of multistep carcinogenesis with multiple genetic alterations in human colorectal carcinoma is a well-known hypothesis.[1,2] During tumor progression, the inactivation of tumor suppressor genes or the activation of oncogenes contributes to the development of colorectal carcinoma.[1,2] Inactivation of tumor suppressor genes plays a major role in colorectal carcinogenesis; for example, in the *APC*, *p53*, *DCC*, and *SMAD4*/*DPC4* genes.[1–3] Although the roles of *APC* and *p53* in colorectal carcinogenesis are well-established, the roles of the latter two genes (*DCC* and *SMAD4*/*DPC4*) are less well understood.[4] However, a recent in vitro study of *SMAD4*/*DPC4* mutant mice has shown that *SMAD4*/*DPC4* gene alterations are closely associated with colorectal carcinogenesis.[5] In addition, previous studies utilizing human samples have demonstrated that at least one allele of chromosome 18q (loss of heterozygosity, LOH) is lost in over 70% of sporadic colorectal carcinomas.[6,7] Three candidate tumor suppressor genes, *DCC*, *SMAD4*/*DPC4*, and *SMAD2*, map to this region.[8] Although there has been no evidence that alteration of the *SMAD2* gene is associated with colorectal carcinogenesis, loss of *DCC* and *SMAD4*/*DPC4* expression has been reported in advanced colorectal carcinomas.[8,9] In particular, these changes are associated with metastasis in colorectal carcinoma.[8] This finding suggests that these two genes (*DCC* and *SMAD4*/*DPC4*) play a role in the progression of late-stage colorectal carcinomas.[3,8] Although the *SMAD4*/*DPC4* gene has been implicated as playing a prominent role in advanced disease, this gene has not been considered a major player, as the *APC*, *Ki-ras*, and *p53* genes, in colonic neoplasia.[6]

In previous studies,[6–8] tumors at different stages have been used to analyze tumor progression. However, this method of analysis is not suitable for investigating tumor progression. Instead, it is necessary to establish a model of tumor progression using tissue from within the same tumor. For example, if adenomatous and carcinomatous cells are found in close proximity, this sample would be a good model to examine tumor progression. Another good model of progression would be cancer cells with low-grade atypia coexisting with cells having high-grade atypia. However, such samples can be difficult to find during practical histological examination. Therefore, a new progression model, using single tumors, must be developed to analyze the roles of gene alterations.

DNA multiploidy is defined as the coexistence of diploid and aneuploid populations in the same area.[10,11] In a previous study, we demonstrated that aneuploid tumor cells of multiploid colorectal carcinomas evolved from diploid tumor cells of the multiploid colorectal carcinomas.[10,12] Therefore, multiploidy is a novel model to study tumor progression within the same tumor. Multiploidy is identified by the crypt isolation method, which is used to obtain only tumors and crypts from tumor tissue.[10] Conventional methods used to isolate tumor cells do not remove all of the normal cells, and thus cannot be used to distinguish tumor from non-tumor diploid populations.[10,12] Therefore, the crypt isolation method is a superior technique that may be used to verify the existence of both diploid and aneuploid populations within a single tumor.[10,12] Cytometric sorting of cells from DNA multiploid tumor areas can be used to determine differences in the genetic alterations of diploid and aneuploid populations that develop during neoplastic progression within an individual cancer.

To determine the role of *SMAD4*/*DPC4* gene alterations in the development and/or progression of colorectal carcinomas, we examined this gene for mutations and allelic losses in diploid and aneuploid fractions of primary multiploid colorectal carcinomas. In addition, hypermethylation of the promoter region was analyzed to determine whether inactivation of gene expression occurred in these samples.

Subjects, materials, and methods

Thirty sporadic colorectal carcinomas obtained by colectomy from 30 patients (21 men and nine women), ranging in age from 46 to 90 years (mean, 66.0 years) were analyzed for genetic alterations of the *SMAD4*/*DPC4* gene, using tumor cells isolated by the crypt isolation method. The clinicopatholigical findings for the 30 patients are shown in Table 1. The pathological diagnosis and staging were performed according to a combination of a Japanese classification[13] and a modified Dukes' classification.[14] In total, 6 tumors were classified histologically as well-differentiated and 24 as moderately differentiated. According to the modified Dukes' stage, 2 carcinomas were stage A, 11 were stage B, 10 were stage C, and 7 were stage D.

Table 1. Clinicopathological findings for 30 patients with multiploid colorectal carcinomas

Total no. of patients	30
Age (years; mean)	46–90 (66.0)
Sex (M/F)	21/9
Tumor lesion (R/L)	9/21
Microscopic type	
WDA	6
MDA	24
Dukes' stage	
A	2
B	11
C	10
D	7

R, right side (cecum, ascending colon and transverse colon); L, left side (descending colon, sigmoid colon, and rectum); WDA, well differentiated adenocarcinoma; MDA, moderately differentiated adenocarcinoma

Crypt isolation technique for tumor-cell isolation

Fresh tumor and normal tissue samples were obtained from surgical specimens that were removed during surgery for colon carcinoma. The tumor samples were obtained primarily from the central area of the tumor ulcerations, and included the most invasive layer of the tumor. A sample of normal colonic mucosa was removed from a site distant from the lesion. Crypt isolation was used to enrich tumor cells taken from the colorectal tumor tissue, because pure tumor cells are required in order to analyze genetic alterations accurately.

Crypt isolation from the tumor and normal mucosa was performed as previously described.[15] Briefly, fresh mucosa and tumor were minced with a razor into minute pieces, then incubated at 37°C for 30 min in calcium-, and magnesium-free Hanks' balanced salt solution (CMF), containing 30 mmol/l ethylenediaminetetraacetic acid (EDTA). Following this procedure, the tissue was then stirred in CMF for 30–40 min. The isolated crypts were immediately fixed in 70% ethanol and stored at 4°C until used for DNA extraction. The fixed isolated crypts were examined under a dissection microscope (SZ60; Olympus, Tokyo, Japan). Normal crypts were clearly distinguishable from tumor crypts, based on their characteristic features, as reported elsewhere.[10,12,13,15]

The isolated crypts were routinely processed for histopathological analysis to morphologically confirm their

isolated nature. No contamination (such as interstitial cells) was observed in any of the 30 samples.

Flow cytometric sorting

Aneuploid and diploid populations were isolated from each sample, using a DNA flow cytometer. Nuclei were sorted into diploid and aneuploid cell populations using a cell sorter (EPICS Altra; Coulter, CA, USA).[11]

DNA extraction

Genomic DNA from diploid and aneuploid fractions of multiploid tumor cells and nontumor cells were extracted and prepared from the isolated crypts as described previously.[10,11]

Analysis of allelic imbalance at 18q21

Analysis of allelic imbalance was carried out using two fluorescence-labeled microsatellite markers (D18S1156 and D18S363). The primer pairs used were as follows: D18S1156 (sense), 5′-CCTGCAAGTTNA CTGGC and (antisense), 5′-CAATGACAACCTGTT GTTGG; D18S363 (sense), 5′-TTGGGAACTGCTC TACATTC and (antisense), 5′-GCTTCATTCTCTCA CTGGAT.

These two markers were closely linked to the *SMAD4/DPC4* locus on 18q21.[6] PCR reactions were performed using a thermal cycler (GeneAmp PCR System 9600; Applied Biosystems, Perkin-Ermer Cetus, Norwalk, CT, USA) with genomic DNA as template, 1 µM of each primer and 1 × Ampli Taq Gold Master Mix (Applied Biosystems), in a final reaction volume of 25 µl. Samples were processed for 25 to 30 cycles, with each cycle consisting of 30 s at 94°C, 1 min at 55°C to 58°C, and 2 min at 72°C, followed by a final 10-min extension at 72°C. For quantitative detection of the allelic imbalance at each locus, polymerase chain reaction (PCR)-loss of heterozygosity (LOH) analysis was performed as described previously.[10,11] A 1-µl aliquot of the PCR product was added to 3-µl formamide and a 0.5-µl TAMRA 500 size standard (Applied Biosystems), loaded on a 6% polyacrylamide-8-M urea gel, and run for 2–6 h in a PRISM 310 Automated Sequencer (Applied Biosystems) at a constant power of 30 W. The data were collected automatically and analyzed by GeneScan 3.1 software (Applied Biosystems). Allelic imbalance was scored by calculating the ratios of the peak areas of the constitutional alleles, as previously described (q value).[16,17] A tumor was considered to have allelic imbalances if the allele peak ratio was less than or equal to 0.60, representing an allelic signal reduction of at least 40%. We interpreted this allelic imbalance as allelic loss, with the realization that, in some cases, changes in the allele peak ratio may have resulted from allelic amplification.

Mutations of the SMAD4/DPC4 gene

Sequencing of PCR-amplified products was used to detect mutations in exons 8–11 of the *SMAD4/DPC4* gene in patient-derived normal mucosa and tumor DNA samples. PCR conditions and sequencing of mutations were performed as described previously, with slight modifications.[8] Automated direct DNA sequencing was performed using fluorescently labeled dideoxynucleotide triphosphates (PRISM 310 sequencer; Applied Biosystems).

DNA methylation analysis

Sodium bisulfite conversion of genomic DNA from colorectal cancers was performed as described previously, followed by PCR amplification using SMAD-S1 (5′-GGGAAAAGGGTTAAATTTTGA-3′) and SMAD-AS1 (5′-CCTAACTCCCCTCACCC-3′) oligonucleotide primers, using 30 cycles at 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min. The primers designated for this analysis are shown, underlined, in Fig. 1. The genomic sequence of the promoter region (Accession no. AC 091551) of the *SMAD4/DPC4* gene was obtained from the Genome Database (http://

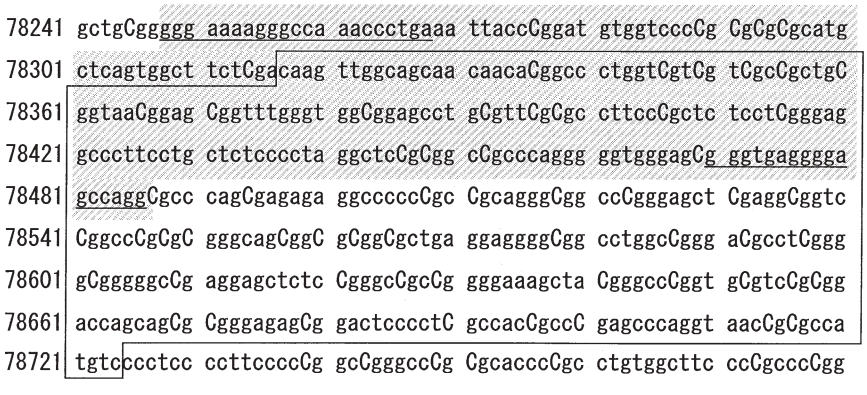

Fig. 1. Genomic sequence of the *SMAD4/DPC4* promoter region that was analyzed for methylation. The primers designated for this analysis are *underlined*. *Gray shading* depicts the polymerase chain reaction (PCR) product analyzed. *Enclosed areas with a white background* indicate the PCR product reported by Roth et al.[28]

Table 2. Detailed data of clinicopathological and genetic analysis

Sample no.	Age (years)	Sex	Tumor lesion	Microscopic type	Dukes' stage	D18S1156		D18S363		Mutation	Methylation
						D	A	D	A		
1	65	M	S	MDA	C	N	N	N	N	–	–
2	90	F	R	MDA	B	–	+	–	+	–	–
3	46	M	R	MDA	D	–	+	–	–	–	–
4	68	M	A	MDA	C	N	N	+	+	+	–
5	53	M	R	MDA	B	–	+	–	–	–	–
6	79	M	S	MDA	B	N	N	–	+	–	–
7	46	M	S	MDA	D	N	N	–	+	–	–
8	77	F	S	MDA	B	+	+	+	+	–	–
9	61	F	R	MDA	C	N	N	–	+	–	–
10	61	F	De	MDA	C	N	N	–	+	–	–
11	53	F	R	MDA	B	N	N	–	+	–	–
12	55	M	R	MDA	C	N	N	–	+	–	–
13	76	M	A	MDA	B	–	–	–	–	–	–
14	74	M	R	MDA	D	N	N	–	+	–	–
15	65	F	R	MDA	B	–	+	–	–	–	–
16	66	M	De	WDA	D	N	N	–	+	–	–
17	55	F	R	MDA	C	–	+	–	+	–	–
18	63	M	T	MDA	D	N	N	–	+	–	–
19	80	M	A	WDA	B	N	N	–	+	–	–
20	55	M	R	WDA	B	N	N	N	N	–	–
21	72	M	S	MDA	D	N	N	N	N	–	–
22	72	M	R	MDA	B	+	+	+	+	–	–
23	56	F	R	MDA	C	–	–	–	+	–	–
24	74	M	R	WDA	A	N	N	–	+	–	–
25	73	F	A	WDA	C	N	N	–	+	–	–
26	71	M	T	MDA	A	N	N	–	+	–	–
27	65	M	A	WDA	C	N	N	+	+	–	–
28	83	M	A	MDA	D	+	–	+	–	–	–
29	56	M	R	MDA	B	N	N	–	+	–	–
30	69	M	A	MDA	C	N	N	–	+	–	–

A, ascending colon; T, transverse colon; De, descending colon; R, rectum; S, sigmoid colon; D, diploid; A, dneuploid; WDA, well-differentiated adenocarcinoma; MDA, moderately differentiated adenocarcinoma

gdbwww.gdb.org/gdb/). The PCR products were sequenced using the sense primer with a PRISM 310 DNA sequencer (Applied Biosystems). Bisulfite conversion was confirmed to be complete, because thymine was substituted for all cytosine residues that occurred at non-CpG sites.

Statistical analysis

The data were analyzed using the χ^2 test with the aid of StatView-IV software (Abacus Concepts, Berkeley, CA, USA). Samples were determined to be significantly different when the *P* value was less than 0.05.

Results

Flow cytometric analysis

The coefficient of variation (CV) of diploid populations ranged from 2.1 to 4.9 (mean, 3.41). Aneuploid DNA Index (DIs) ranged from 1.25 to 1.89 (mean, 1.63).

Allelic imbalance at the SMAD4/DPC4 locus

All samples showing allelic imbalance were subjected to repeat analysis involving at least two independent PCR amplifications. In all cases, replicate tests yielded identical results. Microsatellite instability was not detected in this study.

Two microsatellite markers (D18S1156 and D18S363) were used to detect the18q 21 locus that is closely linked to the *SMAD4*/*DPC4* gene. The latter (D18S363) was a highly informative marker (27/30 cases; 90%) to detect allelic imbalance in multiploid colorectal carcinomas. Detailed results showing the allelic alterations that were revealed by each marker are listed in Table 2. When an allelic imbalance was observed in at least one locus of the two markers examined, imbalance of the 18q locus was confirmed. The allelic imbalances of diploid and aneuploid populations are shown in Table 3. The aneuploid populations that we evaluated demonstrated an allelic imbalance in 25 of 27 informative cases (92.6%), although a few imbalances were also detected in the corresponding diploid

Table 3. Frequencies of allelic imbalances, mutations of the *SMAD4*/*DPC4* gene, and hypermethylation of the promoter region in 30 multiploid carcinomas

			Multiploid (%)	
	Total	Informative cases	Diploid	Aneuploid
Allelic imbalance	30	27	5 (18.5)	25 (92.6)
Mutation	30		0	1 (3.3)
Methylation	20		0	0

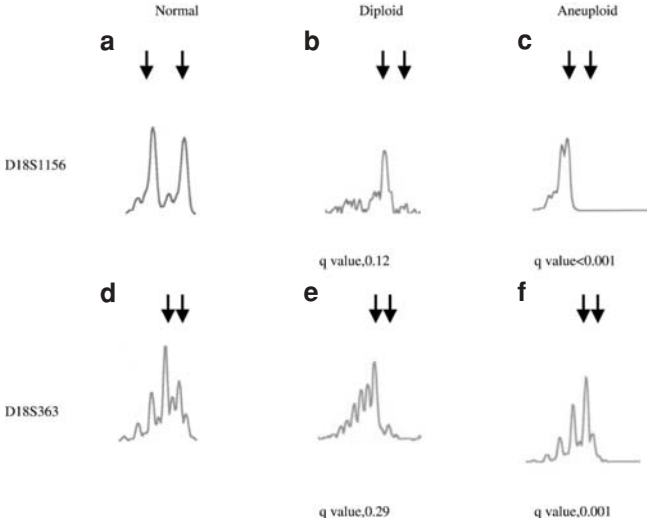

Fig. 2. Allelic imbalances at the *SMAD4*/*DPC4* locus detected in sorted diploid and aneuploid populations. *Arrows*, two alleles for each chromosomal locus. One of the two alleles was lost in b, c, e, and f

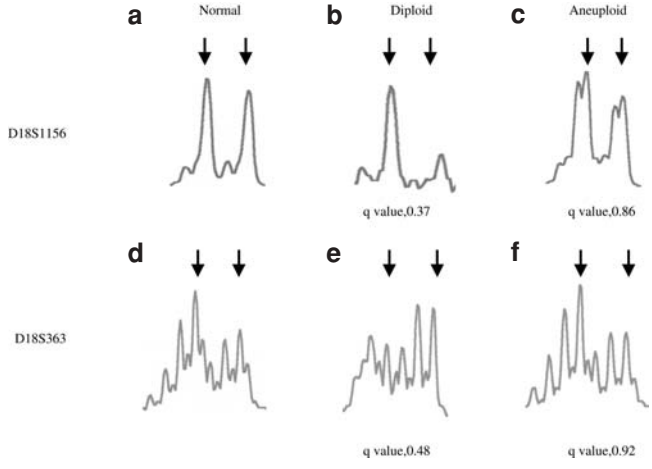

Fig. 3. A representative example of allelic imbalances at *SMAD4*/*DPC4* in a diploid population of multiploid colorectal carcinomas. Allelic imbalances at the *SMAD4*/*DPC4* locus were found in a diploid population of multiploid colorectal carcinomas (b and e), but not in the corresponding aneuploid population (c and f)

populations (5/27; 18.5%; Table 3). A representative example of allelic imbalance at the *SMAD4*/*DPC4* locus is shown in Fig. 2. There was a significant difference in the frequency of allelic imbalances at the *SMAD4*/*DPC4* locus between diploid and aneuploid populations of multiploid colorectal carcinomas ($P < 0.01$). Four of five diploid samples showing allelic imbalance at the 18q locus had an allelic imbalance at the same allele in the corresponding aneuploid populations. However, only one of the five diploid tumor-cell populations revealing an 18q allelic imbalance showed no allelic imbalance in the corresponding aneuploid population (Fig. 3).

SMAD4/DPC4 gene mutations

The frequency of *SMAD4*/*DPC4* mutations is summarized in Table 3. *SMAD4*/*DPC4* gene mutations were observed in only one aneuploid population of the 30 multiploid carcinomas (3.3%), but not in the corresponding diploid population. A representative example of a *SMAD4*/*DPC4* gene mutation is shown in Fig. 4. A transition from C to T in the first nucleotide of codon 441 (exon 10) was observed. The normal sequence was lost in the aneuploid population, suggesting loss of the other allele.

Hypermethylation of the SMAD4/DPC4 gene promoter region

In the present study, the methylation status of the *SMAD4*/*DPC4* gene promoter was analyzed using direct sequencing after sodium bisulfite conversion. Using this method, we evaluated the methylation status of the *SMAD4*/*DPC4* promoter region in 20 available samples of 30 colorectal carcinomas. The presence or absence of methylated CpG islands at the promoter region was determined and compared with the results for the corresponding normal mucosa. Conversion from C-to-T after sodium bisulfite treatment was found in all samples that were examined. Therefore, we could not detect methylation of the *SMAD4*/*DPC4* gene promoter region, as shown in Table 3.

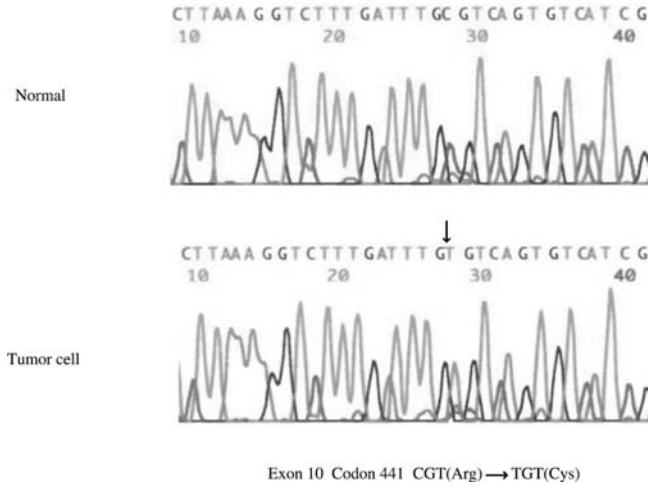

Fig. 4. Mutation analysis of the *SMAD4*/*DPC4* gene. A point mutation of the *SMAD4*/*DPC4* gene was observed in the aneuploid population, but not in the corresponding diploid population. Note that a normal sequence of the other allele was not detected in the aneuploid population of multiploid colorectal carcinomas, indicating allelic loss at the other allele. *Arrows* show a mutation

Clinicopathological correlations

Allelic imbalances in the *SMAD4*/*DPC4* gene were assessed for their association with tumor location, histology, and tumor stage. Overall, no statistically significant associations were identified.

Discussion

The evolution from diploid tumor cells to a single aneuploid cell population and then to multiploidy is a common occurrence in colorectal carcinomas. DNA multiploidy is, as mentioned above, defined as the coexistence of diploid and aneuploid populations in the same area.[10,11] Multiploidy is a novel model to analyze genetic alterations during tumor progression. This progression model rests on the assumption that an aneuploid population of multiploid colorectal carcinoma originates from a diploid population of multiploid colorectal carcinoma within the same area. This hypothesis is supported by a previous study showing that all seven diploid samples showing allelic loss at the 17p locus had allelic loss at the same allele as the corresponding aneuploid populations.[11] In addition, our previous studies[12,18] revealed that diploid tumor populations showing *Ki-ras* or *p53* mutations had the same mutations in the corresponding aneuploid populations of multiploid carcinomas, with a very few exceptions. However, the present study showed that, in one diploid population of multiploid colorectal carcinoma with allelic imbalance, both alleles were not lost in the corresponding aneuploid population. This finding suggests that at least a very few multiploid tumor cell populations consist of different diploid and aneuploid populations. However, we believe that most diploid tumor cells of multiploid colorectal carcinoma evolve into aneuploid cells of multiploid colorectal carcinoma, as has been shown in previous studies.[10,12,18] Therefore, we suggest that multiploidy can be used as a novel progression model to examine genetic alterations of tumor cells, in cases where the transition of diploid fractions of multiploidy into the aneuploid fraction of the multiploid carcinomas is confirmed.

In the present study, whereas the frequency of allelic imbalance at 18q21 in diploid populations of multiploid colorectal carcinoma was low (5/27 cases), the frequency in aneuploid populations of multiploidy was very high (25/27 cases). A high frequency of LOH on 18q21 has previously been recognized in the progression of colon carcinomas.[8] For example, 18q LOH is associated with metastatic ability and may have prognostic significance in colorectal carcinomas.[19] Two candidates for tumor suppressor genes at 18q21, *DCC* and *SMAD4*/*DPC4*,[20,21] have been cloned, and a recent study suggested that the *SMAD4*/*DPC4* gene may deserve to be another target gene at this locus.[5] The present study shows that allelic imbalance at the *DPC4* gene locus plays a major role in the transition from diploid populations of multiploid colorectal carcinomas to aneuploid populations of multiploid colorectal carcinomas. We suggest that LOH of the *SMAD4*/*DPC4* gene is closely associated with tumor progression in colorectal carcinoma.

Although mutations of the *SMAD4*/*DPC4* gene were examined in 30 multiploid colorectal carcinomas, using PCR-single-strand conformation polymorphism (SSCP) analysis, coupled with direct sequencing, a mutation was found in only one aneuploid line of multiploidy. Mutation analysis was restricted to exons 8, 9, 10, and 11 of the *SMAD4*/*DPC4* gene, which span the entire conserved C-terminal *SMAD4*/*DPC4* homology region.[22] Approximately 90% of the *SMAD4*/*DPC4* gene mutations that have been reported are located in this region.[19,22] Therefore, we believe that additional mutations of the *SMAD4*/*DPC4* gene are unlikely to be found outside these four exons (exons 8–11). The mutation rate of the *SMAD4*/*DPC4* gene is correlated with the tumor stage and/or progression of colorectal tumors.[8] Miyaki et al.[8] analyzed 176 colorectal tumors at varying stages, using PCR-SSCP. They found *SMAD4*/*DPC4* mutations in 6 of 17 (35%) primary carcinomas with distant metastases, compared with 0/40 in adenomas, 4/39 (10%) in intramucosal carcinomas, and 3/44 (7%) in invasive carcinoma without distant metastasis.[4,8] These findings suggest that the frequency of

SMAD4/*DPC4* gene mutations increases with the progression of carcinogenesis. On the other hand, some investigators have reported that the frequencies of *DPC4* gene mutation are low in advanced colorectal carcinomas,[3,23–27] though such data originated from relatively small studies. In the present study, we used multiploid carcinomas as a novel tumor progression model to examine *SMAD4*/*DPC4* gene mutations in colorectal carcinomas. Numerous genetic alterations were accumulated in aneuploid populations of multiploid colorectal carcinomas, compared with diploid populations of the tumor cells. Two repeat analyses, using PCR-SSCP coupled with direct sequence analysis, were performed. Therefore, we believe that the data presented here are reliable and reproducible. We suggest that mutations of the *SMAD4*/*DPC4* gene play a minor role in tumorigenesis of colorectal multiploid carcinomas.

We can suggest two possible explanations for the different frequencies of *SMAD4*/*DPC4* gene mutations found in our study, compared to previous studies.[4,8] The first explanation is that the *SMAD4*/*DPC4* gene mutation frequency may depend on the tumor stage of the colorectal carcinomas.[4,8] In the present study, a mutation of the *SMAD4*/*DPC4* gene was present in 1 of 17 tumors from patients with lymph node metastasis. A larger study will be needed to clarify the role of *SMAD4*/*DPC4* gene mutations. Such a study should use multiploid carcinomas with metastasis, which correspond to late progression of colorectal carcinoma within the same tumor. A second possible explanation is that only carcinomas showing multiploidy were selected in the present study, because a progression model using tissues from the same tumor was used. Thus, this study did not include diploid and pure aneuploid carcinomas, which constitute one-third of advanced colorectal carcinomas.[10] The lack of diploid and pure aneuploid carcinomas may be responsible for the lower frequency of *SMAD4*/*DPC4* gene mutations seen in this study.

In the present study, we examined whether hypermethylation of the promoter region could be an alterative mechanism for *SMAD4*/*DPC4* inactivation. Although we attempted to perform methylation analysis of the *SMAD4*/*DPC4* gene, using a methylation-sensitive PCR (MSP) that was previously reported by Roth et al.,[28] reliable and reproducible results of the MSP experiments could not be obtained. Therefore, the CpG island within the promoter region (near noncoding exon 1) of the *SMAD4*/*DPC4* gene was selected for this analysis (Fig. 1). This region contains many CpG islands, and using it, reliable and reproducible results were obtained. Twenty diploid plus the corresponding aneuploid populations of the multiploid carcinomas were examined, and no methylation of this region was found, consistent with the results of the study of Roth et al.[28] Our results confirmed that hypermethylation of the *SMAD4*/*DPC4* gene promoter region was not associated with the transformation of diploid fractions to the corresponding aneuploid fractions of multiploid colorectal carcinomas. Therefore, we suggest that hypermethylation of the region plays no fundamental role in the progression of colorectal carcinogenesis.

The frequencies of *SMAD4*/*DPC4* gene allelic imbalance were not correlated with the mutation rates of the *SMAD4*/*DPC4* gene in the multiploid carcinomas that we examined. In addition, no methylation was observed in these carcinomas. These findings do not appear to fulfill the criteria for a classical tumor suppressor model, because loss of both alleles is required to inactivate gene expression. However, a recent study has shown that loss of heterozygosity (LOH) by itself contributes to human carcinogenesis, even if the other allele is retained (haploid insufficiency model).[29] In the present study, expression of *SMAD4*/*DPC4* was not examined. Although a previous study has shown that loss of gene expression in colonic adenocarcinomas correlates with metastatic stage,[9] it is still not clear whether LOH without inactivation of the other allele is associated with the loss of *SMAD4*/*DPC4* expression. Whereas previous studies have shown that LOH is related to reduced gene expression,[30,31] Pilozzi et al.[32] indicated that LOH was not related to the expression of the *CDX1* gene. On the other hand, another report showed that a specific combination of LOH was correlated with the expression of certain subclasses of breast cancer.[33] We speculate that an insufficient amount of the *SMAD4*/*DPC4* gene product (half of the normal gene product) caused by LOH may influence expression of the gene. In addition, heterogeneity of genetic alterations or gene expression within the same tumor should be considered, given that such a finding is frequently observed in human tumors.[34,35] We suggest that heterogeneity within a tumor is an important factor related to the observed difference in expression.

In summary, we examined allelic imbalance, mutations of the *SMAD4*/*DPC4* gene, and methylation of the promoter region, in order to clarify the role of tumor progression in 30 multiploid carcinomas. Our results indicate that allelic imbalance at the *SMAD4*/*DPC4* gene locus plays an important role in the progression of multiploid carcinomas. However, mutations of the *SMAD4*/*DPC4* gene and hypermethylation of the region are not associated with the conversion of diploid fractions to aneuploid fractions of multiploid colorectal carcinomas.

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References

1. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, et al. Genetic alterations during colorectal tumor development. N Engl J Med 1988;319:525–32.
2. Fearon ER, Vogelstein, B. A genetic model for colorectal tumorigenesis. Cell 1990;61:759–67.
3. Takagi Y, Kohmura H, Futamura M, Kida H, Tanemura H, Shimokawa K, et al. Somatic alterations of the *DPC4* gene in human colorectal cancers in vivo. Gastroenterology 1996;111: 1369–72.
4. Miyaki M, Kuroki T. Role of Smad4 (DPC4) inactivation in human cancer. Biochem Biophys Res Commun 2003;11;306:799–804.
5. Takaku K, Oshima M, Miyoshi H, Matsui M, Seldin MF, Taketo MM. Intestinal tumorigenesis in compound mutant mice of both *Dpc4* (*Smad4*) and *Apc* genes. Cell 1998;92:645–56.
6. Salovaara R, Roth S, Loukola A, Launonen V, Sistonen P, Avizienyte E, et al. Frequent loss of SMAD4/DPC4 protein in colorectal cancers. Gut 2002;51:56–9.
7. Hadija MP, Kapitanovic S, Radosevic S, Cacev T, Mirt M, Kovacevic D, et al. Loss of heterozygosity of *DPC4* tumor suppressor gene in human sporadic colon cancer. J Mol Med 2001;79:128–32.
8. Miyaki M, Iijima T, Konishi M, Sakai K, Ishii A, Yasuno M, et al. Higher frequency of *Smad4* gene mutation in human colorectal cancer with distant metastasis. Oncogene 1999;20:18:3098–103.
9. Maitra A, Molberg K, Albores-Saavedra J, Lindberg G. Loss of *Dpc4* expression in colonic adenocarcinomas correlates with the presence of metastatic disease. Am J Pathol 2000;157:1105–11.
10. Sugai T, Habano W, Nakamura S, Sato H, Uesugi N, Takahashi H, et al. Genetic alterations in DNA diploid, aneuploid and multiploid colorectal carcinomas identified by the crypt isolation technique. Int J Cancer 2000;88:614–9.
11. Sugai T, Habano W, Nakamura S, Sato H, Uesugi N, Orii S, et al. Allelic losses of 17p, 5q and 18q loci in diploid and aneuploid populations of multiploid colorectal carcinomas. Hum Pathol 2000;31:925–30.
12. Sugai T, Habano W, Uesugi N, Jiao Y-F, Nakamura S, Yoshida T, et al. Frequent allelic imbalance at the ATM locus in DNA multiploid colorectal carcinomas. Oncogene 2001;20:6095–101.
13. Japanese Society for Cancer of the Colon and Rectum. Japanese classification of colorectal carcinoma, first English edition. Tokyo: Kanehara; 1997. p. 30–63.
14. Turnbull RB, Kyle K, Watson FR, Spratt J. Cancer of the colon; the influence of the no-touch isolation technique on survival rates. Ann Surg 1967;166:420–7.
15. Nakamura S, Goto J, Kitayama M, Kino I. Application of the crypt-isolation technique to flow-cytometric analysis of DNA content in colorectal neoplasms. Gastroenterology 1994;106:100–7.
16. Habano W, Sugai T, Nakamura S, Yoshida T. A novel method for gene analysis of colorectal carcinomas using a crypt isolation technique. Lab Invest 1996;74:933–40.
17. Sugai T, Habano W, Nakamura S, Yoshida T, Uesugi N, Sasou S, et al. Use of crypt isolation to determine loss of heterozygosity of multiple tumor suppressor genes in colorectal carcinoma. Pathol Res Pract 2000;196:145–50.
18. Sugai T, Habano W, Nakamura S, Jiao Y-F, Higuchi T, Inomata M, et al. Analysis of Ki-*ras* gene mutations associated with DNA diploid, aneuploid and multiploid colorectal carcinomas using a crypt isolation technique. Cytometry 2001;46:345–50.
19. Martinez-Lopez E, Abad A, Font A, Monzo M, Ojanguren I, Pifarre A, et al. Allelic loss on chromosome 18q as a prognostic marker in stage II colorectal cancer. Gastroenterology 1998;114: 1180–7.
20. Fearon ER, Cho KR, Nigro JM, Kern SE, Simons JW, Ruppert JM, et al. Identification of a chromosome 18q gene that is altered in colorectal cancers. Science 1990;247:49–56.
21. Hahn SA, Schutte M, Hoque AT, Moskaluk CA, da Costa LT, Rozenblum E, et al. *DPC4*, a candidate tumor suppressor gene at human chromosome 18q21.1. Science 1996;271:350–3.
22. Mamot C, Mild G, Reuter J, Laffer U, Metzger U, Terracciano L, et al. Infrequent mutation of the tumour-suppressor gene *Smad4* in early-stage colorectal cancer. Br J Cancer 2003;88:420–3.
23. Lipton L, Halford SE, Johnson V, Novelli MR, Jones A, Cummings C, et al. Carcinogenesis in MYH-associated polyposis follows a distinct genetic pathway. Cancer Res 2003;63:7595–9.
24. Lei J, Zou TT, Shi YQ, Zhou X, Smolinski KN, Yin J, et al. Infrequent *DPC4* gene mutation in esophageal cancer, gastric cancer and ulcerative colitis-associated neoplasms. Oncogene 1996;13:2459–62.
25. Hoque AT, Hahn SA, Schutte M, Kern SE. *DPC4* gene mutation in colitis associated neoplasia. Gut 1997;40:120–2.
26. Akiyama Y, Arai T, Nagasaki H, Yagi OK, Nakahata A, Nakajima T, et al. Frequent allelic imbalance on chromosome 18q21 in early superficial colorectal cancers. Jpn J Cancer Res 90:1329–37.
27. Koyama M, Ito M, Nagai H, Emi M, Moriyama Y. Inactivation of both alleles of the *DPC4*/*SMAD4* gene in advanced colorectal cancers: identification of seven novel somatic mutations in tumors from Japanese patients. Mutat Res 1999;406:71–7.
28. Roth S, Laiho P, Salovaara R, Launonen V, Aaltonen LA. No *SMAD4* hypermethylation in colorectal cancer. Br J Cancer 2000;83:1015–9.
29. Tang B, Bottinger EP, Jakowlew SB, Bagnall KM, Mariano J, Anver MR, et al. Transforming growth factor-beta 1 is a new form of tumor suppressor with true haploid insufficiency. Nat Med 1998;4:802–7.
30. Habano W, Sugai T, Nakamura S, Uesugi N, Higuchi T, Terashima M, et al. Reduced expression and loss of heterozygosity of the *SDHD* gene in colorectal and gastric cancer. Oncol Rep 2003;10:1375–80.
31. Semczuk A, Marzec B, Roessner A, Jakowicki JA, Wojcierowski J, Schneider-Stock R. Loss of heterozygosity of the retinoblastoma gene is correlated with the altered pRb expression in human endometrial cancer. Virchows Arch 2002;441:577–83.
32. Pilozzi E, Onelli MR, Ziparo V, Mercantini P, Ruco L. *CDX1* expression is reduced in colorectal carcinoma and is associated with promoter hypermethylation. J Pathol 2004;204:289–95.
33. Wang ZC, Lin M, Wei LJ, Li C, Miron A, Lodeiro G, et al. Loss of heterozygosity and its correlation with expression profiles in subclasses of invasive breast cancers. Cancer Res 2004;64:64–71.
34. Sugai T, Habano W, Uesugi N, Jiao Y-F, Nakamura S, Abe K, et al. Three independent genetic profiles based on mucin expression in early differentiated-type gastric cancers—a new concept of genetic carcinogenesis of early differentiated-type adenocarcinomas. Mod Pathol 2004;17:1223–34.
35. Sugai T, Habano W, Jiao Y-F, Suzuki M, Takagi R, Otsuka K, et al. Analysis of allelic imbalances at multiple cancer-related chromosomal loci and microsatellite instability within the same tumor using a single tumor gland from colorectal carcinomas. Int J Cancer 2005;114:337–45.