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Original Article

Expansion of CpG Methylation in the *SFRP2* Promoter Region during Colorectal Tumorigenesis

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***Secreted frizzled-related protein 2*, (*SFRP2*) is a Wnt inhibitor whose promoter CpGs were recently found to be methylated at high frequency in colorectal cancers (CRCs). We hypothesized that the pattern of *SFRP2* methylation may differ throughout the promoter during progressive tumorigenesis. Using combined bisulfite restriction analysis (COBRA), two methylation-sensitive regions (Regions A and B) of the *SFRP2* promoter were investigated in 569 specimens of colorectal tissue: 222 CRCs, 103 adenomatous polyps (APs), 208 normal colonic mucosa from CRC patients (N-Cs), and 36 normal colonic mucosa from subjects with no evidence of colorectal neoplasia at colonoscopy (N-Ns). Extensive (including both Regions A and B) and partial (either Region A or B) *SFRP2* methylation levels were found in 61.7% and 24.8% of CRCs, 8.7% and 37.9% of APs, 3.9% and 39.9% of N-Cs, and 0% and 30.6% of N-Ns, respectively. Extensive methylation of the *SFRP2* promoter was present primarily in CRCs, while partial methylation was common in APs. Whereas APs with the *KRAS* mutant showed no correlation to any pattern of *SFRP2* methylation, extensive methylation of the *SFRP2* promoter was significantly associated with *KRAS* mutant CRCs ($p < .0001$), suggesting that genetic alteration in the RAS-RAF pathway might precede the spread of CpG methylation through the *SFRP2* promoter, which is observed in over 60% of advanced colorectal tumors.**

Key words: *BRAF/KRAS* mutations, promoter methylation, colorectal cancer

Aberrant WNT pathway signaling is an early progression event in approximately 90% of CRCs and occurs through mutations mainly of *APC* and, less often, of *CTNNB1* (encoding beta-catenin). These mutations allow ligand-independent WNT signaling that culminates in abnormal accumulation of free beta-catenin in the nucleus [1–3]. Secreted frizzled-related proteins (SFRPs) possess a domain similar to one in the WNT-receptor frizzled proteins and can inhibit WNT receptor binding to downregulate pathway signaling during development [4]. Recently, gene silencing of *SFRP*s, which consist of 5 members (SFRP1-5), by the hypermethylation of their promoter was identified in CRCs and other malignancies [5–10]. Especially, *SFRP2* methylation was detected with a high frequency of over 60% of CRCs, including Lynch syndrome, which suggests that *SFRP2* methylation might potentially be useful as a promising

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sensitive screening marker for the stool-based detection of CRCs and premalignant lesions, as our previous study demonstrated [11–13]. Additionally, we also demonstrated that methylation in the *SFRP2* promoter was more likely to be found in CRCs with the *KRAS* mutant [8]. Although the detailed mechanisms underlying hypermethylation are still unclear, we have recently described the gradual expansion of methylated CpG residues in the *MGMT* promoter in a normal-adenoma-carcinoma sequence [14]. Herein we show that *SFRP2* methylation also expands methylated CpG residues throughout the promoter in a stepwise manner through the adenoma-cancer multistep cascade.

Materials and Methods

Tissue samples. Tissue specimens from 222 CRCs and 208 adjacent normal colorectal mucosa ("normal from cancer patients", or N-C) were obtained from patients with colorectal cancer who had undergone curative surgery at Okayama University Hospital, Okayama, Japan, between 1994 and 2007. We excluded CRCs that occurred in patients with a family history of CRC. A total of 103 APs and colonic biopsy specimens from 36 subjects with no evidence of colorectal neoplasia at colonoscopy ("normal from non-neoplastic colons" or N-N) were obtained from subjects who had received a colonoscopy at Chikuba Hospital and Okayama University Hospital, Okayama, Japan, from 2002 to 2007, as described previously [8]. The tumor/node/metastasis (TNM) classification system was used for cancer staging [14]. APs were divided into two subsets: advanced polyps (*i.e.*, polyps with high-grade dysplasia, villous architecture, and tubular adenomas ≥ 1 cm in diameter: 82 specimens), and minor polyps (simple tubular adenomas < 1 cm in diameter: 21 specimens). Institutional review board approval was granted, and informed consent was obtained from all subjects to use their tissues.

DNA isolation and bisulphite modification. DNA was extracted from CRC, N-C, and N-N tissues by standard procedures described previously [15]. All DNA from APs was extracted from formalin-fixed, paraffin-embedded archival materials. For each AP, DNA was extracted using a TaKaRa DEXPAT kit (Takara Bio Inc., Otsu, Japan). Then, 200 to 1,000 ng of genomic DNA was subjected to sodium bisulfite modification. Bisulfite modification was carried out using a CpGenome DNA Modification Kit (Intergen Co., Purchase, NY, USA).

Combined bisulfite restriction analysis (COBRA) assays for SFRP2 methylation. We designed the COBRA to examine both 'Region A' and 'Region B' of the *SFRP2* promoter (Fig. 1A). Primer sequences for "Region A" and "Region B" COBRA for the *SFRP2* promoter region were: (*a*) Region A-F (5'-GTYGGAGTTTTTYGGAGTTG-3') and Region A-R (5'-AACCCRCTCTCTTCRCTAAATAC-3'), and (*b*) Region B-F (5'-GGTTGTTAGTTTTTYGGGGT TT-3') and Region B-R (5'-CAACIAACCAAAACCC TACAACAT-3'), generating fragment lengths of 139 and 153 bp, respectively. COBRA was carried out in a 25 μl PCR mixture containing 12.5 μl of HotStarTaq Master Mix kit (Qiagen). The PCR products derived from Region A were digested with *BssH*II (New England Biolabs, Ipswich, MA, USA) at 50℃ for 16h, and those from Region B were digested with *Hha*I (New England Biolabs) at 37℃ for 16h. The digested DNA was separated on 3% agarose gels in 1x TAE buffer and stained with ethidium bromide. We used a Gel Logic 200 Imaging System (Eastman Kodak Co., Rochester, NY, USA) to perform densitometric analyses on all gels. Band intensities were quantified using Kodak 1D analysis software (Eastman Kodak Co.). The methylation levels (ratios of methylated to unmethylated DNA) were determined from the relative intensities of cut and uncut PCR products to quantitate methylation.

Microsatellite instability (MSI) testing and BRAF/KRAS mutation analysis. MSI testing and *BRAF* V600E mutation/*KRAS* mutations at codons 12 and 13 were determined by methods described previously [8, 11].

Statistical analysis. The methylation statuses of both regions in the *SFRP2* promoter as determined by COBRA were analyzed as categorical variables (positive: methylation level ≥ 1%, negative: methylation level < 1% by using Kodak 1D analysis software). Methylation levels of specimens classified as methylation-positive were analyzed as a continuous variable, and these data were expressed on a log scale by calculating Spearman's rank correlation coefficients (ρ). A correlation between subjects was considered strong when $|\rho| \geq 0.7$, but the correlation was weaker when $|\rho|$ was between 0.5–0.7. Differences in frequency were evaluated by the χ^2-test. All reported p values

were two-sided, and a p value < 0.05 was considered statistically significant.

Results

Tissue characteristics. To clarify the features of *SFRP2* methylation through colorectal tumorigenesis, we collected and analyzed colorectal tissues obtained from 36 N-Ns, 208 N-Cs, 103 APs, and 222 CRCs. Table 1 shows the clinical information on 569 colorectal tissues. There were no differences in age or gender among all colorectal subsets, or in tumor location between APs and CRCs in the study cohort.

Methylation profiles in the SFRP2 promoter. COBRA for *SFRP2* was designed to examine the methylation status in 2 regions of the *SFRP2* promoter (Regions A and B) as shown in Fig. 1A. By analogy, we divided the human *SFRP2* promoter into these 2 regions. We chose Region A based on sequence homology to the mouse *sfrp2* promoter sequence, which suggested that this region might be a binding site for the transcriptional factor *pax2* [16]. We chose Region B based on previous studies in which we showed that this promoter region was aberrantly methylated at high frequency in CRC specimens [8]. When we consider methylation-positive as methylation level $\geq 1\%$ by COBRA, *SFRP2* methylation (partial or extensive) was observed in 30.6% of N-Ns, 43.8% of N-Cs, 46.6% of APs, and 87.6% of CRCs (Table

Table 1 Clinical features of colorectal specimens

Variable		Normal colonic mucosa without Neoplasia (N-N; n = 36)	Normal colonic mucosa from CRC patients (N-C; n = 208)	Adenomatous polyp (AP; n = 103)	Colorectal cancer (CRC; n = 222)	*p*-value
Age	≥ 65	50.0 (18)	51.0 (106)	51.5 (53)	52.7 (117)	0.98
	< 65	50.0 (18)	49.0 (102)	48.5 (50)	47.3 (105)	
Gender	Female	44.4 (16)	35.6 (74)	26.2 (27)	36.4 (79)	0.16
	Male	55.6 (20)	64.4 (134)	73.8 (76)	63.4 (138)	
Location	Proximal	–	–	32.0 (33)	30.7 (63)	0.82
	Distal	–	–	68.0 (70)	69.3 (142)	

All *p*-values are analyzed by χ^2-test.

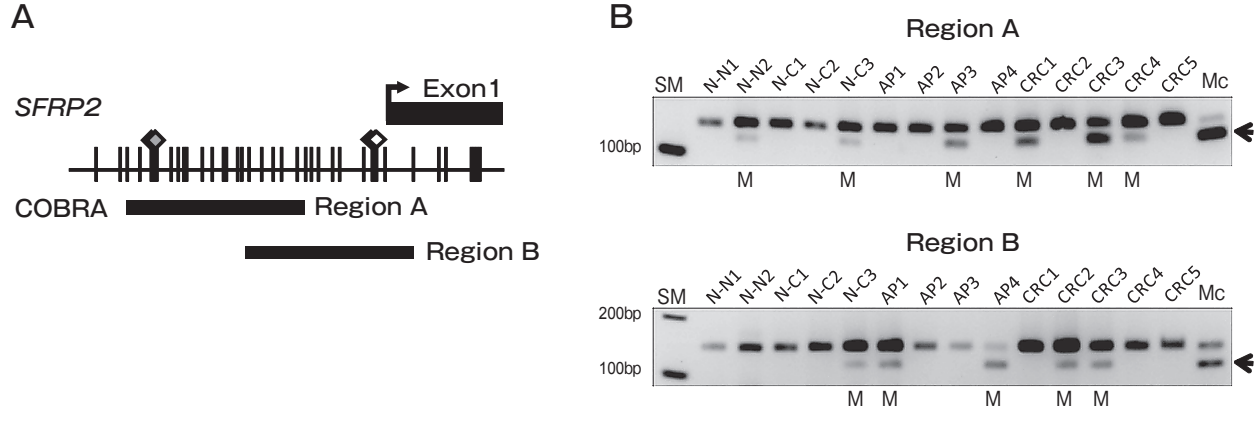

Fig. 1 **(A)** Schematic representation of SFRP2 gene promoter regions analyzed by combined bisulfite restriction analysis (COBRA). Black squares represent the coding exon 1 region; arrows on the squares indicate transcriptional start sites. Vertical lines indicate CpG sites. White diamonds represent the restriction sites for *BssH*II, and gray diamonds represent restriction sites for *Hha*I. Thick horizontal lines depict the locations of COBRA products, **(B)** Representative results of COBRA in the 2 regions (Regions A and B) of SFRP2. Arrows indicate methylated alleles. Mc denotes methylated control. SM denotes a size marker.

2). However, extensive (including both Regions A and B) *SFRP2* methylation was observed in only 0% of N-Ns, 3.9% of N-Cs, 8.7% of APs, and 61.7% of CRCs ($p < .0001$). Thus, although approximately half of normal colonic mucosa possesses *SFRP2* methylation, its methylation pattern is partial methylation (either Region A or B), observed mainly in Region A.

We next analyzed the relationships between levels of methylation in both regions of the *SFRP2* promoter in each subset as a continuous variable. As shown in Fig. 2, there was a lack of correlation between the levels of methylation of Region A and that of Region B in N-Cs and N-Ns ($p = -0.1030$; $p = 0.55$, $p = 0.1801$; $p = .0092$, as well as in APs ($p = 0.1515$; $p = 0.13$). However, there was a significant positive correlation for methylation at both regions in CRCs ($p = 0.6000$; $p < .0001$).

Correlations of SFRP2 methylation with clinical and pathological features. Tables 3 and 4 present the correlations between the methylation status of *SFRP2* and the clinical and pathological features in CRCs and APs, respectively. In CRCs, methylation of the *SFRP2* Region B promoter was detected more frequently in elderly patients than in younger patients ($p = .03$). We observed no association among the frequencies of *SFRP2* methylation (partial or extensive) and gender, tumor location, TNM staging, or histology in CRCs. We next compared the *SFRP2* methylation status with MSI status. We observed that the *SFRP2* Region A promoter region was fully (100%) methylated in CRCs showing MSI.

Among APs, methylation of the *SFRP2* Region B promoter was detected more frequently in tubular adenoma with villous architecture than in tubular adenoma without such architecture ($p = .03$). There were no significant differences between *SFRP2* methylation status and the other clinical factors. Interestingly, extensive methylation was rarely observed in minor APs but primarily present in advanced APs.

We also examined whether or not any associations

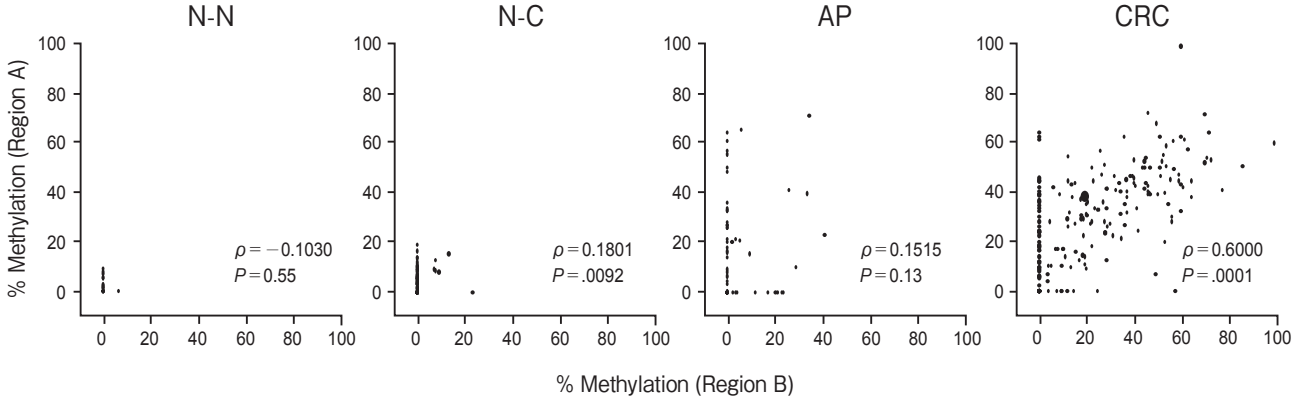

Fig. 2 The relationships among methylation levels of Regions A and B in N-Ns, N-Cs, APs, and CRCs.

Table 2 Frequency of *SFRP2* methylation*

Subset (no)	*SFRP2* Methylation Status- % (no)									
	Non-methylation	Partial methylation	Extensive methylation	*p*-value	Region A methylation		*p*-value	Region B methylation		*p*-value
					1.0～5.0%	> 5.0%		1.0～5.0%	> 5.0%	
N–N (n = 36)	69.4 (25)	30.6 (11)	0 (0)	< .0001	25.0 (9)	2.8 (1)	< .0001	2.8 (1)	0 (0)	< .0001
N–C (n = 208)	56.3 (117)	39.9 (83)	3.9 (8)		23.6 (49)	17.8 (37)		4.3 (9)	2.6 (4)	
AP (n = 103)	53.4 (55)	37.9 (39)	8.7 (9)		2.9 (3)	35.0 (36)		5.8 (6)	11.7 (12)	
CRC (n = 222)	13.5 (30)	24.8 (55)	61.7 (137)		4.1 (9)	79.7 (177)		3.2 (7)	61.3 (136)	

*Methylation of specimens was determined by COBRA. A specimen was considered positive for methylation if any methylated product was detected (*i.e.*, a methylation level ≥ 1%). Methylation-positive cases were divided into 2 subsets by their methylation degree: an 1.0～5.0% methylated group and an over 5.0% methylated group.

Table 3 Clinicopathological features of CRC patients by *SFRP* promoter methylation status

Variable		*SFRP2* Methylation Status - *% (no)*							
		Non-methylation	Partial methylation	Extensive methylation	*p*-value	Region A methylation	*p*-value	Region B methylation	*p*-value
Age	≥ 65 (n = 117)	11.1 (13)	20.5 (24)	68.4 (80)	0.1	86.3 (101)	0.28	70.9 (83)	0.03
	< 65 (n = 105)	16.2 (17)	29.5 (31)	54.3 (57)		81.0 (85)		57.1 (60)	
Gender	Female (n = 79)	13.9 (11)	22.8 (18)	63.3 (50)	0.91	83.5 (66)	0.97	65.8 (52)	0.76
	Male (n = 138)	13.8 (19)	25.4 (35)	60.9 (84)		83.3 (115)		63.8 (88)	
Location	Proximal (n = 63)	11.1 (7)	19.1 (12)	69.8 (44)	0.3	85.7 (54)	0.41	73.0 (46)	0.1
	Distal (n = 142)	16.2 (23)	25.4 (36)	58.5 (83)		81.0 (115)		61.3 (87)	
T	T1 & T2 (n = 35)	14.3 (5)	20.0 (7)	65.7 (23)	0.85	83.9 (29)	0.93	68.6 (24)	0.6
	T3 & T4 (n = 169)	14.8 (25)	24.3 (41)	61.0 (103)		82.3 (139)		63.9 (108)	
N	Node metastasis (−) (n = 89)	11.2 (10)	23.6 (21)	65.2 (58)	0.45	86.5 (77)	0.17	67.4 (60)	0.48
	Node metastasis (+) (n = 115)	17.4 (20)	23.5 (27)	59.1 (68)		79.1 (91)		62.6 (72)	
M	Distance metastasis (−) (n = 164)	12.8 (21)	22.6 (37)	64.6 (106)	0.17	84.8 (139)	0.07	67.1 (110)	0.15
	Distance metastasis (+) (n = 40)	22.5 (9)	27.5 (11)	50.0 (20)		72.5 (29)		55.0 (22)	
Stage	I &II (n = 91)	9.9 (9)	28.6 (26)	61.5 (56)	0.27	87.9 (80)	0.14	63.7 (58)	0.9
	III & IV (n = 127)	16.5 (21)	22.1 (28)	61.4 (78)		80.3 (102)		64.6 (82)	
Histology	Wel (n = 40)	12.5 (5)	25.0 (10)	62.5 (25)	0.21	82.5 (33)	0.63	67.5 (27)	0.67
	Mod (n = 149)	14.1 (21)	25.5 (38)	60.4 (90)		83.2 (124)		63.1 (94)	
	Poor or Muc (n = 15)	26.7 (4)	0 (0)	73.3 (11)		73.3 (11)		73.3 (11)	
MSI Status	MSI (n = 15)	0 (0)	33.3 (5)	66.7 (10)	0.26	100 (15)	0.08	66.7 (10)	0.85
	Non-MSI (n = 207)	14.5 (30)	24.2 (50)	61.4 (127)		82.6 (171)		64.3 (133)	
BRAF/KRAS Mutation Status	*BRAF* Mutant (n = 20)	0 (0)	45.0 (9)	55.0 (11)	0.0002	100 (20)	0.0008	55.0 (11)	0.0017
	KRAS Mutant (n = 73)	4.1 (3)	17.8 (13)	78.1 (57)		93.2 (68)		80.8 (59)	
	Wild-type (n = 129)	20.9 (27)	25.6 (33)	53.5 (69)		76.0 (98)		56.6 (73)	

T, invasion by the primary tumor; N, regional node involvement; M, distal metastasis; Wel, well-differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma; Muc, mucinous adenocarcinoma. All *p*-values were analyzed by the χ^2-test.

Table 4 Clinicopathological features of AP patients by *SFRP* promoter methylation status

Variable		*SFRP2* Methylation Status - *% (no)*							
		Non-methylation	Partial methylation	Extensive methylation	*p*-value	Region A methylation	*p*-value	Region B methylation	*p*-value
Age	≥ 65 (n=53)	47.2 (25)	43.4 (23)	9.4 (5)	0.42	43.4 (23)	0.23	18.9 (10)	0.7
	< 65 (n = 50)	60.0 (30)	32.0 (16)	8.0 (4)		32.0 (16)		16.0 (8)	
Gender	Female (n = 27)	48.2 (13)	33.3 (9)	18.5 (5)	0.11	40.7 (11)	0.13	29.6 (8)	0.05
	Male (n = 76)	55.3 (42)	39.5 (30)	5.3 (4)		36.8 (28)		13.2 (10)	
Location	Proximal (n = 33)	45.5 (15)	45.5 (15)	9.1 (3)	0.52	39.4 (13)	0.83	24.2 (8)	0.21
	Distal (n = 70)	57.1 (40)	34.3 (24)	8.6 (6)		37.1 (26)		14.3 (10)	
Size	≥ 1 cm (n = 82)	53.7 (44)	35.4 (29)	11.0 (9)	0.23	37.8 (31)	0.98	19.5 (16)	0.28
	< 1 cm (n = 21)	52.4 (11)	47.6 (10)	0 (0)		38.1 (8)		9.5 (2)	
Histology	TA (n = 82)	56.1 (46)	36.6 (30)	7.3 (6)	0.44	37.8 (31)	0.98	13.4 (11)	0.03
	TA with villous architecture (n = 21)	42.9 (9)	42.9 (9)	14.3 (3)		38.1 (8)		33.3 (7)	
KRAS Mutation status*	*KRAS* Mutant (n = 21)	47.6 (10)	47.6 (10)	4.8 (1)	0.52	38.1 (8)	0.98	19.1 (4)	0.83
	Wild-type (n = 82)	54.9 (45)	35.4 (29)	9.8 (8)		37.8 (31)		17.1 (14)	

TA, tubular adenoma. *There is no BRAF V600E mutant.
All *p*-values were analyzed by the χ^2-test.

existed between patient age and the degrees of methylation in the discrete regions of the *SFRP2* promoter in normal mucosa. However, no such correlations were detected in these analyses (data not shown).

Correlation of SFRP2 methylation with KRAS mutations in CRCs. We found the *BRAF* V600E mutation in 20 CRCs (9.0%) and in none of the APs (0%). On the other hand, we found *KRAS* mutations in 73 CRCs (32.9%) and 21 APs (25.3%). Region A was more frequently methylated in CRCs with *BRAF* (100%) or *KRAS* (93.2%) mutation than in CRCs showing the wild type of both genes (76.0%; $p = .0008$), whereas the Region B *SFRP2* methylation was higher in *KRAS* mutant CRCs (80.8%) compared to *BRAF* mutant CRCs (55.0%) or both wild-type CRCs (56.6%; $p = .0017$). Therefore, when we compared the complete spectrum of *BRAF/KRAS* mutation with the pattern of *SFRP2* methylation, extensive methylation was the highest in CRCs with *KRAS* mutation (78.1%) compared to CRCs with *BRAF* mutation (55.0%) or the wild type of both genes (53.5%; $p = .0002$; Table 3). However, in APs, there were no associations among the *SFRP2* methylation pattern and *KRAS* mutant APs (Table 4).

Discussion

The current study clearly demonstrates for the first time the biological significance of methylation in discrete regions of the *SFRP2* promoter in a large cohort of specimens obtained from the colon and rectum, including neoplasia. Aberrant methylation in gene promoters has been established as a key mechanism for the inactivation of tumor suppressor genes in human malignancies, including CRCs [17]. Clinically, the identification of genes that are prone to abnormal methylation and that consequently become downregulated is of critical importance, since this is considered to provide a good source of novel biomarkers [18, 19]. The family of *SFRP* genes, functionally acting as Wnt signaling inhibitors, was recently shown to be a common target of promoter hypermethylation in numerous tumor entities. Indeed, we and others have demonstrated that aberrant methylation of the *SFRP2* promoter region is a potential biomarker with which to screen CRCs by analyzing fecal DNA [11, 12].

Generally, the methylation pattern in CpG islands is sometimes considered to be uniformity (either entirely methylated or unmethylated). However, in the *SFRP2* promoter region, our results clearly demonstrate that methylated alleles do not possess methylated cytosine consistently. In addition, by quantifying the methylation levels in discrete regions of the *SFRP2* promoter, we also illustrated an accumulation of aberrant methylation according to the adenoma-carcinoma sequence. During this stepwise progression, the methylation level of both regions in the *SFRP2* promoter increased gradually and the existence of cytosine methylation expanded widely. Almost none of the nonmalignant specimens, including normal colonic mucosa, showed extensive methylation in the *SFRP2* promoter. More importantly, extensive methylation is a characteristic of a malignant colorectal tumor while partial methylation is a feature of an earlier stage of colorectal tumor, including benign polyps. In this study, we frequently found N-Ns and N-Cs with low density methylation (under 5.0%) in Region A (Table 2). Therefore, when we considered from 1.0% to 5.0% methylation density as methylation-positive, approximately 25% of normal colorectal mucosa showed low-level (1.0%～5.0%) methylation in the S*FRP2* Region A promoter. On the other hand, Region B did not show such lower-level (1.0%～5.0%) methylation in either normal or neoplastic lesions. Thus, the majority of methylated normal colorectal mucosae showed partial methylation (Region A), and there were fewer methylated cells than there were in neoplastic lesions with *SFRP2* methylation. It is unclear why a quarter of normal colonic mucosae had small populations of *SFRP2* Region A methylated cells. One explanation for this lower-density methylation is an association with aging. Indeed, methylation in *estrogen receptor*, *hMLH*1, and *O^6-methylguanine-DNA methyltransferase* (*MGMT*) gene promoter observed in normal colonic mucosa is associated with aging [20–22]. However, by precise analysis of our cohort of normal colorectal mucosa, we could not confirm any correlation between the methylation level of CpG sites we studied and age. When we looked into the pattern of *SFRP2* methylation in colorectal tumors, we found that, as almost all of the tumors showed little partial methylation in Region B, *SFRP2* promoter methylation would come into existence from Region A to Region B consistently. While we could not ascribe any importance to the low level of Region A methylation in

N-Cs or N-Ns, the low level of *SFRP2* Region A methylation might have the potential to act as a 'seed of methylation', which is considered to be emerging as an important precursor for aberrant methylation [23–25].

It has been suggested that V600E *BRAF* mutations are present in sessile serrated polyps and serrated aberrant crypt foci (ACF), whereas *KRAS* mutations are more highly associated with non-serrated polyps and ACFs [26–28]. It is reasonable that, in this study, we confirmed that there was no *BRAF* V600E mutation in our cohort of APs because of the absence of a serrated structure.

In this study, we confirmed that the extensive *SFRP2* methylation was associated with the *KRAS* mutations in CRCs but not in APs. It was reported that APs with the *KRAS* mutation showed some degree of methylation in multiple loci [29]. Although our cohort of APs displayed some degree of *SFRP2* methylation, most of the methylation pattern of the *SFRP2* promoter in APs was that of partial methylation. We hence confirmed that the frequency of extensive *SFRP2* methylation was clearly increased in CRCs (61.7%) compared to APs (8.7%). Interestingly, there was no extensive methylation in APs <1 cm in diameter. This suggests that extensive methylation of the *SFRP2* promoter might be a critical feature of CRC cells and may be necessary to convert normal colorectal epithelia to a malignant formation. Of particular interest is that we observed for the first time a gradual increase in the *SFRP2* methylation density in the 2 promoter regions from the normal-adenoma-carcinoma sequence. Although the detailed mechanisms underlying hypermethylation are still unclear, we can present a new hypothesis to explain tumorigenesis according to the adenoma-carcinoma sequence when we combine the results obtained from this study into the data we previously reported. Fig. 3 shows the schema of our modified adenoma-carcinoma sequence [8, 14, 15]. Our hypothesis is as follows:

1) first, in normal colorectal epithelium, the 'seed of methylation' comes into existence;
2) next, alteration of the RAS-RAF pathway (including *BRAF* or *KRAS* mutations) initiates the direction and characteristics of neoplasia (this alteration may occur in small adenomas);
3) finally, aberrant methylation spreads to certain directions according to the mutation spectra of *BRAF/KRAS* (*i.e.*, if a neoplasia gains the *BRAF* V600E mutation, the neoplasia would transform into polyps with a serrated structure and accumulate dense

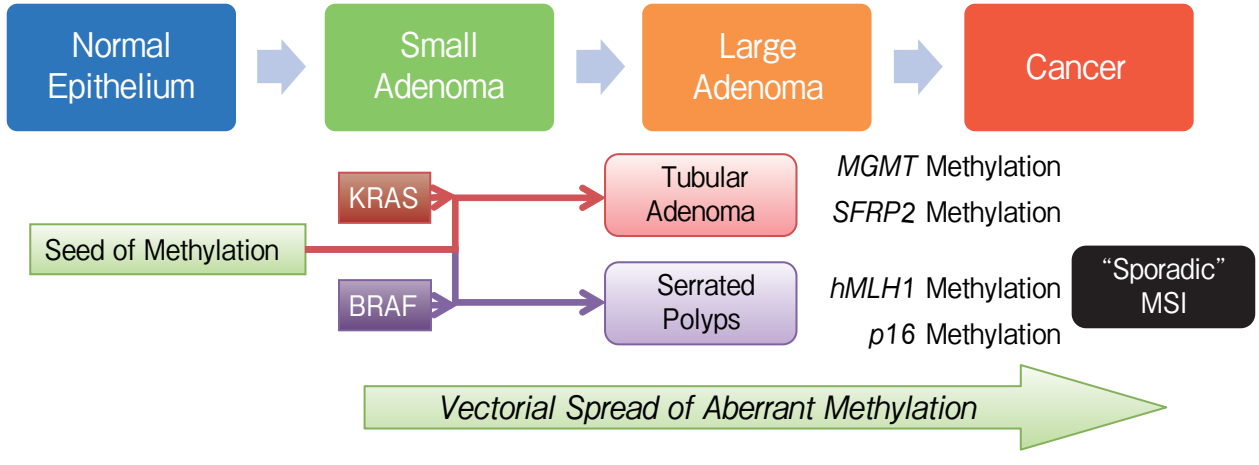

Fig. 3 Epigenetic model of colorectal tumorigenesis. Tumorigenesis proceeds through a series of epigenetic alterations. First, in normal colorectal epithelium, a 'seed of methylation' comes into existence. Second, in small adenoma, alteration of the RAS-RAF pathway (including *BRAF* or *KRAS* mutations) initiates the direction and characteristics of neoplasia. Third, aberrant methylation spreads in certain directions according to the mutation spectra of *BRAF/KRAS* (*i.e.*, if a small polyp gains the *BRAF* V600E mutation, the polyp would transform into polyps with a serrated structure and accumulate dense methylation in the *hMLH1/p16* promoter, and consequently become sporadic MSI cancer [15, 25]. If a small adenoma gains *KRAS* mutations, the adenoma would transform into tubular adenoma with villous architecture and accumulate dense methylation in the *SFRP2/MGMT* promoter, consequently becoming non-MSI cancer [8, 14]).

methylation in the *hMLH1/p16* promoter, and consequently become sporadic MSI cancer [15, 25]. If a neoplasia gains *KRAS* mutations, the neoplasia would transform into tubular adenoma with villous architecture and accumulate dense methylation in the *SFRP2/MGMT* promoter, consequently becoming non-MSI cancer [8, 14]).

In conclusion, the current data clearly suggested that the extensive methylation in the discrete *SFRP2* promoter regions associate with a malignant form of colorectal neoplasia, while partial methylation in those regions, commonly detected in the earlier stage of colorectal neoplasia and also in normal colonic mucosa, may represent a transitional stage that may progress to the advanced stage during colorectal tumorigenesis. These findings are pivotal in this context, as they form a basis on which aberrant methylation of *SFRP2* may be exploited as a potential diagnostic screening marker for subjects at risk for developing colorectal neoplasia as well as for patients with CRC.

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