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DKK1 Promoter Methylation in Colorectal Cancer Is Associated with CIMP and Elevation of *MYC* Expression

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Abstract: *Background:* Carcinogenesis of colorectal cancer (CRC) is influenced greatly by the canonical WNT signaling pathway. Genetically, the secreted protein Dickkopf (Dkk) family is known as an antagonist to the WNT. To clarify the role of DKK1 in the WNT signaling pathway in the colorectal carcinogenesis, we examined the *DKK1* promoter methylation in CRC and analyze the relationship of expression level of *DKK1* and *MYC* in relation with *APC* gene abnormalities. Methods: We integrated clinico-pathological and molecular findings of 41 cases of CRC. We adopted methylation-specific PCR, DNA sequencing, allelic loss analysis, quantitative RT-PCR, and MSI testing for genetic analyses. Results: CRCs with *DKK1* promotor methylation were found in 10 cases (24.4%), which were located predominantly in the proximal colon and frequently showed findings of poorly differentiated adenocarcinoma or mucinous adenocarcinoma. Furthermore, colorectal cancers with *DKK1* promotor methylation showed characteristics of microsatellite instability (MSI)-high (70%) and a *BRAF* mutation (40%), which are known as the CpG island methylator phenotype (CIMP). In the *DKK1* promotor methylation group, the relative expression level of *DKK1* mRNA was significantly reduced in comparison to the *DKK1* promotor un-methylation group ($p < 0.05$). When excluding the impact of APC abnormality, *MYC* expression in the *DKK1* promotor methylation group was significantly elevated compared to that in the *DKK1* promotor un-methylation group ($p < 0.05$). *Conclusions:* It is suggested that DKK1 is one of the regulators involved in *MYC* expression through the WNT signaling pathway and may have a negative effect on carcinogenesis of the colorectum without *APC* abnormalities.

Keywords: *DKK1*, *MYC*, WNT Signaling Pathway, Methylation, Colorectal Cancer, CIMP

1. Introduction

Colorectal cancer (CRC) is one of the most prevalent malignancies throughout the world and the third leading cause of cancer-related deaths in Japan. Therefore, elucidation of its etiology and the development of therapeutic measures are very important [1]. In the past, many studies of CRC development have undergone and shown that the etiology is very complex, because of complicated interactions among various environmental factors and many genetic alterations and/or epigenetic events such as methylation of the promotor region [2]. Genetically, carcinogenesis in CRC is a multi-step process, including the activation of mutations in proto-oncogenes and loss of function of gatekeeper tumor suppressor genes [3]. Moreover, epigenetic silencing of genes is another important mechanism for the inactivation of tumor suppressor genes in

carcinogenesis [4].

The canonical WNT signaling pathway is an intracellular signaling mechanism that is activated through WNT ligands binding to the transmembrane co-receptor complex frizzled (Fz) and LRP5/6. In the activated WNT signaling pathway, β-catenin avoids ubiquitination and proteolytic destruction by the GSK3-β/Axin/APC complex. Subsequently, the transcriptional coactivator β-catenin translocates to the nucleus to regulate expression of genes such as *MYC*, *CCND1*, and *PCBD2* [5]. The secreted protein family Dickkopf (Dkk) is known as an antagonist to the secreted ligand Wnt. In the vertebrates, four kinds of DKK protein are expressed [6]. Dkk1 protein binds to LRP5/6 and blocks its interaction with WNT and Fz and is thus generally considered to inhibit the WNT signaling pathway [7]. The *DKK1* gene, which is located at 10q11.2, encodes the Dkk1 protein. It has been assumed that epigenetic inactivation of *DKK1* lead to activation of the WNT signaling pathway and elevated expressions of target genes. So far, several investigations have been carried out to examine the association of methylation of DKK1 promotor methylation and cancer, especially CRC [8–10]. These reports have revealed that methylation of the *DKK1* promoter region is frequently observed in advanced CRCs. One of the causes of carcinogenesis is the declining expression of WNT antagonist DKK1, and many CRCs with *DKK1* promotor methylation have a microsatellite (MSI)-high status.

We examined the DKK1 promotor methylation in 41 cases of sporadic CRC using the methylation specific PCR (MSP) method and revealed the characteristics of CRCs with DKK1 promotor methylation.

The CpG island methylator phenotype (CIMP), in contrast to the chromosomal instability (CIN) phenotype is caused by a specific mechanism of carcinogenesis in some CRCs. The CIMP CRCs occur frequently in the proximal colon and are associated with MSI through epigenetic silencing of the mismatch repair gene *MLH1*, often due to *BRAF* mutations. Here, we explored the relationship of *DKK1* promotor methylation and CIMP.

The relationship between DKK1 promotor methylation and the expression level of DKK1 was examined using quantitative RT-qPCR. Furthermore, by examining the expression level of MYC, which is a transcriptional factor and a powerful oncogene in the nucleus, we estimated the impact of DKK1 promotor methylation in carcinogenesis of CRC. Although there have been reports investigating the relationship between abnormalities of APC, which is considered to be an important tumor suppressor gene in CRC, and the expression level of MYC [11, 12], this study has revealed for the first time the relationship between DKK1 promotor methylation and MYC expression level.

2. Methods

2.1. Patients and Tissue Samples

Initially, we collected 178 CRC cases and started DNA and RNA extractions for these samples. However, some of them were revealed to be inappropriate for our sensitive MSP analysis and quantitative RT-PCR due to the insufficient quality of DNA and/or RNA, then the remaining 41 cases suitable for molecular analysis were used in the following analysis. The subjects included 41 Japanese patients with CRC who underwent surgery in our hospital. Their clinico-pathological findings are shown in Table 1. In this study, we excluded all the patients related to familial adenomatous polyposis (FAP) or familial CRC accumulation such as Lynch syndrome. This study was approved by the Ethical Institutional Review Board of Hyogo College of Medicine (Approval No. 173) and informed consent was obtained from all patients for molecular analysis of the resected specimen. Tissues of normal colonic mucosa and the cancer were collected from the resected specimen. At that time, samples for DNA analysis were immediately frozen and those for RNA analysis were treated with RNA protect tissue reagent (Qiagen). Then genomic DNA and total RNA were extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden) and a QIAamp RNA Mini Kit (Qiagen, Hilden), respectively.

Table 1. *Clinico-pathological characteristics of 41 subjects.*

	Cases (n = 41)	
	No (%)	
Gender		
Male	27 (65.8)	
Female	14 (34.2)	
Age at surgical treatment (years)		
Mean (min – max)	67.5 (48 – 88)	
<50	2 (4.9)	
≥50	39 (95.1)	
Location		
Proximal colon	17 (41.5)	
Cecum		4
Ascending colon		8
Transverse colon		5
Distal colon and rectum	24 (58.5)	
Descending colon		0
Sigmoid colon		9
Rectum		15
Histology		
Papillary adenocarcinoma	1 (2.4)	
Well differentiated tubular adenocarcinoma	8 (19.5)	
Moderately differentiated tubular adenocarcinoma	28 (68.3)	
Poorly differentiated adenocarcinoma	3 (7.3)	
Mucinous adenocarcinoma	1 (2.4)	
pT		
Tis	0 (0.0)	
T1	3 (7.3)	
T2	10 (24.4)	
T3	22 (53.7)	
T4	6 (14.6)	
pN		
N0	27 (65.9)	
N1	10 (24.4)	
N2	4 (9.7)	
TNM stage		
0	0 (0.0)	
I	11 (26.8)	
II	16 (39.0)	
III	11 (26.8)	
IV	3 (7.3)	

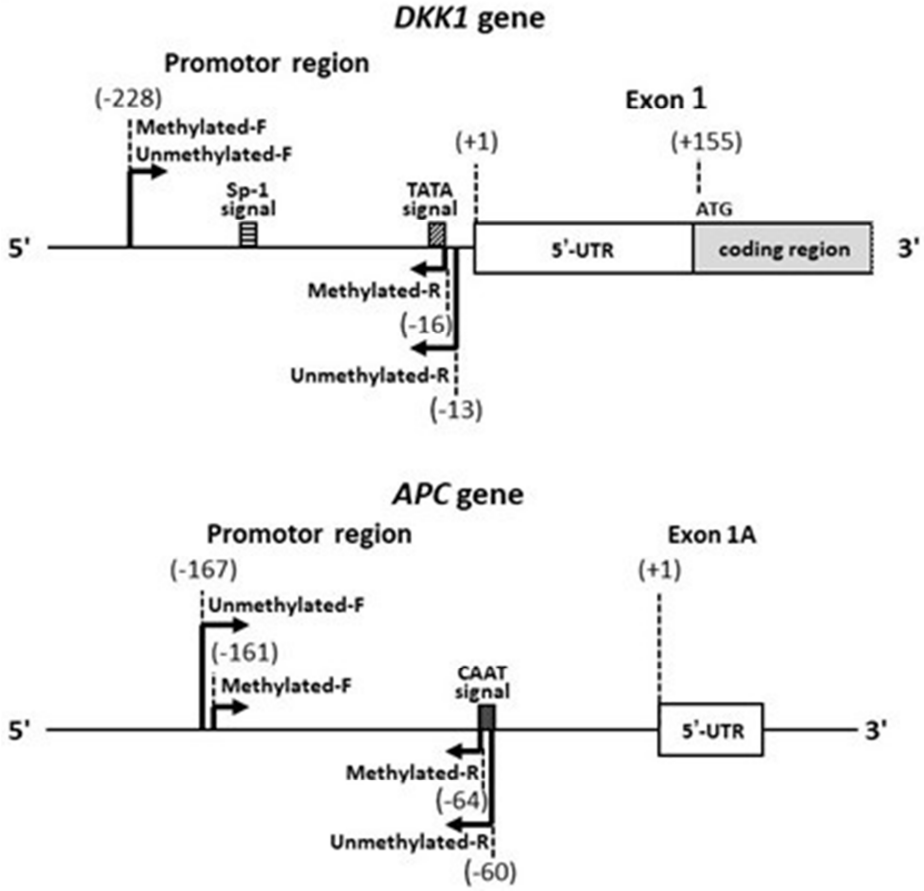

Figure 1. *The relationship between promoter-specific signals in the promoter region of DKK1 gene and APC gene and the primers used for methylation specific PCR analysis was shown. Methylated-F and Methylated-R, forward primers and reverse primers for methylated DNA, respectively; Unmethylated-F and Unmethylated-R, forward primers and reverse primers for unmethylated DNA, respectively; Sp-1, TATA signal, and CAAT signal, promotor specific signals; 5'-UTR, 5' untranslated region; ATG, initial codon; Number in parentheses, nucleotide number starting from the transcription site.*

2.2. Analytical and Statistical Methods

2.2.1. Methylation-Specific PCR (MSP)

The methylation status of the DKK1 and APC promotor region was determined by MSP. First, a bisulfite reaction for the conversion of un-methylated cytosines to thymines via uracils was achieved using the EpiTect Bisulfite Kit (Qiagen, Hilden). Next, the MSP was carried out using the HotStarTaq Master Mix kit (Qiagen, Hilden), with primers specific to either the modified un-methylated DNA (U) or the methylated DNA (M). These primers were designed in consideration of CpG islands and the consensus sequences of the respective promoter regions of *DKK1* and *APC*, as shown in Figure 1. The primer sequences, PCR condition, and size of the amplicons are shown in Table 2. We used DNA derived from blood of a disease-free person as a negative control and the same DNA treated with CpG methyltransferase (New England Biolabs) as a positive control in each Methylation-specific PCR (MSP) experiment. Each of the amplified fragments was observed via 2% agarose gel electrophoresis containing ethidium bromide. When an MSP product was visible, we judged the case as methylation positive. Representative electrophoretic patterns indicative of the MSP state are shown in Figure 2.

Table 2. *PCR primers and conditions used this study.*

Region	Primer sequences	Annealing temperature (°C)	Predicted fragment size (bp)
Methylation Specific PCR for *DKK1* promotor			
Methylated	5'-CGCTCGCTGGTAGCCTTCACCCCGA-3' 5'-GCGGCTGCCTTTATACCGCGGG-3'	65	213
Unmethylated	5'-TGTTTGTTGGTAGTTTTTATTTTGA-3' 5'-ACCACAACTACCTTTATACCACAA-3'	58	216
Methylation Specific PCR for *APC* promotor			
Methylated	5'- CACTGCGGAGTGCGGGTC -3' 5'- TCGGCGGGCTCCCGACGG -3'	64	98
Unmethylated	5'- GTGTTTTACTGTGGAGTGTGGGTT -3' 5'- CCAATCGGAGGGCTCCCAACAA -3'	62	108

Region	Primer sequences	Annealing temperature (°C)	Predicted fragment size (bp)
Real time PCR			
DKK1	5'-TCCGAGGAGAAATTGAGGAA-3' 5'-CCTGAGGCACAGTCTGATGA-3'	58	157
MYC	5'-CACCAGCAGCGACTCTGAG-3' 5'-TCCAGCAGAAGGTGATCCA-3'	60	115
ACTB	5'-GCGAGAAGATGACCCAGAT-3' 5'-GAGTCCATCACGATGCCAGT-3'	58	118
DNA sequencing analysis			
APC (MCR)			
codon 1260-1410	5'-AGACTTATTGTGTAGAAGATAC-3' 5'-ATGGTTCACTCTGAACGGA-3'	51	450
codon 1389-1547	5'- TCTGTCAGTTCACTTGATAG-3' 5'-CATTTGATTCTTTAGGCTGC-3'	51	475
BRAF			
V600 mutation search	5'- TGCTTGCTCTGATAGGAAAATGA-3' 5'- CCAAAAATTTAATCAGTGGAAAAATA-3'	55	216
KRAS			
exon 2	5'- AAAAGGTACTGGTGGAGTATTTGA-3' 5'- GGTCCTGCACCAGTAATATGC-3'	55	251
Microsatellite instability (MSI) test			
Bethesda panel*): BAT25, BAT26, D"S123, D5S346, D17S250			

Boland CR, Thibodeau SN, Hamilton SR, et al: Cancer Res 58 (22): 5248-5257, 1998. PMID: 9823339.

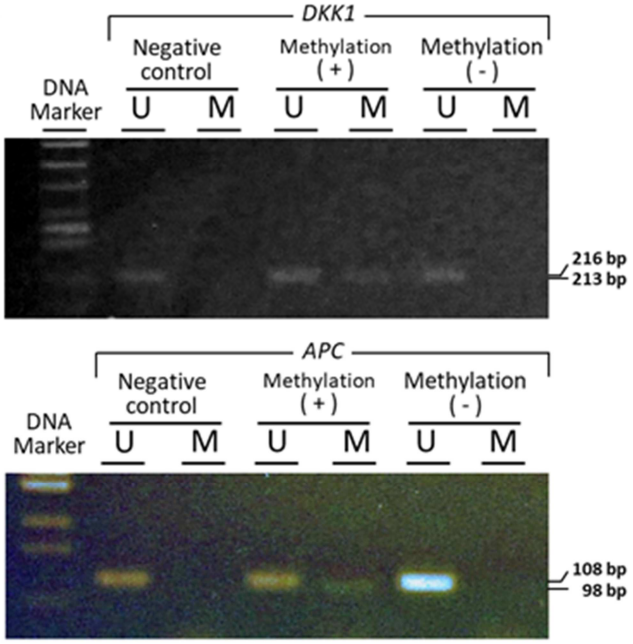

Figure 2. *Methylation-specific PCR with bisulfite-treated samples of colorectal tissue. The representative MSP experiments of DKK1 and APC are shown above and below, respectively. We used DNA derived from blood of a disease-free person as a negative control and the same DNA treated with CpG methyltransferase (New England Biolabs) as a positive control in each Methylation-specific PCR (MSP) experiment, but only negative control sample was presented in Figure 2.*

Control, negative control; Methylation(+), CRC with methylation of the promotor regions; Methylation (-), colorectal cancer without methylation of the promotor regions; U, PCR amplicon using un-methylated primer set; M, PCR amplicon using methylated primer set. DNA marker: ΦX174/*Hinc* II digest. In *DKK1*, a 216 bp band corresponds to PCR amplicon in un-methylated DNA and a 213 bp band corresponds to that in methylated DNA. In *APC*, a 108 bp or a 98 bp band corresponds to PCR amplicon in un-methylated DNA or methylated DNA, respectively.

2.2.2. Search for Mutations in APC, BRAF and KRAS

We searched for mutations using the Sanger method for the mutation cluster region (MCR), which corresponds to the codons 1250-1460 of *APC*, V600E mutation of *BRAF* and codon 12/13 of *KRAS*. Sanger sequencing was performed as described previously [13], and the primers used are shown in Table 2. Mutation analyses of the *APC* gene were performed on both DNA derived from cancer tissues and that derived from the normal mucosa for next allelic loss analyses.

2.2.3. Allelic Loss of APC Gene

To evaluate allelic losses of the *APC* gene, we utilized a loss of heterozygosity (LOH) method with a single nucleotide polymorphism locus, *rs41115*. When DNA derived from normal mucosa was heterozygous and DNA derived from the cancer tissue lacked one of the alleles, as determined through DNA sequencing analyses of the MCR of *APC* gene, the tumor was evaluated as allelic loss positive.

2.2.4. MSI Testing

We performed MSI testing using the following microsatellite markers, *BAT25*, *BAT26*, *D2S123*, *D5S346*, and *D17S250* from the Bethesda panel according to the report of the US National Cancer Institute [14].

2.2.5. Evaluation of Gene Expression of DKK1 and MYC

The cDNA was synthesized using RNA by the High-Capacity cDNA Reverse Transcription Kit (QIAGEN), and then the cDNA analyzed by quantitative RT-qPCR with the SYBR-Green method. The expression level was compared quantified using the β-actin gene (*ACTB*) as an internal control. The primer sequences and PCR conditions with the annealing temperatures are shown in Table 2. The relative expression level of *DKK1* of each sample was calculated with the following formula:

$$\text{Relative expression level of } DKK1 = \frac{(\text{Expression value of } DKK1 \text{ in cancer tissue}) \,/\, (\text{Expression value of } DKK1 \text{ in normal mucosa})}{(\text{Expression value of } ACTB \text{ in cancer tissue}) \,/\, (\text{Expression value of } ACTB \text{ in normal mucosa})}$$

Those of *MYC* was also calculated in the same manner.

$$\text{Relative expression level of } MYC = \frac{(\text{Expression value of } MYC \text{ in cancer tissue}) \,/\, (\text{Expression value of } MYC \text{ in normal mucosa})}{(\text{Expression value of } ACTB \text{ in cancer tissue}) \,/\, (\text{Expression value of } ACTB \text{ in normal mucosa})}$$

2.2.6. Relationship Between DKK1, MYC Expression and DKK1 Methylation

Since the WNT signaling pathway has been known to be strongly downregulated by the molecular complex containing APC, GSK3-β, and AXIN2, we analyzed the relationship between the mRNA level of *DKK1* or *MYC* and the methylation status of *DKK1* according to the presence or absence of *APC* abnormalities, such as promotor methylation, mutation of MCR, and allelic loss.

2.2.7. Statistical Analysis

After analysis of the F test for variance, the t-test or Welch test were used to test the differences in the average of relative expression levels. Associations between clinico-pathological factors and various biological conditions were compared using the χ^2 test, and corrections such as Yates correction or William's correction were added. P-values of <0.05 was considered to be statistically significant.

Table 3. *Clinico-pathological features according to DKK1 methylation and APC status.*

	41 all cases		**25 cases with *APC* abnormalities**		**16 cases without *APC* abnormalities**	
	***DKK1* promotor**		***DKK1* promotor**		***DKK1* promotor**	
	M (n = 10)	**U (n = 31)**	**M (n = 2)**	**U (n = 23)**	**M (n = 8)**	**U (n = 8)**
	No (%)	**No (%)**	**No (%)**	**No (%)**	**No (%)**	**No (%)**
Gender						
Male	5 (50.0)	22 (71.0)	0 (0.0)	15 (65.2)	5 (62.5)	7 (87.5)
Female	5 (50.0)	9 (29.0)	2 (100)	8 (34.8)	3 (37.5)	1 (12.5)
Age at surgical treatment (years)						
Mean	70.2	66.6	73.5	66.7	69.4	66.5
<50	1 (10.0)	1 (3.2)	0 (0.0)	1 (4.3)	1 (12.5)	0 (0.0)
≥50	9 (90.0)	30 (96.8)	2 (100)	22 (95.7)	7 (87.5)	8 (100)
Location	$^{*}p < 0.001$				$^{*}p < 0.01$	
Proximal colon	10 (100)	7 (22.6)	2 (100)	6 (26.1)	8 (100)	1 (12.5)
Distal colon and rectum	0 (0.0)	24 (77.4)	0 (0.0)	17 (73.9)	0 (0.0)	7 (87.5)
Histology	$^{**}p < 0.05$					
Papillary adenocarcinoma	0 (0.0)	1 (3.2)	0 (0.0)	0 (0.0)	0 (0.0)	1 (12.5)
Well differentiated adenocarcinoma	3 (30.0)	6 (19.4)	0 (0.0)	6 (26.1)	3 (37.5)	0 (0.0)
Moderately differentiated adenocarcinoma	3 (30.0)	24 (77.4)	0 (0.0)	17 (73.9)	3 (37.5)	7 (87.5)
Poorly differentiated adenocarcinoma	3 (30.0)	0 (0.0)	1 (50.0)	0 (0.0)	2 (25.0)	0 (0.0)
Mucinous adenocarcinoma	1 (10.0)	0 (0.0)	1 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)
pT						
Tis	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
T1	1 (10.0)	2 (6.5)	0 (0.0)	1 (4.3)	1 (12.5)	1 (12.5)
T2	2 (20.0)	8 (25.8)	0 (0.0)	7 (30.4)	2 (25.0)	1 (12.5)
T3	5 (50.0)	17 (54.8)	2 (100)	13 (56.5)	3 (37.5)	4 (50.0)
T4	2 (20.0)	4 (12.9)	0 (0.0)	2 (8.7)	2 (25.0)	2 (25.0)
pN	$^{*}p < 0.001$					
N0	8 (80.0)	19 (61.3)	2 (100)	14 (60.9)	6 (75.0)	5 (62.5)
N1	0 (0.0)	10 (32.3)	0 (0.0)	8 (34.8)	0 (0.0)	2 (25.0)
N2	2 (20.0)	2 (6.4)	0 (0.0)	1 (4.3)	2 (25.0)	1 (12.5)
TNM stage						
0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
I	3 (30.0)	8 (25.8)	0 (0.0)	6 (26.1)	3 (30.0)	2 (20.0)
II	5 (50.0)	11 (35.5)	2 (100)	8 (34.8)	3 (30.0)	3 (30.0)
III	1 (10.0)	10 (32.3)	0 (0.0)	8 (34.8)	1 (10.0)	2 (20.0)
IV	1 (10.0)	2 (6.5)	0 (0.0)	1 (4.3)	1 (10.0)	1 (10.0)

M: methylation of *DKK1* promotor region, U: un-methylation of *DKK1* promotor region
* Yates correction, ** William's correction.

3. Results

3.1. Characteristic of CRC with DKK1 Promotor Methylation

As shown in Table 3, there are three factors, namely location, histology and rate of metastasis of lymph node in which statistically significant differences were found between the DKK1 promotor methylation (DPM) and DKK1 promotor un-methylation (DPuM) groups. CRCs with DKK1 promotor methylation were located predominantly in the proximal colon (10/10 cases, 100%) and had a higher rate of poor differentiation or mucinous histology (4/10 cases, 40%)

compared to CRCs without DKK1 promotor methylation. Furthermore, the DPM group had significantly lower rate of metastasis of lymph node than the DPuM group. In 16 cases without *APC* abnormalities, 8 cases had the *DKK1* promotor methylation (50%). Clinico-pathological features were similar to that from the analysis of whole cases, however, a statistically significant difference was found only in the cancer location.

The presence or absence of *DKK1* promotor methylation did not exert a significant influence on gender, age, invasion depth, and the stage classification.

3.2. Characteristics of Abnormalities of APC, BRAF, KRAS, and MSI Status

Table 4 shows the relationship between *DKK1* methylation status and the biological markers such as *APC*, *BRAF*, *KRAS*, and MSI status. In 41 sporadic CRCs, any abnormalities of the *APC* gene were detected in 25 cases (61.0%). Only two of the 10 (20.0%) cases of DPM group carried *APC* abnormalities, in which methylation of the *APC* promotor region and mutations in the MCR were detected 2 cases (20.0%), 1 case (10.0%), respectively, and one of these two cases had 2 types abnormalities. No allelic loss of APC was observed in these 10 cases. On the other hand, the rate of APC abnormalities in DPuM group was 23/31 (74.2%), in which methylation of the *APC* promotor region, mutations in the MCR and allelic loss were detected 20 cases (64.5%), 11 case (35.5%) and 1 in 8 informative cases (12.5%), respectively, and 9 cases had 2 types abnormalities. There is a statistically significant difference in the rate of *APC* gene abnormalities in DPM group and DPuM group ($p< 0.01$). Regarding to the *BRAF* V600E mutation, no mutations were detected in the CRCs of the DPuM group, however, mutations were detected in 4 CRCs (40%) of the DPM group ($p < 0.01$). In addition, CRCs with the *BRAF* V600E mutation were limited to only 4 cases without *APC* abnormalities in the DPM group. However, *KRAS* mutations were detected at a lower rate in the CRCs of the DPM group (10%) compared to that of the DPuM group (45.2%), but no statistically significant difference was observed.

Seventy percent of the *DKK1* promotor methylation samples were MSI-high, and it was statistically different from the status in the DPuM group ($p < 0.001$).

3.3. Correlation Between DKK1 Promotor Methylation and Expression of DKK1 or MYC

The relative expression level of *DKK1* mRNA was significantly lower compared to that in DPuM group ($p < 0.05$ Figure 3a). A similar result was obtained either in 25 cases with *APC* abnormalities or in 16 cases without *APC* abnormalities (Figure 3b, 3c).

Furthermore, 36 out of 41 cases that were suitable for mRNA analysis showed an average of 14.8 times higher *MYC* expression level in cancer tissue than the corresponding normal mucosa (data not shown). As for the relationship of *MYC* expression and *DKK1* promotor methylation, there was no significant difference between the relative expression level of *MYC* mRNA and *DKK1* promotor methylation either in all 41 cases or in 25 cases with *APC* abnormalities (Figure 4a). However, in 16 cases without *APC* abnormalities, the relative mRNA expression level of *MYC* mRNA in the DPuM group was statistically lower compared to that in the DMP group ($p < 0.05$ Figure 4c).

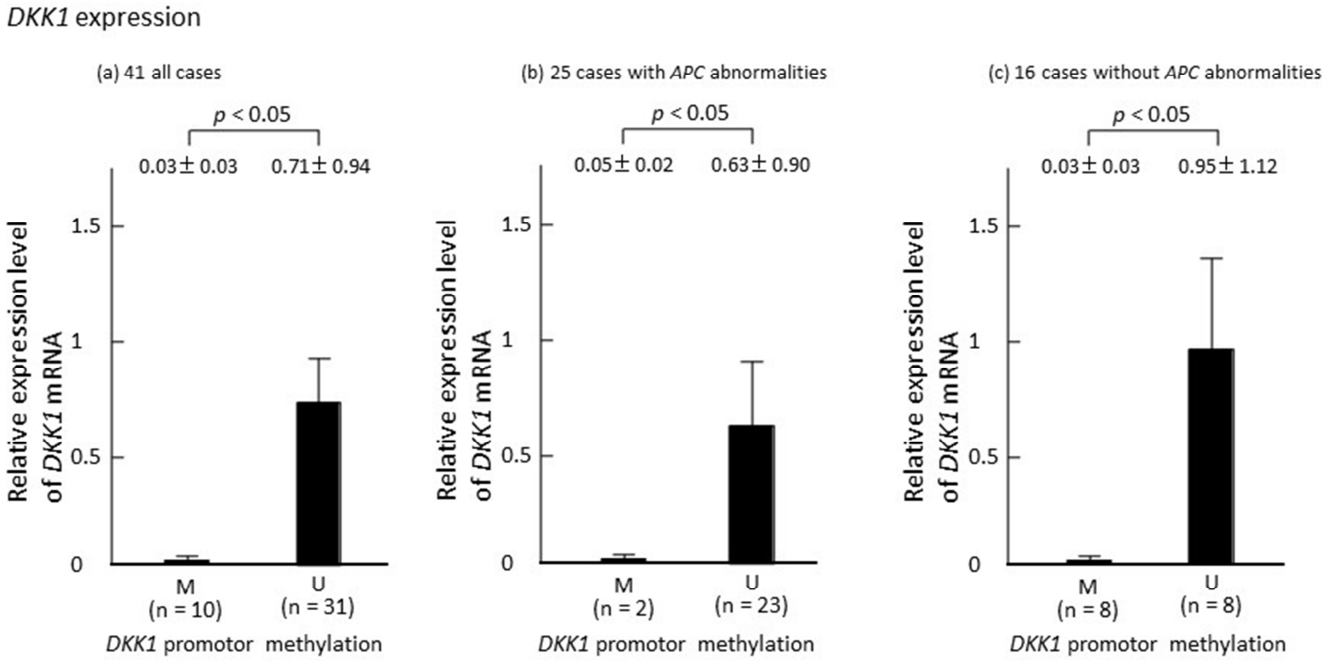

Figure 3. *Relative expression level of DKK1 mRNA. The expression level of DKK1 was normalized to the expression of the β-actin gene (ACTB) as an internal control. M, CRC with methylation of DKK1 promotor region; U, colorectal cancer with un-methylation of DKK1 promotor region. The parentheses at the top of each figure are as the following: (a) Forty-one cases being available for the analysis of mRNA expression, (b) 25 cases with abnormalities of the APC gene, (c) 16 cases without abnormalities of the APC gene.*

Table 4. *Correlation between DKK1 methylation status and other biological markers such as APC abnormalities, BRAF mutation, KRAS mutation, and MSI status.*

Biological features of MSI, *BRAF*, *APC* and *KRAS*			**Methylation status of the *DKK1* promotor region**		
			M	**U**	
			n = 10	**n = 31**	
			No (%)	**No (%)**	
APC abnormalities (any)	positive	n = 25	2 (20.0)	23 (74.2)	*$p < 0.01$
	negative	n = 16	8 (80.0)	8 (25.8)	
Methylation of *APC* promotor region	positive	n = 22	2 (20.0)	20 (64.5)	
	negative	n = 19	8 (80.0)	11 (35.5)	
Mutations in MCR	positive	n = 12	1 (10.0)	11 (35.5)	
	negative	n = 29	9 (90.0)	20 (64.5)	
Allelic loss (informative cases: 11)	positive	n = 1	0 (0.0)	1 (12.5)	
	negative	n = 10	3 (100.0)	7 (87.5)	
BRAF mutation (V600E)	positive	n = 4	4 (40.0)	0 (0.0)	*$p < 0.01$
	negative	n = 37	6 (60.0)	31 (100.0)	
KRAS mutation	positive	n = 15	1 (10.0)	14 (45.2)	*NS
	negative	n = 26	9 (90.0)	17 (54.8)	
MSI status	MSI-high	n = 7	7 (70.0)	0 (0.0)	*$p < 0.001$
	MSS or MSI-low	n = 34	3 (30.0)	31 (100.0)	

M: methylation of *DKK1* promotor region, U: un-methylation of *DKK1* promotor region, MCR: mutation cluster region
* Yates correction.

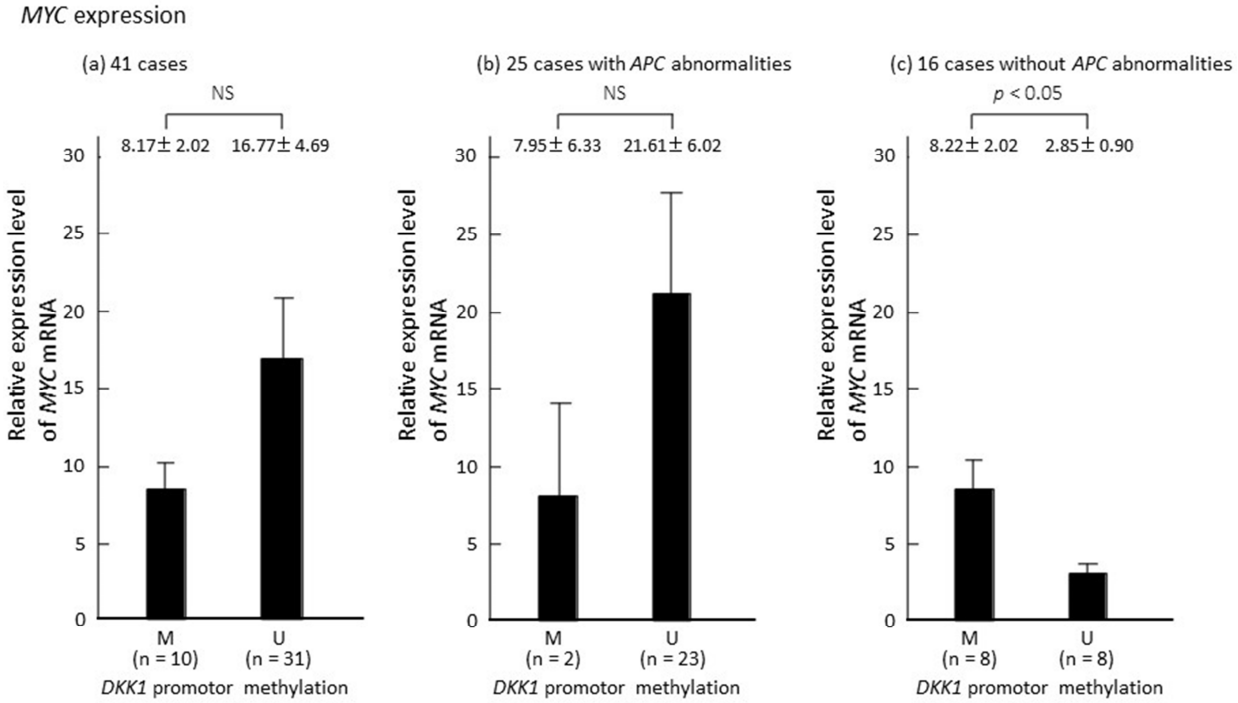

Figure 4. *Relative expression level of MYC mRNA. The mRNA expression level of MYC was normalized to expression of the β-actin gene (ACTB) as an internal control. M, CRC with methylation of DKK1 promotor region; U, CRC with un-methylation of DKK1 promotor region. The parentheses at the top of each figure are as follows: (a) Forty-one cases being available for the analysis of mRNA expression, (b) 25 cases with abnormalities of the APC gene, (c) 16 cases without abnormalities of the APC gene.*

4. Discussion

In the WNT signaling pathway, DKK1 binds competitively to LRP5/6, a co-receptor of WNT and therefore acts as a negative antagonist. We focused on the *DKK1* promotor methylation of colon cancer tissue. If the *DKK1* promotor methylation affects the WNT signaling pathway, the behavior of the cells could be changed. We therefore investigated the frequency of *DKK1* promotor methylation and the clinico-pathological features of the CRCs with such epigenetic conditions. The *DKK1* promotor methylation was observed in 10/41 (24.4%) cases of CRCs. Two studies in Japan have reported that the frequency of the *DKK1* promotor methylation was 12% and 35%, respectively [9, 15]. Since we designed the primers for methylation-specific PCR in consideration of CpG islands and the promotor- specific consensus sequence, it is possible that cases with severe methylation at the *DKK1* promotor region could be detected, and we believe that the adopted detection system could be accurately used in future studies.

There is a significant difference either in tumor location,

histology, rate of metastasis of lymph node between the DPM and DPuM groups. Therefore, although the number of CRCs with DKK1 promotor methylation were relatively small, it suggested that this type of CRC has some specific biological characteristics.

The *DKK3* gene, which encodes Dkk3 of the Dickkopf (Dkk) family, was also examined in the 41 cases. Methylation of the *DKK3* promoter region was detected in more than 51.6% of the cases, but no significant correlations between *DKK3* promotor methylation, clinico-pathological features, and molecular findings were observed (data not shown).

It has been reported that inactivation of APC protein has a significant impact on the WNT signaling pathway due to an accumulation of β-catenin and the activation of WNT target genes such as *MYC*, *CCND1*, and *PCBD2* [11, 12]. Therefore, we classified them into CRCs with *APC* abnormalities and those without *APC* abnormalities, and then compared accurately the amounts of *DKK1* and *MYC* transcripts in each group. If any *APC* abnormalities such as *APC* mutations in the mutation cluster region, *APC* allelic loss, or *APC* promotor methylation were detected, we defined such case as “a case with *APC* abnormalities”. In 16 cases without *APC* abnormalities, 8 cases had the *DKK1* promotor methylation (50%). The clinico-pathological features were similar to those of the whole group, except for a significant difference in tumor location (Table 3).

Next, we have examined V600E mutations of the *BRAF* gene, *KRAS* mutations, MSI. In the DPM group, the V600E *BRAF* mutation and MSI-high were detected in 40% and 70% of the cases, respectively, and there was a significant difference when compared to the DPuM group. On the other hand, the frequency of *APC* abnormalities and *KRAS* mutations was lower compared to the DPuM group. This finding was consistent with the differences between "Hypermutated tumors", including CIMP, and "Non-hypermutated tumors", as revealed by The Cancer Genome Atlas Network (TCGA, https://cancergenome.nih.gov/) based on comprehensive molecular characterization [16]. In 1999, CIMP was first reported where aberrant methylation of the promoter region and CpG islands were associated with transcriptional inactivation of tumor suppressor genes such as *p16, THBS1*, and *MLH1* [17]. CIMP underlies sporadic MSI and is tightly associated with *BRAF* mutations in CRC [18]. Therefore, the methylator characteristics of CRCs with the *DKK1* promotor methylation shown in this study were very similar to CIMP. The *DKK1* promotor methylation may also be used as a biomarker to detect a subset of CRCs with the so-called CIMP.

CRC is a heterogeneous disease that develops through different pathways, and is classified into several subtypes based on such as CIMP, MSI, *BRAF* mutations, *APC* abnormalities, *KARS* mutations [19-21]. These classifications have been proposed to have significance as prognostic markers for CRCs, such as the combination of MSI-high, CIMP positive, *BRAF* mutation positive, and *KARS* mutation negative, which have been reported to be the best prognosis subtype [20]. CRCs with *DKK1* promotor methylation are similar to this subtype. Although still controversial, CRCs with MMR deficiency and/or CIMP positive features have been known not to be beneficial as suggested from 5-FU-based adjuvant chemotherapy [22, 23]. Furthermore, the effect of the immune checkpoint blockade on MMR-deficient CRC has been reported [24]. The *DKK1* promoter methylation test might be useful for screening the CRCs with MMR deficiency and/or CIMP, and probably has potential for being used for prognosis and when choosing treatment options.

We revealed that the *DKK1* promotor methylation affected to the WNT signaling pathway through accurate examination of the expression level of *MYC*, which is one of the target oncogenes of the WNT signaling pathway. The *MYC* expression level in cancer tissues were elevated approximately 14.8-fold compared to that in normal mucosa. Among 16 cases without *APC* abnormalities, the average level of *MYC* expression had been reduced in 8 cases of CRCs without *DKK1* methylation (Figure 4c). This result suggested that DKK1 is involved in downregulating the *MYC* expression in the cellular environment in which APC function is proficient.

Both APC and DKK1 act as negative regulators in the WNT pathway. From the viewpoint of a tumor suppressor gene in the carcinogenesis mechanism, it is considered that *APC* abnormality and *DKK1* methylation are complementary to each other, that is, basically, one of them should occur in colorectal cancer. In the present study, the result that *APC* abnormalities were detected in 61% (25/41) of the CRC but were found only in 20% (2/10) of *DKK1* methylated cases shown in Table 3 is consistent with this notion. Also, Figure 4 clearly shows that *DKK1* mRNA expression reduces the *MYC* mRNA expression only in CRC without *APC* gene abnormality.

In order to further confirm the putative suppressive function of DKK1 in the WNT signaling pathway on protein expression levels, we performed an immunohistochemical analysis of DKK1 and β-catenin in CRC tissues. However, a clear result showing the correlation between the expression of both molecules could not be obtained, probably due to the relatively low expression of DKK1 protein in colon tissue (data not shown).

5. Conclusions

Since abnormalities of APC function have a significant impact on the WNT signaling pathway, functional evaluation of DKK1 might be limited when APC is functioning normally. We considered the hypothesis that the *DKK1* promotor methylation contributes to the upregulation of *MYC* expression, and then facilitates cell proliferation, which is the initial step of multi-stage carcinogenesis of the colon and the rectum [25]. The present study is the first report that *DKK1* promotor methylation affects the *MYC* expression based on the precise expression analysis. In conclusion, it is suggested that DKK1 is one of the regulators involved in *MYC* expression through the WNT signaling pathway and may have a negative effect on carcinogenesis of

the colorectum without *APC* abnormalities. Taken together with the result of the correlation of CRC with *DKK1* methylation and molecular characteristics of CIMP, *DKK1* promotor methylation, which is one of many epigenetic alterations in the WNT signaling pathway, may have affected cell growth factors such as *MYC*.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding this study.

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