The Significance of Molecular Biomarkers on Clinical Survival Outcome Differs Depending on Colon Cancer Sidedness

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Abstract. *Background/Aim:* This retrospective study focused on the correlation between molecular markers and prognostic outcomes of colon cancer patients depending on sidedness. *Materials and Methods:* A total of 117 stage I-III colon cancer patients who underwent colectomy were enrolled. Novel methylation markers (*KIF1A*, *PAX5* and *VGF*) were selected for epigenetic evaluation and *p53* and *ERCC1* protein expression was examined for the investigation of genetic alterations. *Results:* High frequency of methylation was observed in 68.2% of right-sided and 39.7% of left-sided colon cancer cases ($p=0.004$). Abnormal *p53* was identified in 52.3% of right-sided and 75.3% of left-sided cases ($p=0.015$). In right-sided cases, highly methylated genes demonstrated significantly favorable disease-free survival ($p=0.049$). Regarding left-sided cases, advanced T stage ($p=0.028$) and abnormal *p53* ($p=0.028$) were revealed to be significant predictive factors of the disease-free survival outcome. *Conclusion:* Molecular alterations, as significant prognostic factors, might differ depending on the sidedness of colon cancers.

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the fourth leading cause of cancer deaths in men and women worldwide (1). Generally, CRC incidence and mortality rates remain at the highest level in countries with a high or very high human development index, such as Australia, France, Iceland, New Zealand, United States, and Japan (2). Statistics show that CRC is increasing due to the intake of red or processed meat (3), obesity (4), and alcohol consumption (5). Especially for

advanced cases, multimodal treatment strategies should be adequately selected. For further development and improvement of tailored medicine, molecular prognostic markers are required in clinical practice.

Recently, molecular differences dependent on sidedness have been intensively examined. The CpG island methylator phenotype (CIMP) typically frequently occurs in the right-sided colon cancers of aged females (6) and is associated with *BRAF* mutations (7-9). Cohen *et al.* observed methylation of *CACA1G*, *IGF2*, *NEUROG1*, *RUNX3* and *SOC1* in right-sided CRCs that were closely associated with *BRAF* mutations (10). Cao *et al.* reported that left-sided CRCs had a higher rate of *p53*-positive expression (11). According to Wang *et al.* (12), the abnormal *p53* expression was an unfavorable prognostic predictor in CRC patients. To examine the prognostic molecular markers of colon cancer, we also have to focus on chemotherapy-associated markers including *ERCC1*, which is reported to be correlated with oxaliplatin resistance (13).

Though several researchers have reported disparities in molecular features according to location (10, 14), prognostic significance dependent on sidedness has been rarely discussed. In this research, we aimed to examine how several methylation and mutation markers impact on the prognosis of postoperative colon cancer patients and to establish prognostic factors of recurrence-free survival according to sidedness.

Materials and Methods

Sample collection. Colorectal cancer patients who underwent surgery with adequate lymphadenectomy at Nagoya University Hospital (Nagoya, Japan) between March 2008 and September 2013 were included. Cancers located in the transverse colon, rectum, and anal canal were excluded to define the sidedness of colon cancers clearly (15). Stage IV cases were also excluded due to the inconsistency in the perioperative chemotherapy given. Finally, 117 cases of stage I-III colon cancer cases were selected according to the TNM (tumor, nodes and metastases) classification 7th Edition

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(Union for International Cancer Control) at the time of surgery. Patients' characteristics were retrospectively collected from medical records and summarized in Table I. This study was performed according to the Helsinki Declaration and the protocol was accepted by the Review Board of Nagoya University. Written informed consent for the experimental usage of surgical samples was obtained from all patients.

DNA extraction and bisulfite treatment. DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The presence of >70% of cancer cells in all tumor samples was histologically confirmed in slides taken before and after sample harvesting. Bisulfite treatment was conducted following the protocol of the BisulFlash DNA Modification Kit (Epigentek, Farmingdale, NY, USA) using 1 µg of DNA.

Selection of novel methylation markers. Candidate genome-wide and cancer-wide promoter methylation markers were reported by Hoque *et al.* in 2008 (16). They identified 28 common cancer-specific methylation markers in 13 cancer types. Guerrero-Preston *et al.* also performed a genome-wide analysis in head and neck cancers, and reported ten important tumor suppressor genes that were inactivated by both promoter methylation and somatic mutations (17, 18). Based on the above results by our previous collaborators, we chose *KIF1A*, *PAX5*, and *VGF* genes as candidate methylation markers in colon cancer because these genes were methylated in various cancer types and no previous reports showed frequent methylation in colon cancer. For each gene, the chromosomal locations, functions, and methylation frequencies of various cancer types are described in Table II.

Quantitative methylation-specific PCR (QMSP). StepOnePlus (Thermo Fisher Scientific, Waltham, MA, USA) was used to perform QMSP in triplicate. The final reaction volume was 10 µl, which contained 600 nmol forward and reverse primers, 200 nmol/l probe, 0.6 unit Platinum Taq DNA Polymerase (Thermo Fisher Scientific), dATP, dCTP, dGTP, and dTTP in a concentration of 200 µmol each, 6.7 mmol/l MgCl₂, and 1.5 µl bisulfite-modified DNA. The PCR settings were as follows: initial denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 3 sec and 60°C for 10 sec. Bisulfite-Converted Universal Methylated Human DNA Standard (Zymo Research, Irvine, CA, USA) to construct the standard curve and ultrapure water as a positive control were added to each plate. Mean values were calculated from triplicate samples and used for analyses. The QMSP value is expressed as the percentage of fluorescence intensity for the PCR products of the target gene compared with that of *ACTB* (reference gene), multiplied by 100 for easier tabulation. The oligonucleotide sequences of the primers and probes are described in Table III. All primers and probes were from Hokkaido System Science (Sapporo, Japan).

Immunohistochemical staining (IHC). Formalin-fixed paraffin-embedded tumor tissues were sliced (4 µm each) and used to validate the expression levels of p53 and ERCC1 proteins. An additional slide without a monoclonal antibody was used as a negative control. Slides were deparaffinized in xylene and rehydration was performed through 100% ethanol for 5 min, three times. Endogenous peroxidase activity was blocked with 3% H₂O₂. For antigen retrieval, heat-induced epitope retrieval was conducted by 10-min boiling in Tris-EDTA buffer pH 8.0. The sections were incubated for one h at room

Table I. Clinicohistological characteristics of 117 colon cancer cases.

Characteristics		Number of cases (%)
Age	<70	58 (49.6)
	≥70	59 (50.4)
Gender	Female	70 (59.8)
	Male	47 (40.2)
Sidedness	Right	44 (37.6)
	Left	73 (62.4)
Differentiation	Well, Moderately	111 (94.9)
	Poorly, Mucinous	6 (5.1)
T stage	T1-3	93 (79.5)
	T4	24 (20.5)
N stage	N0	71 (60.7)
	N1-2	46 (39.3)
Stage	I-II	71 (60.7)
	III	46 (39.3)
Lymphatic invasion	Absent	38 (32.5)
	Present	79 (67.5)
Venous invasion	Absent	63 (53.8)
	Present	54 (46.2)
Adjuvant chemotherapy	No	64 (54.7)
	Yes	53 (45.3)

temperature with anti-p53 mouse monoclonal antibody (1:50; Santa Cruz Biotechnology, Dallas, TX, USA) or anti-ERCC1 rabbit monoclonal antibody (1:50; Cell Signaling Technology, Danvers, MA, USA). The secondary antibody (Dako EnVision and System-HRP Labelled Polymer, Agilent, Santa Clara, CA, USA) was used for 30 min at room temperature followed by incubation in 3,3'-diaminobenzidine for 2.5 min for p53 and 10 min for ERCC1, and then hematoxylin counterstaining was applied for 15 min. Slides were dehydrated in 70%, 80%, 90%, and 100% ethanol followed by two changes of xylene and then mounted.

For the evaluation of p53, we defined the weak expression as normal and absent, or strong expression as abnormal according to McGregor *et al.* (19). In detail, samples were classified into four grades according to the percentage of p53-positive cells (Figure 1). Scores 0 and 3 were regarded as aberrant expression, while scores 1 and 2 were normal expression. The percentage of ERCC1-positive cells was also categorized into four grades (Figure 2). Scores 0 and 1 were defined as normal, and scores 2 and 3 were defined as strong staining. Slides were analyzed independently by two cancer pathologists without any information about tumor sidedness and survival outcomes.

Statistical analysis. The Mann-Whitney *U*-test was performed to evaluate the differences in continuous variables, whereas the Fisher's exact test was used for categorical variables. The Cox proportional hazards model was used for univariable and multivariable analyses. Disease-free survival (DFS) was defined as the time from surgery to the time of first documentation of any disease recurrence. Survival curves were drawn using the Kaplan-Meier method, and the difference was calculated by log-rank test. Two-sided *p*-values less than 0.05 were considered statistically significant. All statistical analyses including receiver operating characteristic (ROC) curves were performed using JMP version 14.0.0 (SAS Institute Inc., Cary, NC, USA).

Table II. Details of selected novel methylation markers.

Gene	<i>KIF1A</i>		<i>PAX5</i>		<i>VGF</i>	
Location on chromosomes	2q37.7		9p13.2		7q22.1	
Protein function	Transporter of membranous organelles along axonal microtubules		Activator of B cell differentiation, neural development, and spermatogenesis		Expressed in neuroendocrine cells and up-regulated by nerve growth factor	
Methylation frequencies in cancer cell lines	HNSCC	2/3 (67%)	HCC	8/12 (67%)	Bladder cancer	4/7 (57%)
	Breast cancer	8/9 (89%)	Esophageal cancer	8/9 (89%)	Ovarian cancer	2/7 (29%)
			Gastric cancer	7/8 (88%)		

HNSCC: Head and neck squamous cell carcinoma; HCC: hepatocellular carcinoma.

Table III. List of primers and probes used for QMSP.

Gene	Forward 5'-3' (primer)	Probe	Reverse 5'-3' (primer)	Temperature	Amplicon
<i>ACTB</i>	TGGTGATGGAGGAGG TTTAGTAAGT	(FAM-5', 3'-TAMRA) ACCACCAC CCAACACACAATAACAAACACA	AACCAATAAAACCTA CTCCTCCCTTAA	60°C	133 bp
<i>KIF1A</i>	GCGCGATAAATTAGTT GGCGATT	(FAM-5', 3'-BHQ) CCTCCCGAAA CGCTAATTAACACGCG	CTCGACGACTACTCT ACGCTAT	60°C	140 bp
<i>PAX5</i>	GCGTAAGAGAGACGA AGGTAAG	(FAM-5', 3'-TAMRA) AGAGGTTT GCGTAGTTTCGTCGG	ATATTCGCGAACACC TCTACTAC	60°C	112 bp
<i>VGF</i>	GGATAGCGTTCGTAGG CG	(FAM-5', 3'-BHQ) GCGCCCAAA AACGACGTAAACCTAAATAC	AAAAACCGAATTCCC CACCCCG	60°C	202 bp

QMSP: Quantitative methylation-specific PCR.

Results

Measurement of QMSP values. Tumors and adjacent normal tissues of 117 colon cancer samples were applied to QMSP assays. QMSP values of all three genes are shown in Figure 3. Methylation frequencies in tumor tissues were significantly higher than those in adjacent normal tissues in *KIF1A* ($p<0.001$), *PAX5* ($p<0.001$), and *VGF* ($p<0.001$). Next, we calculated the cut-off values by receiver operating characteristic (ROC) curves between QMSP values of tumors and adjacent normal tissues (Figure 4). Relatively high area under the curve values were obtained (0.721 in *KIF1A*, 0.916 in *PAX5*, and 0.479 in *VGF*). According to the cut-off values, all cases were classified into high QMSP cases and low QMSP cases. Consequently, we divided 117 cases into 59 methylation frequency-high cases (gray color, high QMSP in 2-3 genes) and 58 methylation frequency-low cases (white color, low QMSP in 0-1 gene) (Figure 5).

Methylation frequency and clinicohistological factors. The clinicohistological backgrounds of all 117 colon cancer cases are summarized in Table I. Right-sided colon cancers derived from the midgut were found in 44 (37.6%) cases, while left-sided colon cancers derived from hindgut were found in 73

cases (62.4%). The association of these factors with methylation frequency is shown in Table IV. High frequency of methylation was significantly associated with aged patients ($p=0.010$) and right-sided colon cancers ($p=0.004$), while no significant association was found with tumor factors including T and N stages, and lymphatic invasion.

IHC scores of p53 and ERCC1. IHC scores of p53 and ERCC1 are shown in Table V. Abnormal expression of p53 and ERCC1 was observed in 66.7% (78 cases) and 22.2% (26 cases) of all cases, respectively. The associations of IHC abnormalities with methylation frequency and sidedness are summarized in Table VI. Abnormal expression of p53 was highly observed in left-sided colon cancers ($p=0.015$), but did not correlate with methylation status. As for the expression of ERCC1, no correlation was observed with both methylation status and tumor sidedness.

Prognostic factors depending on sidedness. Kaplan-Meier survival curves of DFS demonstrated that a high frequency of methylation was significantly related to better prognosis in right-sided colon cancer patients ($p=0.042$), whereas no correlation was found in left-sided colon cancers ($p=0.967$) (Figure 6a, b). Interestingly, abnormal p53 expression

Figure 1. Immunohistochemical staining for p53. The expression of p53 was evaluated at a $\times 200$ magnification. In accordance with the percentage of p53-positive cells, samples were categorized into four grades: score 0 (0%), score 1 (0-10%), score 2 (10-50%) and score 3 (50-100%).

showed completely opposite behaviors between right and left cases; the worse prognosis was observed in right-sided colon cancers ($p=0.034$) and favorable prognosis was found in left-sided colon cancers $p=0.043$) (Figure 6c, d).

Univariable analysis of DFS was performed using the proportional hazard model for individual prognostic factors of each side (Table VII). Besides advanced T stage (HR=5.31; 95%CI=1.31-21.48; $p=0.019$), high methylation frequency (HR=0.25; 95%CI=0.06-1.06; $p=0.060$) and abnormal p53 expression (HR=6.99; 95%CI=0.86-56.88; $p=0.069$) were marginally significant prognostic factors in the right-sided colon cancers. On the other hand, in left-sided colon cancers, advanced T stage (HR=3.04, 95%CI=0.96-9.60; $p=0.058$) and abnormal p53 expression (HR=0.33; 95%CI=0.11-1.02; $p=0.055$) were marginally significant.

In the multivariable analysis of DFS, we included all factors with p -values below 0.10 (Table VII). In right-sided colon cancers, high methylation frequency was statistically significant (HR=0.23; 95%CI=0.05-0.99; $p=0.049$), while in left-sided colon cancers, advanced T stage (HR=3.72; 95%CI=1.15-12.05; $p=0.028$) and abnormal p53 expression (HR=0.27; 95%CI=0.09-0.87; $p=0.028$) were significant prognostic factors.

Discussion

Genome-wide hypermethylation of colon cancers is reported to be predominant in aged and female cases (6), mainly located in the right-sided colon (6, 20), and has a better prognosis (21). Inoue *et al.* (22) revealed that accumulated gene methylation in sessile serrated adenoma/polyp (SSA/P) is closely associated with the development of cancers. Also, *BRAF* mutation is common in CIMP-positive CRC tumors (7-9). The *BRAF* mutation, V600E, is considered to initiate a serrated pathway that promotes the progression of normal mucosa to precancerous tissues (23). Although *BRAF* mutation is reported to be one of the poor prognostic factors in advanced colon cancers, it does not influence the survival outcomes among early-stage colon cancers (24).

The significance of mutated *TP53* on colon cancer survival remains controversial. In this study, we examined p53 protein expression, which is reported to be more discriminative than *TP53* mutation status (25). The majority of *TP53* mutations are missense and have an oncogenic role by gain-of-function, while the loss of wild-type *TP53* (loss-of-function) is a minor event, but also affects the tumor invasion process (26). Then, we included all mutation types into abnormal p53 cases and

Figure 2. Immunohistochemical staining for ERCC1. The expression of ERCC1 was validated at a $\times 200$ magnification. In accordance with the percentage of ERCC1-positive cells, samples were classified into four grades: score 0 (0%), score 1 (0-10%), score 2 (10-50%) and score 3 (50-100%).

Figure 3. Quantitative methylation-specific PCR (QMSP) values of normal and tumor tissues. Results of QMSP assays in 117 colon cancer samples are shown. QMSP values of tumors were significantly higher than those of adjacent normal tissues in KIF1A (a, $p < 0.001$), PAX5 (b, $p < 0.001$), and VGF (c, $p < 0.001$).

Figure 4. Receiver operation characteristic (ROC) curves. ROC curves were used to calculate the cut-off values between tumors and adjacent normal tissues.

Table IV. Association of clinicohistological characteristics with methylation frequency.

Characteristics	Methylation frequency		p-Value
	High (59 cases)	Low (58 cases)	
Age group			
<70	22 (37.9%)	36 (62.1%)	0.010
≥70	37 (62.7%)	22 (37.3%)	
Gender			
Female	33 (47.1%)	37 (52.9%)	0.452
Male	26 (55.3%)	21 (44.7%)	
Sidedness			
Right	30 (68.2%)	14 (31.8%)	0.004
Left	29 (39.7%)	44 (60.3%)	
Differentiation			
Well, Moderately	56 (50.5%)	55 (49.5%)	1.000
Poorly, Mucinous	3 (50.0%)	3 (50.0%)	
T stage			
T1-3	46 (49.5%)	47 (50.5%)	0.820
T4	13 (54.2%)	11 (45.8%)	
N stage			
N0	40 (56.3%)	31 (43.7%)	0.132
N1-2	19 (41.3%)	27 (58.7%)	
Stage			
I-II	40 (56.3%)	31 (43.7%)	0.132
III	19 (41.3%)	27 (58.7%)	
Lymphatic invasion			
Absent	22 (57.9%)	16 (42.1%)	0.325
Present	37 (46.8%)	42 (53.2%)	
Venous invasion			
Absent	36 (57.1%)	27 (42.9%)	0.140
Present	23 (42.6%)	31 (57.4%)

Figure 5. Distribution of cases according to methylated genes. Methylation frequency-high group (2-3 genes, gray area) included 59 cases, while 58 cases belonged to the methylation frequency-low group (0-1 gene, white area).

compared them with normal p53 cases. Previously, Paluszkiwicz *et al.* (27) showed that p53 abnormal accumulation was correlated with a lower survival rate of patients with left-sided CRCs. Conversely, a few reports suggested that p53 overexpression is a favorable prognostic factor, especially in the distal colon (28). For example, Soong *et al.* examined over 500 colorectal cancer cases and demonstrated that p53 accumulation was correlated with better

Table V. IHC scores of p53 and ERCC.

IHC scores		0	1	2	3
p53	Number of cases (%)	16 (13.7)	18 (15.4)	21 (17.9)	62 (53.0)
	Definition of expression	Abnormal	Normal	Normal	Abnormal
ERCC1	Number of cases (%)	48 (41.0)	43 (36.8)	13 (11.1)	13 (11.1)
	Definition of expression	Normal	Normal	Abnormal	Abnormal

IHC: Immunohistochemical staining.

Table VI. Association of molecular markers with methylation frequency and sidedness.

Markers	Expression	Methylation frequency		<i>p</i> -Value	Sidedness		<i>p</i> -Value
		High (59 cases)	Low (58 cases)		Right (44 cases)	Left (73 cases)	
p53	Normal	23 (59.0%)	16 (41.0%)	0.240	21 (53.8%)	18 (46.2%)	0.015
	Abnormal	36 (46.2%)	42 (53.8%)		23 (29.5%)	55 (70.5%)	
ERCC1	Normal	44 (48.4%)	47 (51.6%)	0.506	31 (34.1%)	60 (65.9%)	0.170
	Abnormal	15 (57.7%)	11 (42.3%)		13 (50.0%)	13 (50.0%)	

prognosis in distal cancers but not proximal cancers (29). Our results are in accordance with this study; abnormal p53 expression was associated with better prognosis in left-sided colon cancers and poor prognosis in right-sided colon cancers.

Subgroup analyses of our cohort showed that advanced T stage, advanced N stage, and lymph-vascular invasion-positive cases were dominant in p53 abnormal cases on both sides. Thus, abnormal p53 cases were expected to show a poor prognosis for right-sided colon cancers (Figure 6c). However, in left-sided colon cancers, abnormal p53 cases demonstrated better survival than normal p53 cases (Figure 6d). Especially among those left-sided cases who received adjuvant chemotherapy, abnormal p53 cancers indicated significantly favorable DFS than normal p53 cancers ($p=0.003$, Figure 7). In cases without adjuvant chemotherapy, we could not identify any survival difference between normal and abnormal p53 cancers on both sides. These findings imply the possibility that p53 abnormal expression-related chemosensitivity influences survival outcomes. Certainly, Zaanani *et al.* highlighted that sensitivity to oxaliplatin seems to be increased in p53 abnormally-overexpressing cases (30). They demonstrated that the survival of FOLFOX-treated cases had significantly better survival than 5-FU/leucovorin-treated cases in p53-overexpressing cases, whereas no survival difference was found in p53 normal cases. Since p53 abnormal cases are usually dominant in left-sided colon cancers [75.3% (55/73) in left, 52.3% (23/44) in right, Table VI] and oxaliplatin-based regimens were frequently used for advanced cases in this study as adjuvant chemotherapy (44.1% (15/34) in left, 33.3% (4/12) in right), abnormal p53

cases might have longer survival than normal p53 cases with left-sided colon cancers.

Otherwise, normal p53 cancers in left-sided cases should have other critical oncogene mutations and/or microsatellite instability (MSI), which are more harmful than *TP53* mutations. Lorre *et al.* demonstrated that genetic alterations have an asymmetrical distribution (15). For example, alterations in *SMAD4*, *PIK3CA*, *PTEN*, and *BRAF*^{V600E} are dominantly located in right-sided cases, whereas *APC* and *KRAS/NRAS* mutations exist on both sides. Since each gene alteration may have individual characteristics regarding malignancy and chemoresistance, each-sided tumors must have different characteristics and prognoses. Several researchers advocate molecular criteria for adjuvant chemotherapy. Sho and colleagues suggested that a five-gene mutation panel could be useful for choosing adjuvant chemotherapy induction (31). Park *et al.* reported that hypermethylation of *TFAP2E* is an independent prognostic factor for patients with adjuvant chemotherapy (32). These data suggest the significance of possible molecular biomarkers for the guidance of multimodal cancer treatment.

There are some limitations in the current study. First, the sample size was too small to identify statistically persuasive results. Second, we could not collect enough information about the *KRAS/BRAF* mutational status of surgical specimens, hence why we could not include them as candidate prognostic markers.

In summary, *KIF1A*, *PAX5*, and *VGF* were identified as novel methylation markers of colon cancer. All of them were significantly hyper-methylated in cancer tissues. Frequent

Table VII. Univariable and multivariable analyses for significant prognostic factors of disease-free survival after colon cancer resections in each side.

Covariates	Right-sided (44 cases)				Left-sided (73 cases)			
	Univariable analysis		Multivariable analysis		Univariable analysis		Multivariable analysis	
	HR (95%CI)	p-Value	HR (95%CI)	p-Value	HR (95%CI)	p-Value	HR (95%CI)	p-Value
Age group								
<70	Reference	0.805			Reference	0.919		
70≤	1.20 (0.29-5.02)				0.94 (0.30-2.97)			
Gender								
Female	Reference	0.837			Reference	0.994		
Male	0.86 (0.21-3.60)				1.00 (0.32-3.16)			
T stage								
T1-3	Reference	0.019	Reference	0.136	Reference	0.058	Reference	0.028
T4	5.31 (1.31-21.48)		3.00 (0.71-12.71)		3.04 (0.96-9.60)		3.72 (1.15-12.05)	
N stage								
N0	Reference	0.432			Reference	0.423		
N1-2	1.78 (0.42-7.44)				1.60 (0.51-5.04)			
Stage								
I-II	Reference	0.432			Reference	0.423		
III	1.78 (0.42-7.44)				1.60 (0.51-5.04)			
Lymphatic invasion								
Absent	Reference	0.428			Reference	0.746		
Present	0.57 (0.142-2.29)				1.24 (0.36-4.59)			
Venous invasion								
Absent	Reference	0.472			Reference	0.506		
Present	1.69 (0.40-7.09)				1.50 (0.45-4.99)			
Adjuvant chemotherapy								
No	Reference	0.457			Reference	0.599		
Yes	1.69 (0.42-6.77)				1.36 (0.43-4.29)			
Methylation frequency								
Low	Reference	0.060	Reference	0.049	Reference	0.967		
High	0.25 (0.06-1.06)		0.23 (0.05-0.99)		1.02 (0.33-3.23)			
p53 expression								
Normal	Reference	0.069	Reference	0.092	Reference	0.055	Reference	0.028
Abnormal	6.99 (0.86-56.88)		6.33 (0.74-54.13)		0.33 (0.11-1.02)		0.27 (0.09-0.87)	
ERCC1 expression								
Normal	Reference	0.278			Reference	0.798		
Abnormal	0.31 (0.04-2.55)				0.82 (0.18-3.74)			

HR: Hazard ratio; CI: confidence interval.

methylation of these genes was one of the significantly favorable prognostic factors of DFS for stage I-III right-sided colon cancers, but it had no association with DFS in left-sided colon cancers. Also, abnormal expression of p53 was related to shorter DFS in right-sided colon cancers but longer DFS in left-sided cases. As the molecular background of colon cancers seems to differ depending on the sidedness, treatment strategies should be considered individually.

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Conflicts of Interest

There are no conflicts of interest related to this study.

Authors' Contributions

Concept and designed research: M.H; Performed research: S.H., S.M. and H.T.; Data analysis: S.H., G.N., N.H. and M.H; Materials/reagents or analytic tools: S.H., M.H, M.Ka., C.T., D.K., S.Y., M.Ko. and M.F.; Wrote the paper: S.H., M.H., M.T., and Y.K.

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a. Right-sided colon cancers by methylation frequency **b. Left-sided colon cancers by methylation frequency** **c. Right-sided colon cancers by p53 expression** **d. Left-sided colon cancers by p53 expression**

Figure 6. Survival curves of disease-free survival (DFS). The influence of methylation frequency and p53 expression on disease-free survival was examined by Kaplan-Meier curves. In right-sided colon cancers, methylation-high cases showed favorable DFS (a, $p=0.042$). However, no difference was observed in left-sided colon cancers (b, $p=0.967$). Abnormal expression of p53 was significantly correlated with poorer prognosis in right-sided colon cancers (c, $p=0.034$), but with better prognosis in left-sided colon cancers (d, $p=0.043$).

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Figure 7. Survival curve of disease-free survival (DFS) in left-sided cases with adjuvant chemotherapy. Abnormal p53 cases showed longer DFS compared to normal p53 cases ($p=0.003$).

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