Tumor p16^{INK4} gene expression and prognosis in colorectal cancer

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Abstract. Hypermethylation of the tumor suppressor gene *p16*^{INK4} (*p16*) promoter is associated with worse prognosis in colorectal cancer (CRC). In the present study, it was investigated whether p16 mRNA expression correlates with the methylation of its promoter, and whether it influences prognosis in patients with CRC. DNA and RNA were extracted from 101 resected tumor specimens. A MethyLight assay was used to quantify *p16* methylation in terms of percentage of methylated reference (PMR), and the expression of p16 mRNA was measured using reverse transcription-polymerase chain reaction. Associations between *p16* methylation or mRNA expression and patient survival were evaluated using Kaplan-Meier analysis and Cox proportional hazards regression. *p16* methylation was detected in 67 cases (66.3%) and the median PMR value was 0.344 (range, 0.00-468.6). Using a cut-off PMR value of 4, high *p16* methylation was observed in 18 cases (17.8%). No significant association was observed between *p16* methylation level and patient prognosis. As expected, a significant inverse association was observed between *p16* methylation and mRNA expression (P=0.034). Amongst the 83 cases with low *p16* methylation, a significantly worse outcome was identified in patients expressing high p16 mRNA expression levels (P=0.026). Multivariate analysis identified that p16 mRNA expression was an independent prognostic factor for worse survival (P=0.011). These results suggested a paradoxical association between high levels of p16 mRNA expression in the tumor and worse prognosis in patients with CRC.

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Abbreviations: CRC, colorectal cancer; 5-FU, 5-fluorouracil; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; PMR, percentage of methylated reference

Key words: CRC, CDKN2A, methylation, prognostic factor, mRNA

Introduction

Colorectal cancer (CRC) is the third most frequent malignancy worldwide and the fourth most common cause of cancer-associated mortalities (1). Globally, ~1.36 million people are diagnosed with CRC each year and approximately one-half will succumb to this disease (1,2). A number of genes that are mutated in the multistep process of colorectal carcinogenesis and progression have been demonstrated to influence the prognosis of patients with CRC and their response to treatment (3-6). In addition to these somatic genetic mutations, epigenetic alterations and particularly the aberrant hypermethylation of gene promoter regions leading to transcriptional silencing are suggested to be important in CRC tumorigenesis (7). This mechanism is responsible for the functional inactivation of numerous tumor suppressor genes in CRC (7,8), including *human MutL homolog 1, tissue inhibitor of metalloproteinase-3, p14, death-associated protein kinase, adenomatous polyposis coli, O-6-methylguanine-DNA methyltransferase* and *p16*^{INK4} *[(p16) or cyclin-dependent kinase (CDK) inhibitor 2a]* (9-11).

In normal cells, p16 and retinoblastoma (Rb) proteins serve an important role in regulating the cell cycle pathway (12,13). Rb is phosphorylated by the cyclin D1-CDK4/6 complex, resulting in its dissociation from transcription E2 factor (E2F) (13). The subsequent transcriptional activation of E2F leads to progression of the cell cycle from G_1 to S phase (14). As p16 interferes with cell cycle progression by inactivating CDK4/6, decreased expression or inactivation of p16 attenuates the ability of Rb to inhibit cell proliferation (15). The p16-Rb pathway is suppressed in a number of cancer types via genetic or epigenetic alterations in Rb and/or p16, through overexpression of the cyclin D1/CDK4 complex, in addition to a number of other mechanisms (12,13,15,16). In particular, deletion or mutation of the *p16* gene is frequently observed in cancer of the biliary tract, lung, pancreas and esophagus and in brain tumors (17-21). Deletion of *p16* has been associated with a late clinical stage in esophageal cancer, and with lymphatic invasion and distant metastasis in pancreatic cancer (22,23). In gall bladder and lung cancer, *p16* deletions and mutations are associated with poor prognosis (24,25).

Decreased p16 expression due to hypermethylation of the *p16* promoter was detected in 32-55% CRC cases (26-30). Although p16 mRNA expression is inversely correlated with tumor size and lymph node metastasis (31), its influence on the prognosis of patients with CRC remains unclear (32). A previous Japanese study identified that p16 promoter hypermethylation in the primary tumors of patients with CRC was associated with a shorter survival (33). This result was supported by a subsequent meta-analysis (34). In the present study, it was investigated whether p16 mRNA expression was associated with methylation of the *p16* gene promoter and with patient prognosis in CRC.

Materials and methods

Patients with CRC and tissues. The present study included 101 patients with primary CRC who underwent surgery at the Kanazawa University Hospital (Kanazawa, Japan) between April 1999 and December 2002. Eligible patients were aged 20 years or older and had histologically proven adenocarcinoma of the colon and rectum. Exclusion criteria included absolute contraindications to general anesthesia and/or surgery. Their clinicopathological characteristics are presented in Table I. The survival status was determined for all patients and the median follow-up period was 54.5 months. In total, 54 patients (53.5%) received postoperative 5-fluorouracil (5-FU)-based adjuvant chemotherapy.

Tumor tissue samples collected from the fresh surgical specimen were cryopreserved in liquid nitrogen and stored at -80˚C for extraction of DNA and RNA. The remaining surgical specimen was fixed with 10% neutral-buffered formalin for 1-2 days at room temperature and embedded in paraffin for histopathological examination. The tumor stage was determined according to the Union for International Cancer Control tumor, node and metastasis (TNM) classification (35). Genomic DNA and total RNA were extracted from the same tumor tissues using the QIAmp DNA Mini kit and the RNeasy Mini kit (both from Qiagen GmbH, Hilden, Germany), respectively, according to the manufacturer's protocols.

The present study was performed in accordance with the Declaration of Helsinki. The design and protocol for the present study were approved by the Kanazawa University Human Genome and Gene Analysis Research Ethics Committee, and written informed consent was obtained from the majority of the patients.

Quantification of p16 methylation by the MethyLight assay. Bisulfite conversion of genomic DNA was performed as previously described (36). DNA was denatured using 0.2 M NaOH and subsequently incubated with bisulfite for 16 h at 50˚C. The bisulfite-converted DNA was purified using the Wizard DNA purification kit (Promega Corporation, Madison, WI, USA) and precipitated with ethanol. The DNA sample was resuspended in water and stored at -30˚C.

MethyLight, a fluorescence-based real-time PCR assay was used to measure the level of *p16* promoter methylation as previously described (37). The sense and antisense primers used for amplifying the bisulfite-converted *p16* promoter were: 5'-TGG AATTTTCGGTTGATTGGTT-3' and 5'-AACAACGTCCGC ACCTCCT-3', respectively (37). These primers were used with the probe 5'-6FAM-ACCCGACCCCGAACGCG-TAMRA-3' to measure CpG methylation of the *p16* promoter region by real-time PCR (38). The specificity for amplification of methylated DNA was confirmed separately using human sperm DNA (unmethylated) and SssI (New England BioLabs, Inc., Ipswich, MA, USA)-treated sperm DNA (fully methylated) in the assay. Actin was amplified as a control for the total amount of DNA using the sense primer, 5'-TGGTGATGGAGGAGGTTTAGT AAGT-3' and antisense primer, 5'-AACCAATAAAACCTA CTCCTCCCTTAA-3'; and probe, 5'-6FAM-ACCACCACC CAACACACAATAACAAACACA-TAMRA-3' (38). The percentage of fully methylated fraction [percentage of methylated reference (PMR)] at a specific gene locus was calculated by dividing the gene:actin ratio of the sample DNA by the gene:actin ratio of the SssI-treated sperm DNA and multiplying by 100 (38). PMR values obtained using MethyLight were classified into high (PMR ≥4) and low (PMR <4) methylation categories, according to previous studies (9,39,40).

Analysis of p16 mRNA expression. Reverse transcription (RT)-PCR was used to measure p16 mRNA expression, as previously described (41). The relative expression of mRNA was quantified using the $2^{-\Delta\Delta Cq}$ method (42). The expression of actin was measured as an internal standard and the level of p16 mRNA expression in each tumor sample was normalized to the expression of actin. The cut-off value for high and low levels of p16 mRNA expression was defined as the median expression level for all tumor samples. Cut-off values for *p16* PMR and mRNA expression were used to compare *p16* promoter methylation and mRNA expression in the same tumors.

Proliferating cell nuclear antigen (PCNA) mRNA expression levels were additionally measured, using actin expression level as the internal standard. p16 mRNA expression relative to PCNA (p16/PCNA) was calculated as the p16 mRNA:actin ratio divided by the PCNA mRNA:actin ratio in the same cDNA sample. The cut-off value for p16/PCNA expression was defined as the median p16/PCNA level for all tumor samples. This was used to investigate the influence of p16 mRNA expression on the survival of patients with CRC.

Statistical analysis. For statistical comparison of tumor *p16* methylation and mRNA expression with clinicopathological factors and tumor stage, the Fisher's exact test was used to study non-continuous variables, and the Mann-Whitney U test and Kruskal-Wallis test were used to study continuous variables. The Mann-Whitney U test was used to compare *p16* methylation with p16 mRNA expression. The survival of patients was evaluated using Kaplan-Meier analysis. Univariate and multivariate analyses of survival were conducted using Cox proportional hazards regression. All statistical analyses were performed with EZR (version 1.29, Jichi Medical University, Shimotsuke, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander designed to add statistical functions frequently used in biostatistics (43).

Results

p16 promoter methylation and p16 mRNA expression in CRC. The levels of *p16* gene promoter methylation and p16

Table I. Association between *p16* methylation status or p16 mRNA expression and clinicopathological features in colorectal cancer.

Clincopathological features	n	*p16* methylation	P-value	p16 mRNA	P-value
Sex					
Male	57	0.455 (0-1.462)	0.383	2.020 (0.960-5.250)	0.584
Female	44	0.060 (0-2.105)		1.950 (0.85-3.548)	
Age, years					
≥65	56	0.271 (0-1.955)	0.900	2.040 (1.023-4.558)	0.494
<65	45	0.398 (0-1.220)		1.860 (0.700-4.460)	
Tumor site					
Proximal	29	0.455 (0-1.917)	0.259	2.220 (0.780-3.630)	0.588
Distal	48	0.127 (0-0.863)		1.825 (1.033-4.520)	
Not known	24	1.068 (0.055-4.814)		2.585 (0.925-9.550)	
Tumor histology					
Well	34	0.161 (0-1.117)	0.018	2.600 (1.240-4.360)	0.066
Moderately	34	0.082 (0-0.575)		1.775 (0.857-4.380)	
Poorly	4	0.162 (0.551-12.930)		0.335 (0.270-0.760)	
Mucinous	4	38.990 (15.750-80.514)		1.940 (1.137-2.557)	
Not known	25	1.028 (0.006-4.002)		3.070 (0.960-9.050)	
T stage					
T2	3	0.000 (0-24.523)	0.438	1.710 (1.025-3.085)	0.929
T3	56	0.150 (0-0.911)		2.085 (0.932-4.187)	
T4	18	0.438 (0-1.008)		1.750 (1.045-4.137)	
Not known	24	1.068 (0.055-4.814)		2.585 (0.925-9.550)	
Stage					
2	36	0.012 (0-0.658)	0.021	1.970 (0.933-4.968)	0.435
3	41	0.455 (0-3.154)		1.860 (0.970-3.380)	
4	0				
Not known	24	1.068 (0.055-4.814)		2.585 (0.925-9.550)	
Adjuvant chemotherapy					
Yes	54	0.494 (0-2.585)	0.169	1.915 (0.850-4.623)	0.833
No	44	0.192 (0-1.000)		1.970 (0.998-4.473)	
Not known	3	0 (0-0.804)		4.330 (2.845-4.640)	

p16 methylation status and p16 mRNA expression are presented as the median value (25-75th percentile). Histological type of the primary tumor was classified into well-, moderately-, poorly- differentiated adenocarcinoma and mucinous adenocarcinoma according to the Union for International Cancer Control classification.

mRNA expression in the tumor samples of all patients with CRC are presented in Table I in association with clinicopathological features. *p16* methylation (PMR >0) was detected in 67 cases (66.3%) and the median PMR value was 0.34 (range, 0.00-468.6; Fig. 1A). A PMR cut-off value of ≥4 was used to define high methylation and PMR <4 for low methylation, according to previous studies (9,38-40). Using this definition, the high methylation group comprised 18 (17.8%) of the 101 patients (Table II), in agreement with previous studies (9,33). The range of relative p16 mRNA expression levels was between 0 and 154.0, with a median expression level of 1.98 (Fig. 1B). As p16 mRNA expression is likely to be associated with cell cycle (44), the p16 mRNA expression level was normalized to that of PCNA expression in the same tumor and designated as p16/PCNA expression. The relative values of p16/PCNA ranged between 0 and 1,957.6, with a median value of 11.46 (Fig. 1C). To evaluate the influence of p16 mRNA expression on survival outcome, patients were divided into two groups (high and low) according to the median value for p16 mRNA or p16/PCNA expression.

Comparison of p16 mRNA expression between the *p16* high and low methylation groups identified a significant inverse association (P=0.034; Fig. 2A). When a higher PMR cut-off value of 10 was used rather than 4, a strong inverse association with p16 mRNA expression was observed between the high (n=12; 11.9%) and low (n=89; 87.1%) *p16* methylation groups (P<0.001; Fig. 2B). Subsequently, p16/PCNA expression was compared between the *p16* high and low methylation groups. Using a PMR value of 4 to classify *p16* methylation, no significant association was observed between *p16* methylation

Table II. Association between *p16* methylation status or p16/PCNA expression and clinicopathological features in colorectal cancer.

		p16 methylation			p16/PCNA mRNA		
Features	n	High, n=18	Low, n=83	P-value	High, n=50	Low, n=51	P-value
Sex							
Male	57	9	48	0.605	29	28	0.842
Female	44	9	35		21	23	
Age, years							
≥65	56	11	45	0.794	29	27	0.690
<65	45	7	38		21	24	
Site							
Proximal	29	7	22	0.090	12	17	0.814
Distal	48	4	44		22	26	
Not known	24	7	17		16	8	
Tumor histology							
Well	34	3	31	0.017	18	16	0.210
Moderately	34	4	30		13	21	
Poorly	4	1	3		0	4	
Mucinous	4	3	1		2	2	
Not known	25	7	18		17	8	
T stage							
T2	3	1	2	0.353	0	3	0.344
T3	56	7	49		25	31	
T4	18	3	15		9	9	
Not known	24	7	17		16	8	
Stage							
2	36	1	35	0.008	16	20	1.000
3	41	10	31		18	23	
4	0	0	0		0	0	
Not known	24	7	17		16	8	
Adjuvant chemotherapy							
Yes	54	11	43	0.611	29	25	0.543
No	44	7	37		20	24	
Not known	3	0	3		1	2	

Histological type of the primary tumor was classified into well-, moderately-, poorly- differentiated adenocarcinoma and mucinous adenocarcinoma, according to the Union for International Cancer Control classification. PCNA, proliferating cell nuclear antigen.

and p16/PCNA expression (P=0.168; Fig. 2C). However, when classified according to the higher PMR cut-off value of 10, a significant inverse association was observed between *p16* methylation and p16/PCNA expression (P=0.006; Fig. 2D).

Tumor p16 methylation and prognosis of patients with CRC. Comparison of tumor *p16* methylation levels with clinical and histopathologic characteristics of patients with CRC is presented in Table II. High *p16* methylation (PMR >4) level was more frequently detected in mucinous (P=0.017) compared with the other histological types and was significantly associated with later clinical stage (P=0.008); however, not with the sex or age of the patient, tumor site, T stage or adjuvant chemotherapy. There was no significant difference in prognosis between the high and low *p16* methylation groups (P=0.94; Fig. 3).

Tumor p16 mRNA expression and prognosis of patients with CRC. No significant differences in p16 mRNA expression levels (high or low) were observed according to sex or age of the patient, or with tumor site, histology, T stage, or adjuvant chemotherapy (Table III). The survival of patients with CRC with high p16 mRNA expression was worse compared with patients with low expression; however, this did not reach statistical significance (P=0.109; Fig. 4A). The majority (83/101; 82%) of patients with CRC demonstrated low levels (PMR <4) of *p16* methylation (Table II; Fig. 2A). When these patients were divided into high and low p16 mRNA

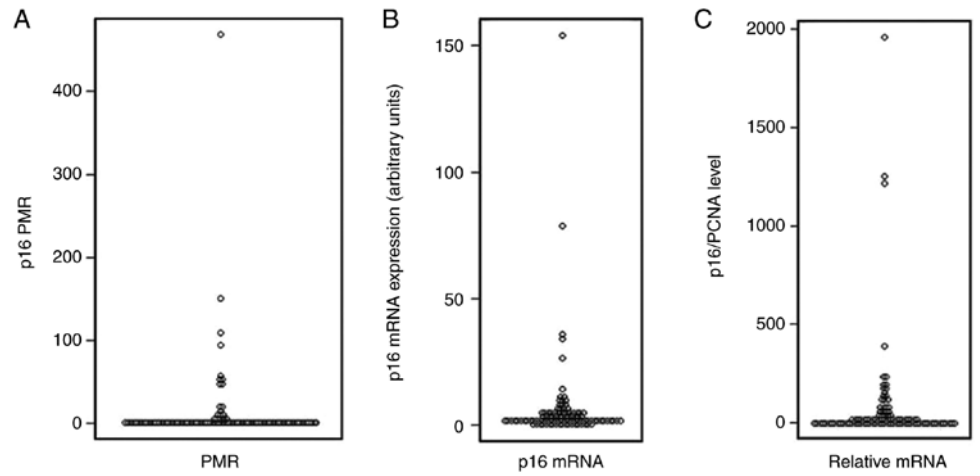

Figure 1. Distribution of *p16* methylation, p16 mRNA and p16/PCNA levels in the primary tumors of patients with colorectal cancer. (A) Distribution of *p16* methylation levels expressed as PMR. The median level for *p16* PMR was 0.34. (B) Distribution of p16 mRNA expression levels. The median level for p16 mRNA expression was 1.98. (C) Distribution of p16/PCNA levels. The median level for p16/PCNA was 11.46. PCNA, proliferating cell nuclear antigen; PMR, percentage of methylated reference.

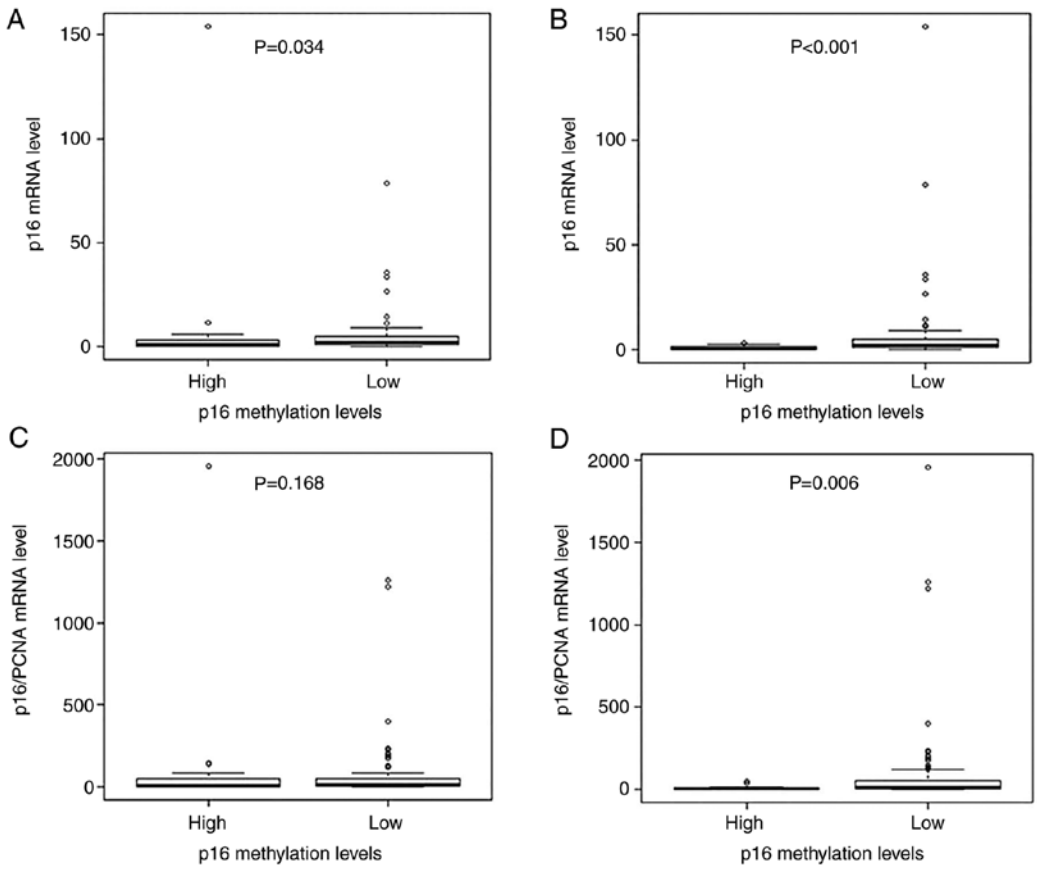

Figure 2. Tumor p16 and p16/PCNA mRNA expression levels in patients with colorectal cancer with high or low *p16* methylation levels. Tumor p16 mRNA expression levels with a PMR cut-off value of (A) 4 and (B) 10. p16/PCNA expression levels with a PMR cut-off value of (C) 4 and (D) 10. Statistical differences between the groups were calculated by the Mann-Whitney U test. PCNA, proliferating cell nuclear antigen; PMR, percentage of methylated reference.

expression groups defined by a median level of value (2.15), the high expression group (n=42) demonstrated a worse outcome compared with the low expression group (n=41; P=0.076; Fig. 4B).

Patients with CRC were additionally divided into p16/PCNA high (n=50) and low (n=51) groups according to the median value (11.46). No differences in any of the clinical and histopathologic parameters were observed between these two groups (Table II), nor was there a significant difference in patient survival between the high and low p16/PCNA groups (P=0.122; Fig. 4C). The 83 patients with low tumor *p16* methylation were further examined by dividing them into

Table III. Association between p16 mRNA expression and clinicopathological features in colorectal cancer.

Features	n	p16 mRNA High, n=51	p16 mRNA Low, n=50	P-value
Sex				
Male	57	29	28	1.000
Female	44	22	22	
Age, years				0.842
≥65	56	29	27	
<65	45	22	23	
Site				
Proximal	29	16	13	0.485
Distal	48	22	26	
Not known	24	13	11	
Tumor histology				
Well	34	19	15	0.668
Moderately	34	15	19	
Poorly	4	1	3	
Mucinous	4	2	2	
Not known	25	14	11	
T stage				
T2	3	1	2	0.507
T3	56	30	26	
T4	18	7	11	
Not known	24	13	11	
Stage				
2	36	18	18	1.000
3	41	20	21	
4	0	0	0	
Not known	24	13	11	
Adjuvant chemotherapy				
Yes	54	27	27	1.000
No	44	22	22	
Not known	3	2	1	

Histological type of the primary tumor was classified into well-, moderately-, poorly- differentiated adenocarcinoma and mucinous adenocarcinoma, according to the Union for International Cancer Control classification.

groups with high or low p16/PCNA expression according to the median value (12.49). In this analysis, patients with high p16/PCNA expression demonstrated a significantly worse survival (P=0.026; Fig. 4D).

The patient group with a low *p16* methylation (PMR <4) was additionally evaluated using Cox regression analysis for the prognostic significance of various clinical and histopathologic features and for p16 mRNA and p16/PCNA expression levels. p16/PCNA mRNA expression was the only

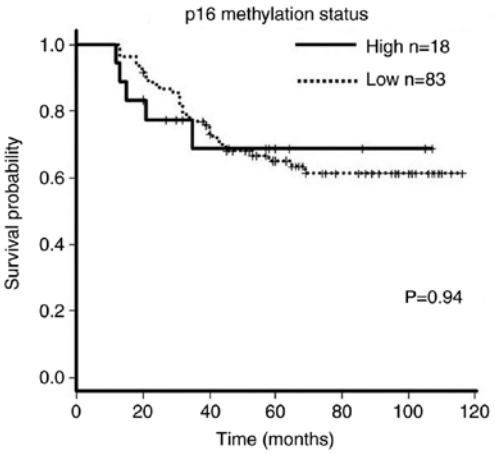

Figure 3. Kaplan-Meier analysis of the overall survival of patients with colorectal cancer stratified, according to their tumor *p16* methylation level (low, PMR <4; high, PMR ≥4). There is no significant difference in outcome between the two patient groups (P=0.94). PMR, percentage of methylated reference.

significant prognostic factor in univariate analysis (Table IV; P=0.026). The influence of T stage, tumor stage and adjuvant chemotherapy on the overall survival of patients with CRC was analyzed using the Kaplan-Meier method. There was no association between any of these factors and the prognosis of the patients (Fig. 5). Multivariate analysis identified that low p16/PCNA expression was an independent factor for better survival in patients with low *p16* methylation (hazard ratio 0.287; 95% confidence interval 0.110-0.747; P=0.011; Table IV).

Discussion

Tumor *p16* methylation has previously been identified as a prognostic factor for a worse outcome in CRC (33,34,41). However, other previous studies demonstrated that *p16* methylation has no impact on prognosis (32) or predicts worse outcome only in patients with poorly differentiated CRC (45). A possible mechanism for the putative association between tumor *p16* methylation and survival of patients with CRC is that expression of p16 protein is diminished, thereby promoting tumor cell proliferation and invasion (46,47). In the present study, however, no significant association was observed between tumor *p16* methylation and the outcome of patients with CRC, thus supporting a previous study (32). A number of technical reasons, in addition to the number of patients examined may account for the inconsistent results demonstrated by different previous studies and the present study. The technical reasons include differences in the tissue samples analyzed (fresh compared with fixed tissues), the methods used to quantify *p16* methylation levels and the cut-off values used in the analyses (32-34,41,45).

p16 promoter methylation is associated with decreased expression of p16 mRNA in clinical samples of CRC (26). In the present study, an inverse association between tumor *p16* methylation and the expression of its transcript was additionally identified. This was most pronounced in tumors with high methylation levels (PMR >10). However, within the high methylation group (PMR ≥4) a number of cases with relatively increased expression of p16 mRNA were identified,

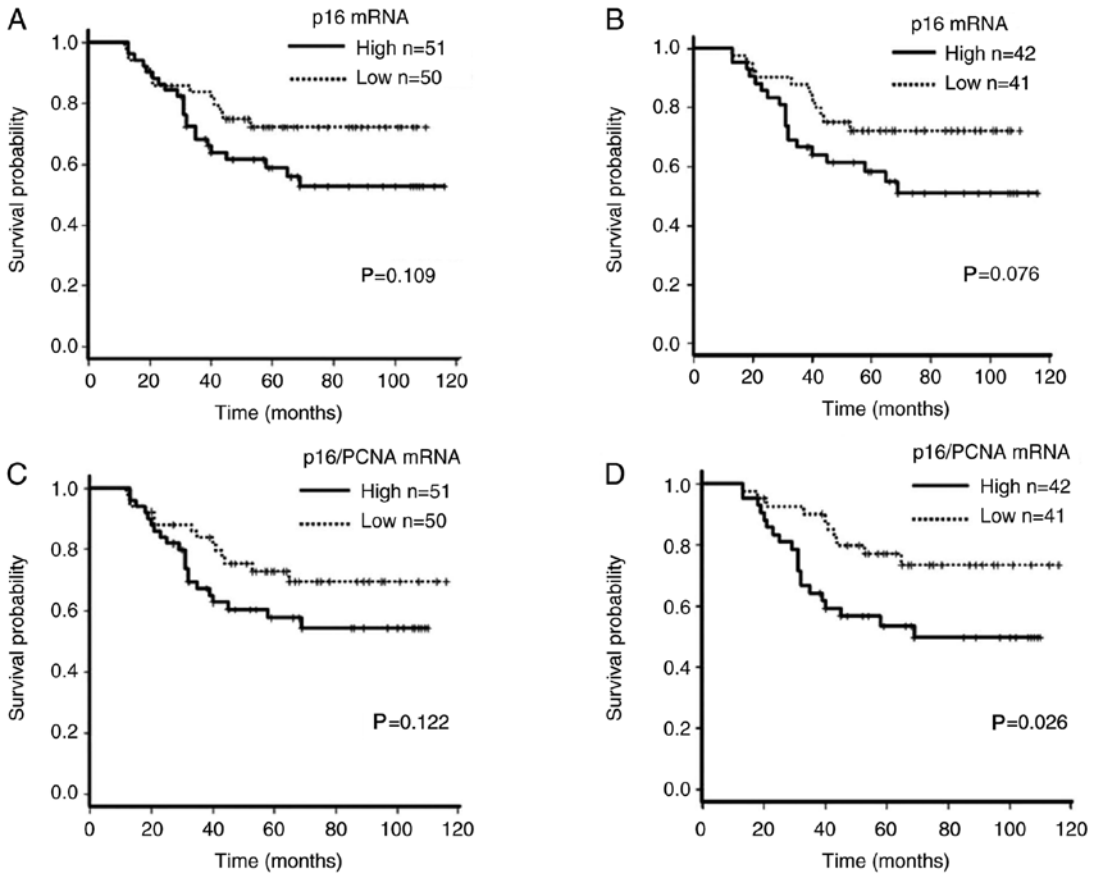

Figure 4. Comparison of the overall survival of patients with colorectal cancer according to their level of p16 mRNA and p16/PCNA mRNA. Kaplan-Meier analysis compares the overall survival of (A) all patients according to their level of p16 mRNA; (B) the patients with low *p16* methylation (PMR <4) according to their level of p16 mRNA; (C) all patients according to their level of p16/PCNA mRNA; and (D) the patients with low *p16* methylation (PMR <4) according to their level of p16/PCNA mRNA. Statistical differences between the groups were calculated using the log-rank test. PMR, percentage of methylated reference; PCNA, proliferating cell nuclear antigen.

Table IV. Univariate and multivariate analysis for the prognostic significance of clinicopathological factors and p16/PCNA mRNA.

Clinicopathological factors	Univariate analysis			Multivariate analysis		
Variables	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Male	0.912	0.443-1.877	0.802	0.660	0.223-1.947	0.451
Age, years	1.026	0.996-1.058	0.095	1.019	0.977-1.063	0.379
Proximal vs. distal	0.516	0.192-1.389	0.190	0.446	0.126-1.574	0.209
Well-differentiated histology vs. others	0.585	0.250-1.370	0.217	0.643	0.231-1.796	0.400
T stage	1.448	0.631-3.332	0.383	1.602	0.604-4.245	0.343
Stage	1.326	0.584-3.007	0.500	1.723	0.677-4.245	0.343
Adjuvant chemotherapy	0.704	0.664-1.481	0.355	0.439	0.144-1.336	0.147
p16/PCNA mRNA	0.422	0.197-0.902	0.026	0.287	0.110-0.747	0.011

CI, confidence interval; PCNA, proliferating cell nuclear antigen.

in agreement with previous studies, which demonstrated that tumor cells with *p16* promoter methylation may express p16 mRNA (31,41). Therefore, p16 mRNA expression may not be controlled exclusively through promoter methylation; however, may additionally be influenced by other factors, including the Ras signaling pathway (48), which is frequently activated in CRC due to *KRAS* proto-oncogene GTPase and *NRAS* proto-oncogene GTPase mutations.

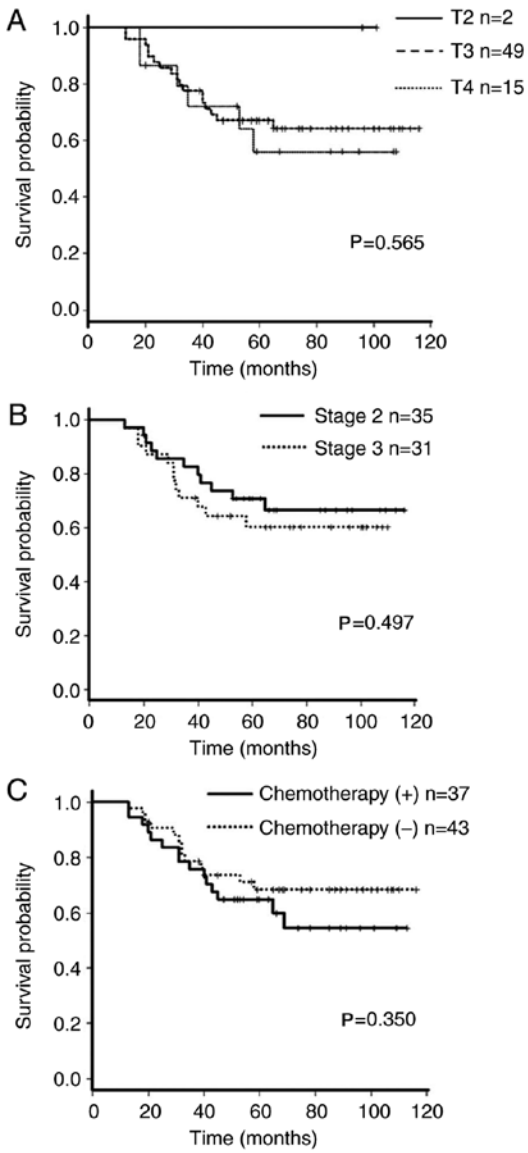

Figure 5. Influence of T stage, tumor stage and adjuvant chemotherapy on the overall survival of patients with colorectal cancer. (A) T stage, (B) tumor stage and (C) adjuvant chemotherapy on the overall survival of patients with colorectal cancer was analyzed using the Kaplan-Meier method.

Previous studies demonstrated that low p16 protein expression in CRC was associated with larger tumor size, lymph node metastasis and faster tumor proliferation (31,49,50), and is thus likely to account for an association with worse prognosis (51). However, little is known regarding the prognostic impact of p16 mRNA expression in patients with CRC. Although an inverse association between tumor *p16* methylation and mRNA expression was observed in the present study, relatively few patients (18/101) demonstrated high levels of *p16* methylation, defined as PMR ≥4. Therefore, patients with low tumor *p16* methylation levels were investigated, as it was hypothesized that p16 mRNA expression may affect patient survival, as those patients may exhibit a wide range of p16 mRNA expression for analysis. An unexpected result of the present study was that patients with high p16/PCNA mRNA expression demonstrated significantly worse survival. Furthermore, this was demonstrated by multivariate analysis to be independent of other factors that potentially influence patient survival, including tumor stage and histological types. Despite its well-recognized tumor suppressor role (52), p16 is overexpressed at the mRNA and protein expression levels in tumor tissues compared with adjacent normal mucosa (31,51). Similar to the present results, patients with breast (53,54) and prostate cancer (55) with high p16 expression were additionally identified to have a worse prognosis. Notably, although p16 is a critical cell cycle regulator and its mRNA expression is likely to be associated with cell cycle (44), none of the previous studies investigating the association of *p16* promoter methylation with prognosis of patients with CRC (32,34) scored a copy number of p16 mRNA. Therefore, the clinical implications of p16 mRNA and protein expression in different cancer types and association with *p16* methylation require further investigation.

Established tumor cell lines with *Rb* deletion demonstrated activated *p16* transcription and increased p16 protein expression (56). It has additionally been demonstrated that cell cycle regulation by p16 is lost in tumor cells with inactivated Rb (57), and that the efficacy of exogenously expressed p16 in cancer cells depends on Rb function (58). Furthermore, the overexpression of transcription factor E2F1 promotes *p16* transcription (59), whereas CDK4 overexpression in sarcoma cells is thought to increase p16 expression through a feedback loop (60). This putative feedback regulation in the expression and function of Rb pathway mediators may explain the paradoxical association observed in the present study and in others between high p16 expression and worse patient survival.

In summary, p16 is a CDK4 inhibitor that counteracts the cell cycle process by sustaining the Rb-mediated pathway. Accordingly, p16 has been recognized as a tumor suppressor that is lost or inactivated through gene mutation, deletion or promoter methylation in various cancer types, including CRC. Previous studies demonstrated an association between *p16* gene promoter hypermethylation and worse prognosis in patients with CRC, in addition to an inverse association between p16 expression and tumor progression. However, the effect of p16 mRNA expression on the prognosis of patients with cancer is controversial. In the present study, it was demonstrated that p16 mRNA expression in the tumors was inversely associated with the levels of *p16* promoter methylation. In addition, multivariate analysis determined that high p16 mRNA expression normalized to PCNA mRNA expression (p16/PCNA) was an independent prognostic factor for poor survival of patients with CRC. These results identified a previously unrecognized and paradoxical association between high expression of p16 mRNA and worse prognosis of patients with CRC, although a similar association has been demonstrated in other cancer types.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HK made substantial contributions to the design and conception of the study, and the acquisition, analysis and interpretation of the data, and drafted the manuscript. HT and TM collected the clinical samples and contributed to the interpretation of the data. TM helped to draft and finalize the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was performed in accordance with the Declaration of Helsinki. As the tissues used in the present study were from the patients diagnosed between 1999 and 2002, written informed consent was obtained from the patients prior to the tissue sample collection. In accordance with Japanese ethical guidelines and law, the study protocol was reviewed and approved by the Kanazawa University Human Genome/Gene Analysis Research Ethics Committee (approval no. 181; Kanazawa, Japan). At the start of the study, it was not possible to directly contact the specific patients to explain the present study. According to the Human Genome/Gene Analysis Research Ethics Committee, the present study was announced on our website, providing these patients an opportunity to opt out of the present study. By the date indicated, none of them refused to be included in the present study. All samples were anonymized before analysis was performed to guarantee the protection of privacy.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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