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KRAS mutations and *CDKN2A* promoter methylation show an interactive adverse effect on survival and predict recurrence of rectal cancer

Maija R. J. Kohonen-Corish[1,2,3], Jason Tseung[4], Charles Chan[4], Nicola Currey[1], Owen F. Dent[5], Stephen Clarke[6], Les Bokey[3] and Pierre H. Chapuis[3,5]

[1] Kinghorn Cancer Centre, Garvan Institute of Medical Research, Darlinghurst, Sydney, NSW, Australia
[2] St Vincent's Clinical School, UNSW Medicine, University of NSW, Sydney, NSW, Australia
[3] School of Medicine, University of Western Sydney, Liverpool, NSW, Australia
[4] Department of Anatomical Pathology, Concord Hospital and Concord Clinical School, University of Sydney, Concord, NSW, Australia
[5] Department of Colorectal Surgery, Concord Hospital and Discipline of Surgery, University of Sydney, Concord, NSW, Australia
[6] Department of Medicine, Concord Hospital and Discipline of Medicine, University of Sydney, Concord, NSW, Australia

Colonic and rectal cancers differ in their clinicopathologic features and treatment strategies. Molecular markers such as gene methylation, microsatellite instability and *KRAS* mutations, are becoming increasingly important in guiding treatment decisions in colorectal cancer. However, their association with clinicopathologic variables and utility in the management of rectal cancer is still poorly understood. We analyzed *CDKN2A* gene methylation, CpG island methylator phenotype (CIMP), microsatellite instability and *KRAS*/*BRAF* mutations in a cohort of 381 rectal cancers with extensive clinical follow-up data. *BRAF* mutations (2%), CIMP-high (4%) and microsatellite instability-high (2%) were rare, whereas *KRAS* mutations (39%), *CDKN2A* methylation (20%) and CIMP-low (25%) were more common. Only *CDKN2A* methylation and *KRAS* mutations showed an association with poor overall survival but these did not remain significant when analyzed with other clinicopathologic factors. In contrast, this prognostic effect was strengthened by the joint presence of *CDKN2A* methylation and *KRAS* mutations, which independently predicted recurrence of cancer and was associated with poor overall and cancer-specific survival. This study has identified a subgroup of more aggressive rectal cancers that may arise through the KRAS-p16 pathway. It has been previously shown that an interaction of p16 deficiency and oncogenic *KRAS* promotes carcinogenesis in the mouse and is characterized by loss of oncogene-induced senescence. These findings may provide avenues for the discovery of new treatments in rectal cancer.

Cancer Genetics

Cancers of the colon and rectum have major differences in molecular and clinicopathologic features.[1,2] Molecular markers such as gene promoter methylation,[3] high microsatellite instability (MSI-H)[4,5] and *KRAS* mutations,[6] are becoming increasingly important in guiding treatment decisions in colorectal cancer. However, their association with clinicopathologic variables and utility in the management of rectal cancer is still poorly understood.

High CpG island methylator phenotype (CIMP-H) features widespread gene silencing and is associated with other molecular defects such as *BRAF* mutations and MSI-H.[7] Several marker gene panels and definitions have been applied for its detection and quantification in tumor specimens, including whole genome approaches.[8] *CDKN2A* is one of the original CIMP marker panel genes that is functionally important in carcinogenesis.[9] It codes for the p16 protein, which is a key negative regulator of the cell cycle. However, there are few studies that have addressed CIMP and *CDKN2A* methylation specifically in rectal cancers. It is emerging that in addition to CIMP-H, an intermediate category of CIMP-low (CIMP-L) may exist, which displays methylation of fewer genes and may be associated with a different set of molecular defects. Although different methodology and marker genes have been used in different studies, the intermediate CIMP phenotype is associated with *KRAS* mutations in colon or colorectal cancer cohorts.[10,11] However, this category of CIMP has not been evaluated specifically in rectal cancer cohorts.

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Correspondence to: Maija Kohonen-Corish, Kinghorn Cancer Centre, Garvan Institute of Medical Research, 370 Victoria St, Darlinghurst, Sydney, NSW 2010, Australia, Tel.: +61293555819, Fax: +61-293555872, E-mail: m.corish@garvan.org.au

What's new?
Though the two are often considered together, rectal cancer and colon cancer behave differently and require different treatments. Fewer prognostic markers are available for rectal cancer, and indicators such as gene methylation, microsatellite instability, and *KRAS* mutations, which help inform treatment decisions, may be considered less often in rectal than colon cancer. The authors investigated these indicators in rectal cancers, and found that a combination of *CDKN2A* gene methylation and *KRAS* mutation may augur a poor outcome, thereby identifying a subgroup of more aggressive rectal cancers that may develop through the KRAS-p16 pathway.

Recently described experimental mouse models of colorectal cancer have highlighted the importance of a functional interaction of p16 deficiency with *KRAS* or *BRAF* mutations in colorectal carcinogenesis. Transgenic mice with either *Kras* or *Braf* oncogenic mutations develop premalignant changes in colon epithelial cells, but in the absence of other gene defects, carcinogenesis does not progress due to the tumor suppressor function of p16. However, if a p16 knockout defect is also introduced, these mice rapidly develop serrated colorectal cancers because oncogene-induced senescence is no longer maintained.[12,13] This may be important in serrated cancers in the proximal colon, characterized by *BRAF* mutations and CIMP-H. *KRAS* and *BRAF* mutations are mutually exclusive but a subset of *KRAS* mutations are found with *CDKN2A* methylation.[14] As *BRAF* mutations are rare in rectal cancer, we postulated that the co-occurrence of *KRAS* mutations and *CDKN2A* methylation might be more important in rectal cancer. Therefore, here we have conducted a detailed analysis of CIMP-H, CIMP-L, *CDKN2A* methylation, *BRAF* and *KRAS* mutations and provide the first clinical evidence for an interaction between methylated *CDKN2A* and mutated *KRAS* in rectal cancer.

Material and Methods

Patients

Clinical data from the patients were collected prospectively. The cohort included 381 rectal cancers, which comprised a subset of the 1,808 colorectal cancers resected at Concord Repatriation General Hospital between January 1988 and December 2001. Patients with colon cancer (1,024), previous colorectal cancer (19), inflammatory bowel disease, polyposis coli or a first degree relative with colorectal cancer (74), stage A or D tumor (290) and those for whom there was insufficient tissue for molecular analysis were all excluded. The clinical and pathological characteristics of these patients are shown in Table 1. The study was conducted after Human Experimentation Review by the Concord Hospital Human Research Ethics Committee.

Clinicopathologic analysis

Resected cancer specimens were analyzed as previously described.[15,16] Blocks were taken to demonstrate maximum direct tumor penetration of the bowel wall. Additional blocks were taken specifically to demonstrate the relationship between tumor and any adherent structure or tissue as well as lines of resection and the free serosal surface. Venous invasion by tumor referred to involvement of thick or thin walled veins, either within or beyond the bowel wall. An apical node was defined as the most proximal of any nodes found within 1 cm of the ligation of a named vessel at the apex of a vascular pedicle. Tumor grade was assessed taking into account the degree of differentiation and anaplasia, the nature of the tumor margin (pushing or infiltrating) and the presence and prominence of vascular invasion. All pathological characteristics were assessed in every specimen. Tumors were staged according to the Australian Clinicopathologic Staging System, which accommodates sub-stages compatible with other clinicopathologic staging systems such as TNM[17] but, importantly, differs in that all lesions with macroscopic or microscopic tumor in any resection margin are coded as stage D and included in analyses as such. These patients, like pTNM stage D patients (who have metastatic residual disease), experience markedly diminished survival.

Follow-up and survival

Details of the follow-up protocol have been described previously.[2] The outcome variables were overall survival, rectal cancer-specific survival and time to any recurrence. Recurrence was defined as clinically or radiologically suspected or biopsy proven tumor in the pelvis or perineal scar or newly diagnosed distant metastasis. Overall survival time was measured from the date of resection to the date of death due to any cause with times censored for patients who were lost to follow-up or who remained alive at the close of study in June 2012. Cancer-specific survival was measured from resection until the date of death due to rectal cancer, the censoring date being the date of last contact for those lost to follow-up or the date of last follow-up for those surviving. The survival times of patients who died of causes other than rectal cancer were measured until the date of death and these patients were classified as being at a competing risk in regression analyses. Time to recurrence was measured until the date of diagnosis of recurrence except for two patients who died of rectal cancer but whose precise recurrence date was not known, in which cases the date of death was substituted. Patients who died without recurrence were classified as being at competing risk in regression analyses.

In the total cohort of 381 patients, 10 (3%) had died before discharge from hospital after their resection and 240 died subsequently with survival times ranging up to 14.4

Table 1. Clinical and pathological features in the cohort of 381 rectal cancer patients

Variable	Category	Number (%) or median (range)
Sex	Male	245 (64)
	Female	136 (36)
Age (years)		68 (29–94)
Tumor distance from anal verge (cm)		10 (2–19)
Type of resection	Anterior resection	275 (72)
	Abdominoperineal excision	66 (17)
	Hartmann's operation	26 (7)
	Other	14 (4)
Tumor max surface dimension (cm)		4.8 (1–19)
Distal clearance margin (cm)		4 (0–17)
Histological type of tumor	Adenocarcinoma	359 (94)
	Mucinous adenocarcinoma	19 (5)
	Signet ring adenocarcinoma	3 (1)
Direct tumor spread	Submucosa (T1)	6 (2)
	Muscularis propria (T2)	29 (8)
	Beyond muscularis propria (T3/T4)	346 (91)
Number of nodes involved	None (N0)	177 (47)
	1–3 nodes (N1)	127 (33)
	>3 nodes (N2)	77 (20)
Tumor stage	Stage B	177 (46)
	Stage C	204 (54)
Tumor grade	Low	20 (5)
	Average	257 (68)
	High	104 (27)
Venous invasion	None	276 (72)
	Mural	14 (4)
	Extramural	70 (18)
	Both	21 (6)
Free serosal surface involved	No	358 (94)
	Yes	23 (6)
Adjacent organ or structure infiltrated	No	372 (98)
	Yes	9 (2)
Preoperative radiotherapy with or without chemotherapy	No	363 (95)
	Yes	18 (5)
Postoperative radiotherapy	No	359 (94)
	Yes	22 (6)
Postoperative chemotherapy	No	320 (84)
	Yes	61 (16)

years (median 3.6 years). In patients remaining alive at the close of the study, survival time ranged from 7.0 years to 19.6 years (median 11.8). Nine patients had been lost to follow-up after a median survival of 4.3 years (range 3.2 months–14.8 years).

Assessment of molecular variables

Cancer specific molecular markers were assessed from formalin fixed paraffin-embedded tissue blocks. Hematoxylin and eosin sections were reviewed by a specialist Histopathologist (JT, CC), who selected representative areas of cancer tissue

for analysis from each patient. Tissue was harvested using an Advanced Tissue Arrayer ATA-100 (Chemicon, Temecula, CA) and DNA was extracted from the tissue cores using the Puregene DNA Isolation Kit (Gentra, Minneapolis, MN). MSI-H was assessed as previously described.[18] *KRAS* and *BRAF* mutations were detected using the SNaPshot Multiplex kit (Applied Biosystems, Foster City, CA) and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).[19] DNA was bisulfite treated using the EpiTect Bisulfite Kit (Qiagen, Valencia, CA) for the assessment of methylated markers. CIMP was determined using the MethyLight protocol and a panel of five markers *CACNA1G*, *IGF2*, *NEURO1G*, *RUNX3* and *SOCS1*.[20–22] CIMP-H was defined when at least three of these markers were methylated and CIMP-L was defined when 1–2 markers were methylated. *CDKN2A* methylation was assessed as previously described.[18,23]

Statistical analysis

The chi-squared test or Fisher's exact test were used to examine the statistical significance of differences in proportions. Comparisons of survival time between strata of covariates were made with the Kaplan-Meier method and log-rank test. As clinicopathologic stage is by far the strongest known predictor of prognosis, associations with survival were examined for stages B and C separately as well as for the two stages combined. Cox regression or competing risk Cox regression and the Lund test were used in multivariable modeling. The assumption of proportional hazards was assessed by examining plots of log cumulative hazard for parallelism and in no case was it materially violated in any variable included in a regression model. In modeling, all variables having an association with recurrence or survival with a p value <0.1 were entered into an initial model which was then reduced by successive elimination of variables with a p value >0.05. The provisional final model thus obtained was further examined by entering separately every excluded variable until a final model containing only variables with a p value <0.05 was obtained. Potential interactions were examined by introducing product terms but only one was found to be statistically significant. The level for two-tailed statistical significance was $p \leq 0.05$ with confidence intervals (CI) at the 95% level. Where multiple testing was involved the level for significance was set more conservatively at ≤ 0.01. Analyses were performed with Stata release 12 (Stata Corporation, College Station, TX, 2011).

Results

Analysis of molecular features

CIMP-H was present in only 4% of patients whereas CIMP-L was more common (25%; Table 2). *CDKN2A* methylation was found in 20% and *KRAS* mutations in 39% of patients. Both *BRAF* mutations (2%) and MSI-H (2%) were rare. There was no statistically significant difference between stage B and stage C patients for any of these markers (Table 2). CIMP-H was associated with the presence of *KRAS* mutation ($p = 0.006$), *BRAF* mutation ($p = 0.012$) and *CDKN2A* methylation ($p = 0.010$; Supporting Information Table S1). In addition, *CDKN2A* methylation was more likely to be present when *BRAF* mutation was present ($p = 0.001$) but there was no significant association between *CDKN2A* methylation and *KRAS* mutation (Supporting Information Table S1). At the conservative criterion of $p \leq 0.01$ (to protect against Type I errors) there were no significant associations between the molecular characteristics and any of the 18 clinicopathologic variables examined (Supporting Information Table S2).

Association of molecular features with survival

The presence of *CDKN2A* methylation was associated with poorer overall survival in stages B and C combined [hazard ratio (HR) = 1.5, $p = 0.008$]. However stratification by stage showed that this was true only in stage C (HR = 1.5, $p = 0.029$); there was no significant association in stage B (Supporting Information Table S3). There was no significant association between CIMP-L or CIMP-H and overall survival (Supporting Information Table S3). *KRAS* mutation predicted poorer survival in stages B and C combined (HR = 1.3, $p = 0.034$) but although a tendency towards this association persisted in stages B and C separately it was not statistically significant in either (Supporting Information Table S3).

Association of clinicopathologic variables with survival

Patients aged ≥ 75 years had poorer overall survival than younger patients (HR = 1.6, $p < 0.001$) and those who had a Hartmann's operation also had poorer survival (HR = 2.4,

Table 2. Frequency of molecular features in the cohort of 381 rectal cancers

	No data	Present	Percent (95% CI)	Present in stage B number (%)	Present in stage C number (%)	Stage B/C $\chi^2 p$ or Fisher's exact p
CIMP-H	82	11/299	3.7 (1.9–6.5)	8/138 (6)	3/161 (2)	0.072
CIMP-L	82	75/299	25.1 (20.3–30.4)	38/138 (28)	37/161 (23)	0.365
CDKN2A methylation	7	73/374	19.5 (15.6–23.9)	29/175 (17)	44/199 (22)	0.177
BRAF mutation	1	6/380	1.6 (0.6–3.4)	3/177 (2)	3/203 (2)	1.000
KRAS mutation	17	143/364	39.3 (34.2–44.5)	68/170 (40)	75/194 (39)	0.794
MSI-high	0	9/381	1.5 (1.1–4.4)	6/177 (3)	3/204 (2)	0.313

Table 3. Multivariable analysis of association between outcome variables and *CDKN2A* methylation in stage C patients with adjustment for clinicopathologic variables

			Death due to any cause				Death due to rectal cancer[1]		Recurrence[2]	
	Patients	Deaths	Bivariate HR (CI)	Bivariate Wald *P*	Multivariable HR (CI)	Multivariable Wald *P*	Multivariable HR (CI)	Multivariable Wald *P*	Multivariable HR (CI)	Multivariable Wald *P*
CDKN2A methylation										
Present	44	36	1.5 (1.05–2.2)	0.029	1.5 (0.99–2.1)	0.055	–	–	–	–
Absent	155	106								
Age $\geq$ 75 yr	52	42	1.5 (1.01–2.1)	0.042	–	–	–	–	–	–
No	147	100								
Hartmann's operation	11	10	2.9 (1.5–5.6)	0.001	2.6 (1.5–5.6)	0.002	–	–	–	–
Other	188	132								
Apical node involved	11	9	2.4 (1.2–4.8)	0.011	2.8 (1.3–5.7)	0.006	–	–	–	–
No	188	133								
$\geq$ 4 nodes involved	77	60	1.5 (1.1–2.1)	0.021	–	–	1.9 (1.3–2.9)	0.002	1.7 (1.1–2.6)	0.014
No	122	82								
High grade	79	58	1.4 (1.02–2.0)	0.036	–	–	1.9 (1.3–2.9)	0.002	1.7 (1.2–2.6)	0.008
No	120	84								
Venous invasion	71	61	2.0 (1.4–2.8)	<0.001	1.9 (1.3–2.6)	<0.001	–	–	1.5 (1.01–2.3)	0.042
No	128	81								
Postoperative chemotherapy	54	29	0.6 (0.4–0.9)	0.009	0.5 (0.3–0.8)	0.004	–	–	–	–
No	145	113								

Features independently associated with death due to any cause were Hartmann's operation, involvement of an apical node, venous invasion and postoperative chemotherapy (negatively). Only involvement of $\geq$ 4 nodes and high grade were independently associated with CRC-specific death, while these two features plus venous invasion were associated with recurrence at any site.
[1]Of 196 patients for whom cause of death of deceased patients was known, 89 died of rectal cancer, 50 died of other causes and 57 were censored.
[2]Of 197 patients for whom recurrence status was known, 98 had a recurrence, 45 died of causes other than rectal cancer and 54 were censored.

Table 4. Multivariable analysis of association between outcome variables and *KRAS* mutation in stage C patients with adjustment for clinicopathologic variables

			Death due to any cause				Death due to rectal cancer[1]		Recurrence[2]	
	Patients	Deaths	Bivariate HR (CI)	Bivariate Wald *P*	Multivariable HR (CI)	Multivariable Wald *P*	Multivariable HR (CI)	Multivariable Wald *P*	Multivariable HR (CI)	Multivariable Wald *P*
KRAS mutation										
Present	75	59	1.4 (0.99–2.0)	0.054	1.3 (0.9–1.9)	0.100	1.7 (1.1–2.5)	0.018	1.5 (1.03–2.3)	0.032
Absent	119	79								
Age $\geq$ 75 years	50	40	1.4 (0.96–2.0)	0.085	–	–	–	–	–	–
No	144	98								
Hartmann's operation	12	11	3.0 (1.6–5.6)	<0.001	2.9 (1.5–5.5)	0.001	–	–	–	–
Other	182	127								
Apical node involved	11	9	2.5 (1.3–4.9)	0.009	2.8 (1.4–5.9)	0.005	–	–	–	–
No	183	129								
$\geq$ 4 nodes involved	75	58	1.4 (1.02–2.0)	0.034	–	–	1.8 (1.2–2.7)	0.009	1.7 (1.1–2.6)	0.011
No	119	80								
High grade	75	55	1.4 (1.01–2.0)	0.044	–	–	2.2 (1.4–3.3)	<0.001	2.0 (1.4–3.1)	0.001
No	119	83								
Venous invasion	68	58	1.9 (1.4–2.7)	<0.001	1.9 (1.3–2.6)	<0.001	–	–	–	–
No	126	80								
Postoperative chemotherapy	51	27	0.6 (0.4–0.9)	0.012	0.6 (0.4–0.9)	0.011	–	–	–	–
No	143	111								

KRAS mutation was independently associated with CRC-specific death, along with involvement of 4 or more nodes and high grade. These three features were also associated with recurrence at any site.

[1]Of 191 patients for whom cause of death of deceased patients was known, 86 died of rectal cancer, 49 died of other causes and 56 were censored.

[2]Of 192 patients for whom recurrence status was known, 95 had a recurrence, 44 died of causes other than rectal cancer and 53 were censored.

Table 5. Multivariable analysis of association between outcome variables and the joint presence of *CDKN2A* methylation plus *KRAS* mutation in stage C patients with adjustment for clinicopathologic variables

			Death due to any cause				Death due to rectal cancer[1]		Recurrence[2]	
	Patients	Deaths	Bivariate HR (CI)	Bivariate Wald *P*	Multivariable HR (CI)	Multivariable Wald *P*	Multivariable HR (CI)	Multivariable Wald *P*	Multivariable HR (C)	Multivariable Wald *P*
CDKN2A methylation and *KRAS* mutation										
Present	17	16	2.5 (1.5–4.2)	<0.001	2.6 (1.5–4.4)	<0.001	2.0 (1.2–3.5)	0.014	1.9 (1.2–3.1)	0.009
Absent	175	120								
Age ≥ 75 years	49	39	1.4 (0.95–2.0)	0.091	–	–	–	–	–	–
No	143	97								
Hartmann's operation	11	10	3.0 (1.6–5.8)	<0.001	3.2 (1.7–6.3)	<0.001	–	–	–	–
Other	181	126								
Apical node involved	11	9	2.5 (1.3–5.0)	0.008	2.5 (1.2–5.3)	0.013	–	–		–
No	181	127								
≥ 4 Nodes involved	75	58	1.5 (1.05–2.1)	0.026	–	–	1.7 (1.1–2.7)	0.014	1.7 (1.1–2.5)	0.015
No	117	78								
High grade	75	55	1.4 (1.03–2.0)	0.035	–	–	2.1 (1.4–3.3)	0.011	2.0 (1.3–3.1)	0.001
No	117	81								
Venous invasion	68	58	2.0 (1.4–2.8)	<0.001	1.8 (1.3–2.6)	<0.001	–	–	–	–
No	124	78								
Postoperative chemotherapy	51	27	0.6 (0.4–0.9)	0.015	0.5 (0.3–0.8)	0.004	–	–	–	–
No	141	109								

Features independently associated with the risk of death from any cause were the joint presence of *CDKN2A* methylation and *KRAS* mutation, Hartmann's operation, apical node involvement, venous invasion and adjuvant chemotherapy (negatively). However only joint *CDKN2A* methylation/*KRAS* mutation, involvement of 4 or more nodes and high grade were independently associated with CRC-specific death and tumor recurrence.

[1]Of 189 patients for whom cause of death of deceased patients was known, 85 died of rectal cancer, 48 died of other causes and 56 were censored.

[2]Of 190 patients for whom recurrence status was known, 94 had a recurrence, 43 died of causes other than rectal cancer and 53 were censored.

$p < 0.001$; Supporting Information Table S3). These differences for stages B and C combined also persisted significantly in the two stages separately. Apical node involvement and ≥ 4 involved nodes were associated with poorer survival in stage C patients where the respective HRs were 2.3 ($p = 0.014$) and 1.4 ($p = 0.043$). Overall, high grade predicted poorer survival (HR = 1.7, $p < 0.001$). Venous invasion was significantly associated with poorer survival in stages B and C combined but this was due only to stage C (HR = 2.0, $p = 0.001$). Postoperative chemotherapy was associated with longer survival only in stage C patients (HR = 0.6, $p = 0.008$).

Multivariable analysis of *CDKN2A* methylation, *KRAS* mutations and survival

Although there was a bivariate association between *CDKN2A* methylation and overall survival this became marginally nonsignificant ($p = 0.055$) after adjustment for clinicopathologic variables associated with survival (Table 3). An equivalent analysis of *KRAS* mutation [because of its near significant ($p = 0.054$) bivariate association with survival] also showed no significant association after adjustment for other variables (Table 4). *CDKN2A* methylation was not associated with either recurrence or death due to rectal cancer or after adjustment (Table 3) but *KRAS* mutation was significantly associated with both of these after adjustment (Table 4).

As *CDKN2A* methylation and *KRAS* mutation had near-significant associations with survival in patients with stage C tumor and because there is a known biological interaction[12] we analyzed the association between survival and the combination of these two features. When *CDKN2A* methylation and *KRAS* mutation were both present, survival was significantly poorer compared to the other three subgroups (all p values <0.04). There was no significant survival difference among these three subgroups (all p values >0.2, Fig. 1). Following from this, survival was significantly poorer in patients with both *CDKN2A* methylation and *KRAS* mutation present than in all other patients combined (HR = 2.5, CI = 1.5 − 4.2, Wald $p < 0.001$). No such association was found in patients with stage B tumor. Multivariable analysis for stage C tumor showed that the association persisted after adjustment for clinicopathologic features ($p < 0.001$) and that the joint presence of both *CDKN2A* methylation and *KRAS* mutation was significantly associated with recurrence and death due to rectal cancer (Table 5).

Discussion

There have been conflicting reports regarding the prognostic significance of *CDKN2A* methylation in colorectal cancer. *CDKN2A* methylation had no prognostic value in a large cohort of 902 colorectal cancer patients,[24] but was associated with poorer disease specific survival in a smaller cohort of rectal cancers.[25] Here, we have shown that the presence of both *CDKN2A* methylation and *KRAS* mutations had an independent adverse effect on overall survival, recurrence and cancer-specific death in rectal cancer. A previous study found that patients with alternate or simultaneous alteration of the *KRAS* and *CDKN2A* genes had a poorer outcome in a colon and rectal cancer cohort.[14] However, here we found that the joint occurrence of these alterations was a stronger independent prognostic factor than either alteration considered separately. Both gene alterations were found in 9% of rectal cancers. A biological interaction between these two alterations has been experimentally demonstrated in a mouse model of colorectal cancer.[12] The mouse strain expressing a colon-specific activated KRAS-G12D mutation develops hyperplastic crypts throughout the entire colon, but these do not progress to malignancy. This is due to oncogene-induced senescence, which is maintained by increased expression of p16 protein and inhibits cell proliferation. When the KRAS-G12D mice were crossed with p16 knockout mice, 50% of the mice developed colon tumors within 12 weeks.[12] Thus, the combination of *KRAS* mutation with p16 deficiency may cause more aggressive tumor development compared with when only one of these alterations is present.

This study also found that some of the established markers of colon/colorectal cancer, such as widespread gene methylation CIMP-H, microsatellite instability MSI-H and *BRAF* mutations are rare in rectal cancer. A striking difference was also the lack of association between MSI-H and CIMP-H, as defined by the widely accepted standard set of markers, which does not include methylation of the *MLH1* or the *CDKN2A* genes.[22] CIMP-H was still associated with *BRAF* mutations similar to colon/colorectal cancer. However, in contrast to colon or colorectal cancer,[10,11] there was no association between CIMP-L and *KRAS* mutations in this cohort. Therefore, these data reveal not only a different

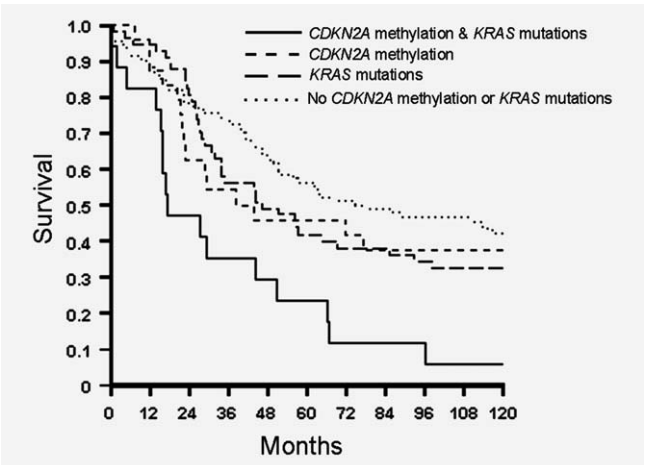

Figure 1. Overall survival by *CDKN2A* methylation and *KRAS* mutation status in Stage C rectal cancer. Overall survival in four patients groups: *CDKN2A* methylation absent and *KRAS* wild type, *CDKN2A* methylation absent and *KRAS* mutated, *CDKN2A* methylation present and *KRAS* wild type, *CDKN2A* methylation present and *KRAS* mutated. Differences among the first three groups were not statistically significant (all p values > 0.2) whereas differences between all of those groups and the fourth group were significant (all p values < 0.04).

profile of molecular markers in rectal cancer but also a lack of marker associations that have been previously well-established in colon/colorectal cancer. This suggests that these associations arose primarily in patients with colon cancer and highlights the heterogeneity of the molecular pathways involved and the need for further studies in pure cohorts of rectal carcinoma.

This study was based on a large consecutive series of patients in a single hospital, with standardized surgical technique and pathology reporting, as well as detailed clinical follow-up. Previous studies on the patients from this hospital established the association of MSI-H with better survival in colorectal cancer[26] and MSI-L with poorer survival in colon cancer.[18] Of the methylation markers, *CDKN2A* methylation was evaluated previously and was not associated with prognosis in colon cancer.[18] Studies on the prognostic and predictive significance of CIMP have been contradictory. CIMP has been associated with poorer survival in colorectal cancer,[27,28] and a better response of patients to 5-FU based chemotherapy.[29] More recently, the presence of CIMP-H in tumors was found to be an independent predictor of low cancer specific mortality in colon cancer[30] and to be associated with a lack of response to 5-FU based adjuvant chemotherapy in colorectal cancer.[3] It is possible that some of these inconsistent results between studies are due to the use of different marker panels for defining CIMP and the inherent differences between cancers in the right and left colon.

In conclusion, this study has provided the first clinical evidence that oncogenic activation of *KRAS* combined with *CDKN2A* promoter methylation identifies a subgroup of more aggressive rectal cancers, which may provide avenues for the discovery of new treatments in rectal cancer.

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