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Colorectal carcinomas with *KRAS* mutation are associated with distinctive morphological and molecular features

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KRAS-mutated carcinomas comprise 35–40% of all colorectal carcinomas but little is known about their characteristics. The aim of this study was to examine the pathological and molecular features of _KRAS_-mutated colorectal carcinomas and to compare them with other carcinoma subgroups. _KRAS_ mutation testing was performed in 776 incident tumors from the Melbourne Collaborative Cohort Study. _O[6]-methylguanine DNA methyltransferase_ (_MGMT_) status was assessed using both immunohistochemistry and MethyLight techniques. Microsatellite instability (MSI) phenotype and _BRAF_ V600E mutation status were derived from earlier studies. Mutation in _KRAS_ codon 12 or codon 13 was present in 28% of colorectal carcinomas. Compared with _KRAS_ wild-type carcinomas, _KRAS_-mutated carcinomas were more frequently observed in contiguity with a residual polyp (38 _vs_ 21%; $P<0.001$), demonstrated mucinous differentiation (46 _vs_ 31%; $P=0.001$) and were associated with different MSI status ($P<0.001$) and with _MGMT_ methylation (47 _vs_ 21%; $P=0.001$). Compared with tumors demonstrating neither _BRAF_ nor _KRAS_ mutation, _KRAS_-mutated carcinomas showed more frequent location in the proximal colon (41 _vs_ 27%; $P=0.001$), mucinous differentiation (46 _vs_ 25%; $P<0.001$), presence of a contiguous polyp (38 _vs_ 22%; $P<0.001$), _MGMT_ methylation (47 _vs_ 26%; $P=0.01$) and loss of MGMT immunohistochemical expression (27 _vs_ 19%; $P=0.02$). _KRAS_-mutated carcinomas were distributed in a bimodal pattern along the proximal–distal axis of the colorectum. Compared with male subjects, female subjects were more likely to have _KRAS_-mutated carcinoma in the transverse colon and descending colon (39 _vs_ 15%; $P=0.02$). No difference in overall survival was observed in patients according to their tumor _KRAS_ mutation status. In summary, _KRAS_-mutated carcinomas frequently develop in contiguity with a residual polyp and show molecular features distinct from other colorectal carcinomas, in particular from tumors with neither _BRAF_ nor _KRAS_ mutation.
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During the past two decades, colorectal carcinoma has evolved from being viewed as a single entity to a heterogeneous group of diseases classified into molecular groups with clinical utility and epidemiological relevance.[1,2] Examples of this are seen in the recognition of Lynch syndrome using specific pathological features,[3] and the roles of lifestyle and

environmental factors in the development of colorectal carcinoma subsets.[4,5] The complex interaction between an individual's genetic background and the environment has led to the concept of tumor uniqueness and molecular pathological epidemiology.[6]

Current colorectal carcinoma classification largely reflects the polyp of origin for each group, various molecular pathways to neoplastic progression and molecular correlates with epidemiological features. Among the most commonly altered molecular pathways in colorectal carcinoma, the *RAS-MAPK* signaling pathway is deregulated in 55% of tumors, comprising mutually exclusive mutations in *BRAF*, *KRAS* and *NRAS*.[7] Colorectal carcinomas with *BRAF* mutation have emerged as a distinct group with a combination of features that sets them apart from the remainder of colorectal carcinoma, including predilection for the proximal colon, female gender, high histological grade, high levels of CpG island methylator phenotype (CIMP-high), an origin in serrated polyps and association with increased body size.[1,8,9] Somatic-activating mutation in the *KR*AS proto-oncogene is observed in 35–40% of colorectal carcinoma.[7,10,11] The overwhelming majority of *KRAS* mutations (90%) occur in codons 12 and 13 of exon 2, with the most frequent alteration being a G>A transition in codon 12.[12] The clinical significance of *KRAS* mutation in colorectal carcinoma patients is controversial; some studies reported no association with survival,[13,14] whereas others suggested that patients with *KRAS*-mutated colorectal carcinoma have poorer outcome for any mutation subtype,[15] mutation in codon 12 only[16,17] or codon 13 only.[11] There is more definitive evidence for a negative predictive value of *KRAS* mutation in patients with advanced stage disease treated with targeted anti-EGFR monoclonal antibody drugs.[18]

In the traditional adenoma–carcinoma pathway of colorectal carcinoma, *KRAS* mutation occurs early in the sequential neoplastic progression, after deregulation of the *WNT* pathway, commonly by *APC* mutation and before *TP53* inactivation.[19] However, *KRAS* mutation is not universally found in tumors harboring *APC* and *TP53* mutations.[20] In addition, some studies have reported that *KRAS*-mutated colorectal carcinomas are associated with other distinctive molecular findings, including inactivation of the DNA repair gene *O*^{6}*-methylguanine DNA methyltransferase* (*MGMT*), and the poorly defined categories of low/indeterminate level of MSI and CIMP.[21,22] Taken together, these observations suggest that some *KRAS*-mutated colorectal carcinomas may develop through a divergent molecular sequence.

Given that new associations have been uncovered when colorectal carcinomas were stratified for the presence of *BRAF* mutation, the possibility exists that *KRAS*-mutated tumors may also show currently unrecognized features and risk factor associations, which set them apart from the balance of colorectal carcinomas. In this report, we provide some important insights into the characteristics and origins of *KRAS*-mutated carcinoma and their gender-related distribution in the large bowel, which may contribute to a better understanding of the molecular epidemiology of colorectal carcinoma.

Patients and methods

Subjects

Participants were enrolled in The Melbourne Collaborative Cohort Study, which is a prospective study of 41 514 people (17 045 male subjects and 24 469 female subjects) recruited between 1990 and 1994, with the aim of examining the role of lifestyle factors in the risk of cancer and heart disease. Participants were between 27 and 75 years of age (almost all were between the ages 40 and 69 years) at baseline.[23] Incident cases of carcinoma of the colon or the rectum were identified by linkage to the population-based cancer registries in Victoria and other Australian states. The study protocol was approved by the Cancer Council Victoria's Human Research Ethics Committee and the Human Research Ethics Committee of the Queensland Institute of Medical Research under protocol P799. Participants gave written consent for participation and for the investigators to obtain their medical records. Clinical data were collected from medical charts, colonoscopy and pathology reports. Location in the colon was designated as proximal colon for tumors located in the cecum, ascending colon and transverse colon, and as distal colon for tumors in the descending colon and sigmoid colon.

Molecular Pathology

All cases underwent standardized histopathology review by a specialist gastrointestinal pathologist (JRJ or CR). When patients received neoadjuvant therapy for a rectal carcinoma, the original biopsy sample was used for analysis. If the biopsy was not available, the case was excluded from the study. Tumors were assessed histologically for the following features: histological type, proportion of tumor with mucinous differentiation, grade, margin (circumscribed or infiltrative), tumor budding (non-quantitative method), the presence of tumor-infiltrating lymphocytes, and the presence and the histological type of a residual polyp adjacent to the carcinoma. Mucinous differentiation in the tumor was defined by the presence of pools of extracellular mucin-containing clusters of carcinomatous cells or individual tumor cells including signet ring cells. When >50% of analyzed tumor demonstrated mucinous differentiation, the tumor was classified as mucinous carcinoma. Histological grading was performed using the latest WHO criteria.[24] For conventional adenocarcinoma ('adenocarcinoma,

NOS'), tumors were classified as low grade if ≥50% gland formation was present and high grade if <50% gland formation was present. For mucinous carcinoma, tumors were graded according to the MSI phenotype: low grade if high level of MSI was present, high grade if no MSI-high was present. Residual polyps with a villous component comprised tubulovillous adenoma, villous adenoma and traditional serrated adenoma.

Somatic mutations in codons 12 and 13 of *KRAS* were screened using real-time PCR with high-resolution melting analysis in the presence of the SYTO9 fluorescent intercalating dye followed by direct Sanger sequencing on cases with differential melting profiles as described previously.[25] *BRAF* V600E mutation analysis was performed using a real-time PCR-based allelic discrimination method.[4] MSI status was determined using a 10-loci panel in tumor DNA and matched normal tissue DNA (BAT25, BAT26, BAT40, MYCL, D5S346, D17S250, ACTC, D18S55, D10S197 and BAT34C4) as described previously.[26] Tumors were classified as microsatellite stable (0% of unstable loci), MSI-low/indeterminate (>0 to <30% of unstable loci) or MSI-high (≥30% of unstable loci). *MGMT* methylation was assessed using techniques described elsewhere.[27]

Immunohistochemical Testing

Immunohistochemistry for MGMT (clone MT3.1, 1:100 dilution; Fisher Scientific, Pittsburgh, PA, USA) was performed on formalin-fixed paraffin-embedded tissue using an automated platform (Dako Autostainer; Dako, Carpinteria, CA, USA). Sections (4μm) were routinely dewaxed and rehydrated, and then subjected to heat-induced epitope retrieval in High pH Target Retrieval solution (Dako) using a pressure cooker, followed by MACH3 mouse HRP detection system (Biocare Medical, Concord, CA, USA). Antigenic sites were developed using DAB+ liquid chromogen, and then the sections were counterstained with hematoxylin before mounting. Histologically normal colonic mucosa served as positive control tissue for MGMT expression. Stained sections were scored by two observers (CR and MDW) blinded to clinical and molecular testing results for cases. MGMT immunohistochemistry was scored as follows: (1) complete loss of expression; (2) reduced expression in tumor cells in comparison with normal epithelial and stromal cells; (3) tumor and non-tumor cells showed equivalent staining intensity; and (4) tumor cells showed increased staining intensity in comparison with non-tumor cells. For the analysis, only tumors showing a score 1 were interpreted as having abnormal loss of MGMT expression.

Statistical Analysis

Statistical analyses were performed with SPSS statistics software version 19.0 (SPSS, Chicago, IL, USA) and Stata version 11.1 (StataCorp LP, College Station, TX, USA). Age is summarized by mean ± s.d. Comparisons for categorical variables were performed using Pearson's χ^2 test or Fisher's exact test where appropriate. Student's *t*-test was used to compare continuous variables between groups. Risk factor associations were initially examined uncorrected for multiple testing. They were then re-examined using a Bonferroni correction to guard against false-positive results.

Kaplan–Meier methods were used to estimate the survival curves by *KRAS* status (wild-type *vs* mutated), with death from all causes as the end point and time measured from the date of surgery until either death or the end of 2008. Survival curves were compared using the log-rank test. Cox proportional hazards multivariable regression models were used to estimate the hazard ratios (HR) associated with *KRAS* (wild-type *vs* mutated), adjusting for: sex, age at diagnosis (<60, 60–70, >70), tumor location (proximal *vs* distal), histological grade (high *vs* low), MSI status (MSI-high *vs* non MSI-high), MGMT expression (normal *vs* absent) and *BRAF* (wild-type *vs* mutated). This Cox model was refitted splitting the *KRAS* mutations into codons 12 and 13. We then excluded patients with a *BRAF* mutation and refitted both these Cox models. Results are presented as estimated HRs with 95% confidence intervals (CIs) and Wald test *P*-values. The proportional hazards assumption was assessed using graphical methods and tests based on Schoenfeld residuals. A two-tailed *P*-value was used for all analyses and values <0.05 were considered to be statistically significant.

Results

By the end of 2009 after excluding 182 subjects with colorectal carcinoma diagnoses pre-baseline, 1098 participants had at least one incident tumor. For subjects with multiple incident tumors, one was randomly selected for analysis. Data were available for 776 subjects (71%). Reasons for subject exclusion from this study were unavailability of tissue sample ($n=228$), insufficient DNA sample ($n=50$) and unsatisfactory results ($n=44$).

KRAS mutation was observed in 28% of colorectal carcinomas (87% in codon 12 and 13% in codon 13; Table 1 and Supplementary Table). The most common variant in codon 12 was the c.35G>A transition (99/190, 52%), followed by the c.35G>T transversion (58/190, 31%). There were four tumors demonstrating mutations in both *KRAS* and *BRAF*; these cases were excluded from the analysis. *KRAS*-mutated carcinomas were compared with non-*KRAS*-mutated carcinomas for age, sex, histological features and molecular characteristics (Table 1). In addition, given that non-*KRAS*-mutated tumors included a distinct subset characterized by *BRAF* mutation, analyses were also performed to compare

Table 1 Summary of clinical, pathological and molecular features for 776 colorectal carcinomas from the Melbourne Collaborative Cohort Study

Feature	*Classes*	*Total*	*KRAS mut CRC*	*KRAS wt CRC*	P-*value (KRAS mut* vs *KRAS wt)*	*BRAF mut CRC*	P *value (KRAS mut* vs *BRAF mut)*	*Null CRC*	P-*value (KRAS mut* vs *null)*
Proportion		776	219 (28)	557 (72)		125 (16)		432 (56)	
Age (years)		68 ± 8	69 ± 8	68 ± 8	0.08	70 ± 7	0.49	68 ± 8	0.02
Sex	Male	405 (52)	126 (58)	279 (50)	0.06	42 (34)	<0.001	237 (55)	0.49
	Female	371 (48)	93 (42)	278 (50)		83 (66)		195 (45)	
Location	Proximal colon	276 (37)	87 (41)	189 (36)	0.33	77 (64)	<0.001	110 (27)	0.001
	Distal colon	197 (26)	51 (24)	146 (27)		21 (18)		124 (30)	
	Rectum	271 (36)	73 (35)	198 (37)		22 (18)		174 (43)	
Histological grade	High	172 (23)	47 (22)	125 (23)	0.75	50 (41)	<0.001	74 (18)	0.21
	Low	581 (77)	166 (78)	415 (77)		72 (59)		340 (82)	
Margin	Circumscribed	505 (72)	157 (77)	348 (70)	0.06	72 (64)	0.01	273 (72)	0.17
	Infiltrating	192 (28)	46 (23)	146 (30)		40 (36)		106 (28)	
Mucinous differentiation	Absent	452 (65)	107 (54)	345 (69)	0.001	53 (48)	0.49	289 (75)	<0.001
	Focal <50%	187 (27)	68 (34)	119 (24)		41 (37)		78 (20)	
	Predominant >50%	57 (8)	23 (12)	34 (7)		17 (15)		17 (5)	
TILs	Present	178 (24)	52 (25)	126 (24)	0.78	47 (39)	0.01	79 (20)	0.14
	Not present	562 (76)	158 (75)	404 (76)		75 (61)		325 (80)	
Tumor budding	Present	240 (36)	83 (42)	157 (34)	0.04	37 (37)	0.41	120 (33)	0.03
	Not present	420 (64)	113 (58)	307 (66)		62 (63)		242 (67)	
Positive nodes	Present	295 (44)	86 (45)	209 (44)	0.72	58 (51)	0.31	148 (41)	0.35
	Not present	373 (56)	104 (55)	269 (56)		55 (49)		212 (59)	
Synchronous CRC	Present	16 (2)	5 (2)	11 (2)	0.78	6 (5)	0.21	5 (1)	0.26
	Not present	718 (98)	199 (98)	519 (98)		112 (95)		402 (99)	
Contiguous polyp	Present	190 (26)	80 (38)	110 (21)	<0.001	22 (18)	<0.001	88 (22)	<0.001
	Not present	546 (74)	128 (62)	418 (79)		97 (82)		317 (78)	
Polyp subtype	TA	20 (11)	1 (1)	19 (17)	0.001[a]	1 (5)	1	17 (19)	0.001[a]
	TVA	125 (65)	63 (79)	62 (56)		8 (36)		54 (62)	
	VA	20 (11)	10 (13)	10 (9)		1 (5)		9 (10)	
	TSA	23 (12)	6 (8)	17 (16)		12 (54)		6 (7)	
	SSA	2 (1)	0 (0)	2 (2)		0 (0)		2 (2)	
MSI	MSI-high	92 (12)	9 (4)	83 (15)	<0.001	52 (42)	<0.001	30 (7)	0.03
	MSI-low/ indeterminate	90 (12)	36 (16)	54 (10)		12 (10)		42 (10)	
	MSS	586 (76)	174 (79)	412 (75)		60 (48)		42 (10)	
MGMT expression	Normal	565 (78)	148 (73)	417 (80)	0.05	91 (76)	0.48	323 (81)	0.02
	Absent	161 (22)	55 (27)	106 (20)		28 (24)		76 (19)	
MGMT methylation	Present	53 (29)	26 (47)	27 (21)	0.001	4 (12)	0.001	23 (26)	0.01
	Absent	128 (71)	29 (53)	99 (79)		29 (88)		67 (74)	

CRC: colorectal carcinoma; mut: mutation; wt: wild type; null: neither *KRAS* nor *BRAF* mutation; TIL: tumor-infiltrating lymphocytes; TA: tubular adenoma; TVA: tubulovillous adenoma; VA: villous adenoma; TSA: traditional serrated adenoma; SSA: sessile serrated adenoma; MSI: Microsatellite instability; MSS: Microsatellite stable.
KRAS-mutated tumors were initially compared with all other tumors. Given that *KRAS* wild-type carcinomas include a distinct subset of tumors characterized by *BRAF* mutation, analyses were also performed to compare *KRAS*-mutated carcinomas with both *BRAF*-mutated carcinomas and the remaining subset of tumors, which demonstrated neither somatic oncogene mutation (null carcinomas).
Results are given in absolute number and (percentage), unless otherwise specified. Age results are given in mean ± s.d.
The *P*-value for significance was adjusted using the Bonferroni correction for multiple hypothesis testing of 17 variables (16 comparisons in Table 1 and one comparison in Table 2) to $P = 0.05/34 = 0.0015$.
[a]*P*-value comparing contiguous polyps with a villous architecture (TVA, VA and TSA) to the other polyp subtypes.

KRAS-mutated tumors with both *BRAF*-mutated tumors and the remaining subset of colorectal carcinomas, which demonstrated neither somatic oncogene mutation (hereafter referred to as 'null carcinomas'). The mean age at presentation for *KRAS*-mutated carcinoma was 69 ± 8 years, which was no significantly different to that for *BRAF*-mutated carcinoma at 70 ± 7 years and null carcinoma at 68 ± 8 years.

Colon Site and Gender Distribution

Gender distribution did not differ significantly between *KRAS*-mutated colorectal carcinomas and null carcinomas. However, women were over-represented in the *BRAF*-mutated carcinoma subset when compared with both *KRAS*-mutated carcinomas and null carcinomas (66% *vs* 42% and 45%, respectively; $P < 0.001$ for *KRAS*- *vs* *BRAF*-mutated

Table 2 Summary of distribution of *KRAS*-mutated carcinomas, *BRAF*-mutated carcinomas and null carcinomas (neither *KRAS* nor *BRAF* mutation) by colonic site for female subjects, male subjects and both genders

Colon site by gender	*KRAS-mutated CRC*	*BRAF-mutated CRC*	*Null CRC*	*Total*	P-*value*[a]
Females					
Cecum	20 (33)	18 (30)	22 (37)	60	
Ascending colon	10 (18)	29 (51)	18 (32)	57	
Transverse colon	**13 (41)**	**9 (28)**	**10 (31)**	**32**	
Descending colon	**6 (35)**	**7 (41)**	**4 (24)**	**17**	
Sigmoid colon	15 (20)	5 (7)	56 (74)	76	
Rectum	27 (25)	11 (10)	70 (65)	108	
Males					
Cecum	23 (46)	7 (14)	20 (40)	50	
Ascending colon	15 (35)	10 (23)	18 (42)	43	
Transverse colon	**5 (16)**	**4 (13)**	**22 (71)**	**31**	
Descending colon	**1 (10)**	**1 (10)**	**8 (80)**	**10**	0.02
Sigmoid colon	29 (32)	8 (9)	55 (60)	92	
Rectum	46 (29)	11 (7)	103 (64)	160	
All patients					
Cecum	43 (39)	25 (23)	42 (38)	110	
Ascending colon	25 (25)	39 (39)	36 (36)	100	
Transverse colon	**18 (29)**	**13 (21)**	**32 (51)**	**63**	
Descending colon	**7 (26)**	**8 (30)**	**12 (44)**	**27**	
Sigmoid colon	44 (26)	13 (8)	111 (66)	168	
Rectum	73 (27)	22 (8)	173 (65)	268	

[a]*P*-value comparing male *vs* female subjects for nadir region *KRAS*-mutated carcinomas.
Results are given in absolute number and (percentage). The nadir region in the transverse colon and descending colon where absolute numbers are in bold.

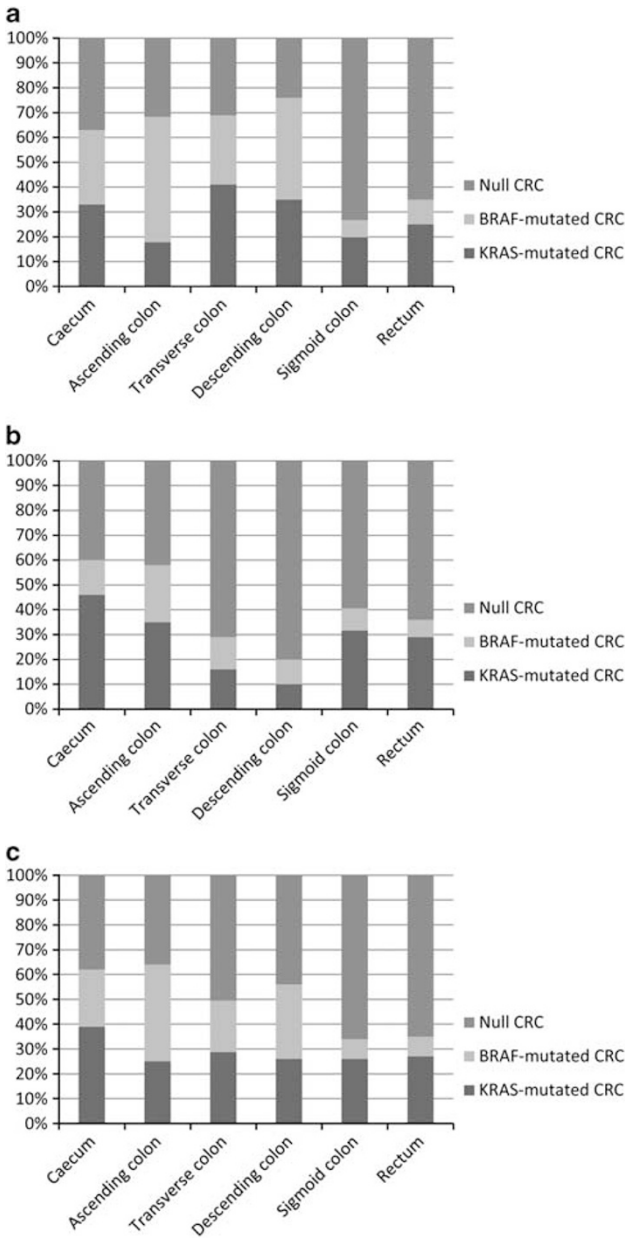

Figure 1 Relative proportions of colorectal carcinomas in different segments of the colorectum in relation to *BRAF* mutation, *KRAS* mutation, non-*KRAS* or *BRAF* mutation (null carcinoma) in female patients (**a**), male patients (**b**) and in all patients (**c**).

tumors) (Table 1). *BRAF*-mutated carcinoma were more likely to be found in the proximal colon than were *KRAS*-mutated or -null carcinomas (64% *vs* 41% and 27%, respectively; $P<0.001$ for *KRAS*- *vs* *BRAF*-mutated carcinoma). In addition, *KRAS*-mutated carcinomas were significantly more proximally located than were the null carcinomas (41 *vs* 27%; $P=0.001$). Distributions with respect to primary sites in the large bowel for all three tumor subgroups (*KRAS*-mutated, *BRAF*-mutated and null carcinomas) are shown in Table 2 and Figure 1, overall and stratified for gender. The absolute number of tumors varied greatly along the proximal–distal axis of the large bowel, following a bimodal distribution with a nadir present in the transverse and descending colon segments. This pattern was also evident for *KRAS*-mutated carcinomas overall and in male subjects. In female subjects, although the absolute numbers of tumors also followed this bimodal pattern, there were differences in proportions when compared with male subjects. The proportion of *KRAS*-mutated carcinomas was greater in female subjects in the transverse colon and the descending colon compared with male subjects (19/49 or 39% *KRAS*-mutated carcinomas in female subjects *vs* 6/41 or 15% in male subjects; $P=0.02$). A significantly increased frequency of *KRAS*-mutated carcinoma was seen in the cecum (39 *vs* 26% in the remaining colorectum; $P=0.01$).

Morphological characteristics

Pathology features are summarized in detail in Table 1. Both *KRAS*- and *BRAF*-mutated carcinomas more frequently demonstrated focal or predominant mucinous differentiation when compared to null carcinomas (46% and 52% respectively *vs* 25%; $P<0.001$ for *KRAS*-mutated carcinomas *vs* null carcinomas). The presence of a contiguous residual polyp was more frequent in *KRAS*-mutated tumors than in other tumor subsets (38% in *KRAS*-mutated carcinomas *vs* 18% in *BRAF*-mutated carcinomas and 22% of null carcinomas; $P<0.001$ for both comparisons). However, the association between *KRAS* mutation and the presence of a residual contiguous polyp was only significant in male subjects. A contiguous polyp was present in 44% *KRAS*-mutated tumors compared with 18% *KRAS* wild-type tumors in male subjects ($P<0.001$), whereas it was found in 31% *KRAS*-mutated tumors and 24% *KRAS* wild-type tumors in female subjects ($P=0.21$). The distribution of residual polyp histological types was different among tumor subsets (Table 1). Contiguous polyps demonstrated a villous component in 99% of *KRAS*-mutated carcinomas compared with 81% of all other carcinomas ($P=0.001$), and 78% of null carcinomas ($P=0.001$). The histological subtype of contiguous polyp in *KRAS*-mutated tumors was tubulovillous adenoma in 79%, villous adenoma in 13%, traditional serrated adenoma in 8%, and tubular adenoma in 1%. *BRAF*-mutated tumors demonstrated a villous component in all but one of the observed contiguous polyps. No significant difference was found between codon 12 *KRAS*-mutated carcinoma and codon 13 *KRAS*-mutated carcinoma for any pathological features ($P>0.05$ in all instances; Supplementary Table).

Molecular Pathology Findings

Molecular pathway-associated findings are listed in Table 1. The partition of colorectal carcinoma into three MSI subclasses resulted in significant differences between *KRAS*-mutated carcinoma and *KRAS* wild-type carcinoma ($P<0.001$), with 16% of *KRAS*-mutated tumors showing MSI-low/indeterminate phenotype compared with 10% in *KRAS* wild-type tumors. There was an increased frequency of *MGMT* methylation in *KRAS*-mutated carcinomas compared with null carcinomas and *BRAF*-mutated carcinomas (47% *vs* 26% and 12%; $P=0.01$ and 0.001, respectively). Loss of immunohistochemical expression of MGMT was present in 22% of all tumors and was also more common in *KRAS*-mutated carcinomas compared with *KRAS* wild-type carcinomas (27 *vs* 20%; $P=0.05$). Of colorectal carcinomas with normal *MGMT* methylation status, 81% demonstrated no loss of immunohistochemical expression of MGMT. This level contrasts significantly with *MGMT* methylation-positive tumors where 43% showed no loss of expression ($P<0.001$). No significant difference was found between codon 12 *KRAS*-mutated carcinoma and codon 13 *KRAS*-mutated carcinoma for any molecular features ($P>0.05$ in all instances; Supplementary Table).

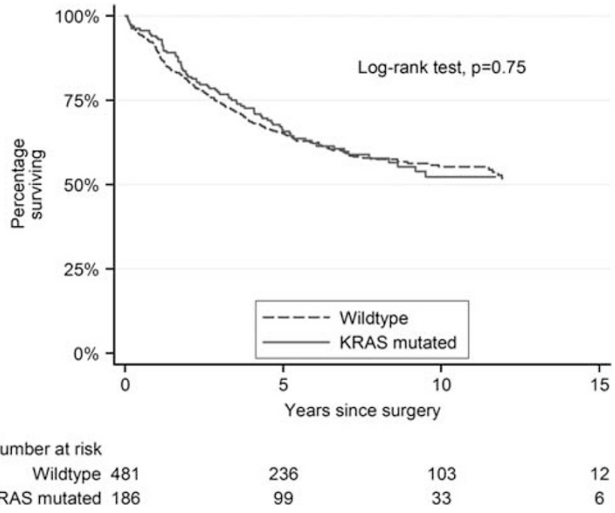

Figure 2 Kaplan–Meier curves comparing overall survival of 667 patients with colorectal carcinoma patients according to *KRAS* status (wild-type and mutated).

Statistical Adjustment for Multiple Testing

The *P*-value for significance was adjusted for multiple hypothesis testing using the Bonferroni correction for comparisons of 17 variables (16 comparisons between *KRAS* mutation and other variables in Table 1 and one additional comparison in Table 2) to $P=0.05/34=0.0015$. The level of significance was considered as borderline if the *P*-value lay between 0.05 and 0.0015 after this correction was applied. The associations that remained significant after the correction was applied between *KRAS*-mutated tumors and one of more other tumor subtypes include mucinous differentiation, presence of a contiguous polyp, different MSI status and *MGMT* methylation (Table 1).

Survival Analyses

Among the 667 (86%) of the 776 patients with data available on all relevant variables, there were 272 deaths during a median follow-up of 7.84 years (range 4.8 months–16.7 years) for censored patients. Figure 2 shows the estimated Kaplan–Meier survival curves by *KRAS* mutation status. There is no evidence of a difference in survival between these groups ($P=0.75$). We found no evidence of a violation of the proportional hazards assumption. In our multivariable Cox regression model, *BRAF* mutation was strongly associated with poorer survival, HR = 1.46 (95% CI: 1.03, 2.06), $P=0.03$.

Conversely, *KRAS*-mutated tumor patients demonstrated no statistically significant difference in overall survival, HR = 0.99 (95% CI: 0.75, 1.32), $P = 0.97$. Further grouping *KRAS* status by codon mutation subtype showed no evidence of an association between *KRAS* status and survival. Excluding patients with a *BRAF* mutation did not affect these conclusions.

Discussion

In this report, we examined the pathological and molecular features of *KRAS*-mutated colorectal carcinoma in a population-based cohort of 776 patients. The observed features were compared with those of *BRAF*-mutated carcinomas and with tumors demonstrating neither *BRAF* nor *KRAS* mutation (null carcinomas). Our exon 2 *KRAS* mutation rate of 28% is in agreement with previous studies when mutations in exon 2 only are considered.[10,11,16,17] Like *BRAF*-mutated carcinomas, *KRAS*-mutated carcinomas showed an increased frequency of mucinous differentiation. This finding has been previously observed by Lin *et al*[28] and more recently by Pai *et al*,[8] who reported that 32% of proximal *KRAS*-mutated carcinoma showed mucinous differentiation compared with 25% of null carcinomas. Maltzman *et al*[29] previously reported that patients with *KRAS* mutated adenomas were significantly older than adenoma patients without a *KRAS* mutation. In our study, we did not find significant age difference between patients with a *KRAS*-mutated carcinoma and other carcinoma patients.

One of the novel findings of our study is the bimodal distribution of colorectal carcinoma frequency along the proximal–distal axis of the large bowel. Consistent with a recent report, we found the highest incidence of site-specific *KRAS*-mutated tumors in the cecum with the frequency of *KRAS*-mutated carcinoma significantly higher in the cecum (39%) than in the rest of the colorectum (26%), although not as high as the figure reported by Yamauchi *et al*[30] (52%). We also found variations in proportions of *KRAS*-mutated carcinoma in different colonic segments between male and female subjects, which did not constitute a continuum. Although we cannot exclude that the observed results are chance findings due to low case numbers, this result raises interesting hypotheses. Epidemiological studies also reported different lifestyle risk factors in male and female subjects with a *KRAS*-mutated colorectal carcinoma.[31] Possible explanations for both distribution variation along the colon and molecular subset variation between genders are likely to be multifactorial and complex. It may include the effect of lifestyle risk factors and host interaction with biochemical and bacterial luminal microenvironments.

It has long been observed that luminal environmental conditions alter from the distal ileum to the rectum, and that these conditions are associated with changes in the physical chemistry of the lumen contents including pH, proportions of short- and branched-chain fatty acids and the products of protein fermentation,[32] as well as those of the microbiota. Colorectal bacterial populations are responsible for important aspects of both health (synthesis of micronutrients such as Vitamin K, protection of the intestine from pathogenic species) and disease (inflammatory reactions and production of carcinogenic metabolites). In relatively recent publications, authors have described associations between species of bacteria, such as *Streptococcus bovis* subtypes,[33] and *Fusobacterium* spp.[34] and colorectal neoplasia. Importantly, the composition of the gut microbiota also varies with gender,[34] and this may be reflected in the distinct distributions of molecular colorectal carcinoma subtypes seen in male and female subjects. A possible explanation for the bimodal distribution of colorectal carcinoma, we observed is that the contact of the large bowel mucosa with luminal contents may last longer in both extremities of the colon than in the mid-colon. Contractions originating in the transverse colon frequently move towards the cecum as well as distally, allowing the proximal colon to act as a braking mechanism on the forward movement of waste products entering from the small intestine, thus ensuring adequate absorption of salts and water.[35] This mechanism might produce areas of stasis at the extremities of the large bowel and may be related to the bimodal distribution of tumors seen in this cohort.

The frequent presence of a contiguous polyp adjacent to *KRAS*-mutated carcinoma has not been reported previously. Differences in tissue specimen handling from multiple sources may partially affect this result. The large number of cases analyzed and the robust statistical association after multiple comparison adjustment suggest a significant finding. However, the biological relevance of this association is unclear, in particular the difference observed between males and female patients. Separate studies need to be performed to verify our results. Possible explanations are that *KRAS*-mutated colorectal carcinoma may require contiguity with their precursor polyps to progress and/or that the malignant component may develop slowly and not rapidly overgrow its precursor lesion. Interestingly, increases in frequency of residual polyp adjacent to carcinoma have also been reported for biallelic and compound heterozygote *MUTYH* mutation carriers.[36] An important feature of the tumors in those particular patients is the high rate of *KRAS* mutation, with over 60% of tumors harboring the c.35G>A transition.[37]

Only a minority of adenomatous and serrated polyps have the capacity for malignant transformation. Adenomas with a villous component comprise a minority of colonic polyps but have an increased risk of malignant transformation.[38] An association

between *KRAS* mutation and the presence of a villous component in adenomas has previously been reported by multiple authors.[19,29,39] Interestingly, the majority of contiguous polyps in *BRAF*-mutated carcinoma from our study were traditional serrated adenoma and tubulovillous adenoma, while none of these tumors demonstrated features of sessile serrated adenoma.

Initial attempts to understand the development of colorectal carcinoma were based on the traditional adenoma–carcinoma model, and were greatly expanded by the molecular analyses of Vogelstein and co-worker in 1988.[19] This was augmented by the slow recognition of an alternative serrated neoplasia pathway over a decade later, initially from mucin expression studies.[40] These canonical pathways are largely linear in nature, and are likely to serve as the foundation of colorectal carcinoma development in the majority of instances. In 2006, Jass *et al*[41] proposed a 'fusion pathway', which combined elements from the canonical pathways. It was suggested that other mutational alterations, such as the inactivation of *MGMT*, may combine with *KRAS* and *TP53* mutations to produce malignant transformation. *KRAS* mutation, as well as low/indeterminate levels of MSI and CIMP, has been associated with methylation of *MGMT* by several authors.[21,22,42,43] In our study, we found that some *KRAS*-mutated carcinomas were associated with MGMT immunohistochemical loss of expression (borderline significance), *MGMT* methylation, and MSI-low/indeterminate phenotype. Of note, we found poor correlation between MGMT immunohistochemical loss of expression and *MGMT* methylation, as reported by others in colorectal carcinoma and glioblastoma.[44,45] This can be secondary to various causes including methodology issues (type of methylation assay, intratumor heterogeneity) and alternative molecular mechanisms that regulate MGMT expression.

Overall, we did not demonstrate any significant effect of *KRAS* mutation subtypes (all mutations, codon 12 or codon 13) on patient survival, whether or not *BRAF*-mutated carcinomas were included in the analysis. This finding is different from Imamura *et al*,[17] who recently reported an association between poor survival and *KRAS* codon 12 mutation in *BRAF* wild-type colorectal carcinoma patients. With multiple studies showing different effects of *KRAS* mutation on patient survival, it is likely that various confounders may interact and that *KRAS* mutation does not represent on its own an important prognostic factor in patients with colorectal carcinoma.

In summary, we uncovered evidence that *KRAS*-mutated carcinomas demonstrate distinctive features from other colorectal carcinomas, in particular from tumors harboring neither *BRAF* nor *KRAS* mutation. To reduce the possibility of our novel associations being false-positive results, we re-examined the comparisons performed using a Bonferroni correction procedure. Our key associations (mucinous differentiation, presence of a contiguous polyp, different MSI status and *MGMT* methylation) were robust to this correction for multiple hypothesis testing. Although some environmental and lifestyle risk factors, such as physical activity, body mass index, tobacco smoke and meat consumption, have been reported to be associated with *KRAS*-mutated colorectal carcinoma,[31,46–49] potential new associations are unlikely to be identified if, as was the case for *BRAF*-mutated carcinomas until relatively recently, *KRAS*-mutated carcinomas continue to be included with the bulk of common colorectal carcinoma. Subsequent studies from different population are required to validate our findings.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)