Pergamon

European Journal of Cancer Vol. 32A, No. 3, pp. 491–497, 1996

Printed in Great Britain
0959-8049/96 $15.00+0.00

0959-8049(95)00567-6

Original Paper

C-KI-RAS Activation and the Biological Behaviour of Proximal and Distal Colonic Adenocarcinomas

J. Elnatan, H.-S. Goh and D.R. Smith

Colorectal Cancer Research Laboratory, Department of Colorectal Surgery, Singapore General Hospital, Outram Road, Singapore 0316, Republic of Singapore

One hundred and forty colonic adenocarcinomas originating on the left side of the colorectum and 70 colorectal carcinomas originating on right side of the colorectum were examined for activating mutations of codons 12 and 13 of the *C-KI-RAS* proto-oncogene. Rates of mutation were significantly different (right colon 43%, 30/70 versus left colon 23%, 32/140; $P = 0.0025$). Adenocarcinomas from the left side of the colorectum showed a significant association between *C-KI-RAS* activation and tumour progression, including the presence of distant organ metastasis at the time of surgery ($P = 0.0039$), and during patient follow-up ($P = 0.00027$), whereas those from the right of the colorectum did not ($P = 0.4$ and $P = 0.5$, respectively). Mutation of the *C-KI-RAS* proto-oncogene was found to be associated with a significantly poorer patient prognosis on the left of the colorectum ($P = 0.0001$ by log rank analysis of Kaplan–Meier plots) but not on the right ($P = 0.7$). These results demonstrate that, not only is the timing and frequency of *C-KI-RAS* activation different between carcinomas originating on the left or right of the colorectum, but also that the biological consequences of such mutations may differ.

Key words: cancer, colorectal, dissemination, *RAS*, site
Eur J Cancer, Vol. 32A, No. 3, pp. 491–497, 1996

INTRODUCTION

THE *RAS* FAMILY of cellular proto-oncogenes code for three closely related proteins of M_r 21,000 which are 188 or 189 amino acids long, and are thought to control cell growth and differentiation by a process of mitogenic signal transduction [1–3]. The *RAS* gene products have been localised to the cell membrane [4] and have been shown to have intrinsic GTPase activity [3]. The three *RAS* genes (termed *HA-RAS, N-RAS* and *KI-RAS*) are converted to active oncogenes in naturally occurring tumours by point mutations in either codons 12, 13 or 61 [1], although there is a strong preference for mutations in codon 12 [2, 5]. These mutations abolish the GTPase activity and result in constitutive stimulation of autonomous growth and contribute to neoplastic development [1–3].

In humans, the particular member of the *RAS* gene family that is found to be activated is characteristic of a particular tumour type: *C-KI-RAS* in colorectal, pancreatic and biliary carcinomas [5–16]; *HA-RAS* in urinary tract tumours [17, 18] and *N-RAS* in haematopoietic and neurogenic tumours [19–21]. Furthermore, the frequency of *RAS* activation varies remarkably with tumour type, varying from negligible in breast tumours [22], to 65% in colorectal carcinomas [5–13, 16], and up to 95% in pancreatic duct carcinomas [23] although this figure is disputed by some authors [24].

The exact frequency of *C-KI-RAS* mutation in colorectal carcinomas is a matter of some dispute, with figures variously reported as 20–25% [5, 11, 25, 26]; 35–41% [6, 7, 13, 27, 28] or greater than 50% [10, 29], with direct comparisons being difficult due to variations in technique. Generally, *RAS* mutation rate is not greater than 50%, suggesting the presence of an alternate pathway or pathways in colorectal carcinogenesis.

The observation that large villous adenomas have a mutation rate approximately equal to that found in carcinomas, and that smaller tubular adenomas show a lower mutation rate of approximately 15% [9], led to the proposal that *C-KI-RAS* mutation was associated with the transition from small benign adenomas to larger more aggressive villous adenomas [30, 31]. This was generally supported by studies which showed that activation of *C-KI-RAS* did not correlate well with clinical parameters, with no apparent association between *C-KI-RAS* activation and either patient age, sex, tumour stage, site, disease dissemination or depth of invasion

Correspondence to D.R. Smith.
Revised 3 Aug. 1995; accepted 23 Aug. 1995.

[26], and *RAS* gene activation on its own did not seem to confer prognostic significance [26]. However, more recent studies on larger sample cohorts show that *C-KI-RAS* activation is associated with patient age, sex and tumour location [32], and two papers have suggested that it is the specific nature of the mutation that may play a role in determining the type of metastatic dissemination of a particular tumour [27, 33]. However, while Moerkerk and his co-workers [33] have shown that tumours with G to A transitions do not progress beyond Dukes' Stage B, Finkelstein and his co-workers ascribe this transition to account for most cases of distant metastasis [27].

Hence, there is still much disagreement about the effects of *C-KI-RAS* activation in human colorectal carcinomas. In Singapore, colorectal neoplasia originating on the right side of the colorectum are relatively rare, accounting for only approximately 15% of all cases [34]. We therefore undertook to examine *RAS* activation in relation to clinicopathological variables in two cohorts, a larger cohort of 140 carcinomas which are representative of the majority of presenting adenocarcinomas, and a smaller cohort of 70 tumours representative of the rarer cases of adenocarcinomas originating on the right side of the colorectum.

PATIENTS AND METHODS

Patients and tumour samples

All samples consisted of single, primary colorectal adenocarcinomas (patients with synchronous tumours were excluded from this study), which were flash frozen in liquid nitrogen at the time of surgery and stored at −80°C until required. Tumours were classified as Dukes' Stage A to D according to Turnbull's modification of Dukes' original staging [35]. Tumours were defined as right sided if the tumour originated in the caecum, ascending colon, hepatic flexure or transverse colon; and left sided if the tumour originated in the splenic flexure, descending colon, sigmoid colon, rectosigmoid colon or rectum.

DNA extraction and in vitro *amplification*

Genomic DNA was extracted from tumour samples containing greater than 70% neoplastic cells (as assessed histochemically) as described previously [36]. A 111 bp fragment of exon 1 of the *C-KI-RAS* gene was amplified using custom synthesised oligonucleotides (New England Biolabs, Beverly, Massachusetts, U.S.A.) of sequence:

5'-ATGACTGAATATAAACTTGT-3' (exon 1; 5'-primer)
5'-CTCTATTGTTGGATCATATT-3' (exon 1; 3'-primer)

Each reaction contained approximately 500 ng of genomic DNA, primers at a final concentration of 1 μM and 2.5 units Taq Polymerase (Promega, Madison, Wisconsin, U.S.A.) in 50 μl of 50 mM KCl, 10 mM Tris–HCl pH 8.8, 2.5 mM $MgCl_2$, 0.1% Triton X-100 and 0.4 mM dNTPs. Cycle times were 94°C for 1 min (denature), 56°C for 1 min (anneal) and 72°C for 1 min (extend) for 35 cycles.

Mismatch specific oligonucleotide hybridisation

Mismatch specific oligonucleotide hybridisation was performed essentially as described by Verlaan-de Vries and associates [37]. Briefly, amplified polymerase chain reaction (PCR) products (20 μl) were denatured with 100 μl of 0.4 M NaOH, 25 mM EDTA solution at 95°C, then made up to 200 μl with 2 M Tris–HCl (pH 7.4). The products were then blotted on to a Nylon filter (Hybond-N, Amersham, U.K.) using a dot-blot apparatus (Hybri-D Manifold, BRL), dried at 80°C and crosslinked under UV light for 1 min on each side. The filter was prehybridised for 1–2 h with 5 × SSPE, 5 × Denhardt's, 0.5% sodium dodecyl sulphate (SDS), 100 mM sodium pyrophosphate; pH 7.5. γ-[^{32}P]-ATP (NEN, Boston, Massachusetts, U.S.A.) labelled oligonucleotide probes were added to the prehybridisation solution and incubated overnight at 37°C. The filter was later washed with 3 M TMAC solution (3 M tetramethylammonium chloride, 50 mM Tris (pH 8.0), 2 mM EDTA, 0.1% SDS) at 61°C. Autoradiography was performed with Fuji X-ray film and exposed at −80°C overnight. Filters were hybridised serially with a panel of primers specific for all possible mutations of the *C-KI-RAS* proto-oncogene in codon 12 or 13 leading to amino acid changes (Muta-Lyzer, Clonetech Laboratories, Palo Alto, California, U.S.A.). Filters were stripped between successive hybridisations.

Single stranded conformational polymorphisms

Single stranded conformational polymorphisms were undertaken essentially as described by others [38, 39] but with minor modifications. After PCR amplification and product purification, an aliquot was end-labelled with polynucleotide kinase as follows: 100 ng of PCR DNA was heat denatured in 5 μl of a solution containing 10 mM Tris–HCl pH 9.5, 1 mM spermidine, 1 mM EDTA. After denaturation, sample volume was increased to 10 μl in 50 mM Tris–HCl pH 9.5, 10 mM $MgCl_2$, 5 mM DTT and containing 2 units polynucleotide kinase (New England Biolabs) and 10 μCi of γ-[^{32}P]-ATP (NEN) and the sample was incubated for 45 min at 37°C. Two microlitres were then removed and added to 4 μl of formamide loading dye (96% formamide, 10 mM EDTA, 0.125% (w/v) Bromophenol blue, 0.125% (w/v) Xylene cyanol). The sample was heat denatured and 1.5 μl loaded on to a 12% polyacrylamide non-denaturing gel containing 5% glycerol in TBE buffer (90 mM Tris, 90 mM boric acid, 2.5 mM EDTA). The gel was run at a constant temperature of 30°C using the Stratatherm™ Cold regulator (Stratagene, La Jolla, California, U.S.A.) until the Bromophenol blue was at the bottom of the gel. After electrophoresis, autoradiography was performed.

Mutant allele enriched PCR

Enrichment for mutant *C-KI-RAS* alleles was undertaken essentially as described by Kahn and associates [40]. Briefly, first round PCR amplification was undertaken on 1 μg of genomic DNA in 50 μl final volume, containing 50 mM KCl, 10 mM Tris–HCl pH 9.0, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 2.5 units Taq DNA polymerase (Promega). Primer concentrations were 10 ng each of K-ras 5'- and K-ras 3'-WT, with sequences as described [39]. Cycle times were 94°C for 48 s, 56°C for 90 s and 72°C for 2 min 35 s for 15 cycles. Five microlitres of this reaction mixture was then digested with 20 units Bst NI (New England Biolabs), in a final concentration of 20 μl as recommended by the manufacturer. After an overnight digestion at 60°C, 10 μl of this reaction mix were subjected to second round PCR under conditions similar to the first round PCR. Primer concentrations were 200 ng of K-ras 5'-WT and K-ras 3'-WT. Cycling times were as above, for 30 cycles. After PCR and product purification, samples were sequenced using the Taq DNA

Polymerase Cycle Sequencing Kit (Gibco BRL, Gaithersburg, Maryland, U.S.A.) as recommended by the manufacturer.

Statistical analysis

Two by two tables were analysed by Fisher's exact test. Kaplan–Meier [41] plots were analysed by log rank analysis using the SPSS computer package (SPSS Inc., Chicago, Illinois, U.S.A.). Logistic regression and Cox regression models were constructed using the SPSS programme. Data was entered into the model using the Forward:Wald method, and entry was conditional upon $P < 0.05$.

RESULTS

Detection of point mutations

Genomic DNA was extracted from 210 colorectal adenocarcinomas comprising of 140 tumours originating on the left of the colorectum and 70 originating on the right of the colorectum. Exon 1 of the *C-KI-RAS* proto-oncogene was amplified by polymerase chain reaction. All samples were analysed for mutations occurring in codons 12 or 13 by mismatch specific oligonucleotide hybridisation (Figure 1a). Samples were considered to be positive for mutation if the signal was 3-fold or greater than background. A subset of 144 samples were analysed independently for mutation by single stranded conformational polymorphisms (Figure 1b). Concordance between the two techniques was 94% (135/144), with nine templates showing evidence of point mutation by mismatch specific oligonucleotide hybridisation but not by single stranded conformational polymorphisms. The technique of Kahn and associates [40] was employed to specifically enrich for mutant alleles, followed by direct sequencing of non-concordant templates. In each case, a result identical to that obtained by mismatch specific oligonucleotide hybridisation was obtained (Figure 1c).

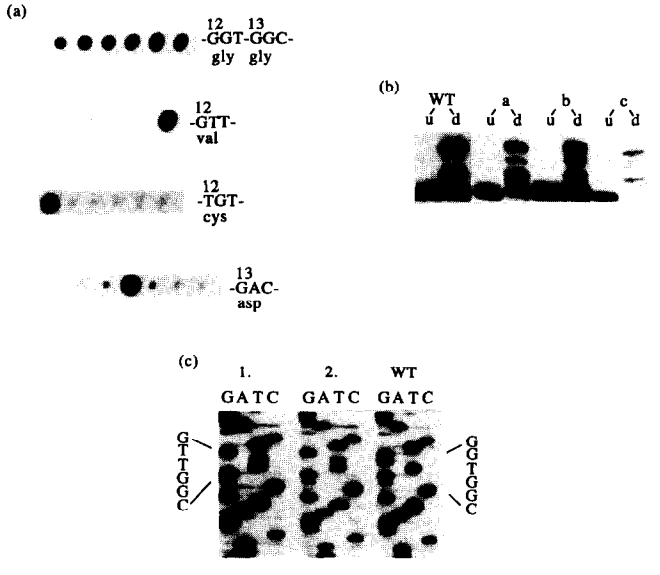

Figure 1. Representative autoradiographs showing detection of point mutations in codons 12 and 13 of the *C-KI-RAS* proto-oncogene. (a) Mismatch specific oligonucleotide hybridisation. PCR products corresponding to exon 1 of the *C-KI-RAS* proto-oncogene were bound to solid matrix and then hybridised successively with probes specific for all possible single base mutations leading to amino acid substitutions. Negative probes are not shown. (b) Single stranded conformational polymorphisms. PCR products corresponding to exon 1 of the *C-KI-RAS* proto-oncogene were heat denatured and analysed on a 12% polyacrylamide non-denaturing gel run at constant temperature. Undenatured product (u) and denatured product (d) are shown for each sample. (c) "Enriched" PCR. PCR products enriched for mutant alleles by the method of Kahn and associates [40] were sequenced by DNA cycle sequencing and analysed on 8% polyacrylamide denaturing gel.

Rate and distribution of mutant C-KI-RAS *alleles*

A total of 32 mutant alleles in 32 carcinomas were detected (Table 1) in the cohort of 140 carcinomas originating on the left of the colorectum giving a mutation rate of 23% (32/140). Seventy-eight per cent of mutations (25/32) occurred in codon 12 and 22% (7/32) in codon 13. Sixty-six per cent (21/32) of mutations lead to Asp12, Asp13 and Val12. Mutations characteristic of alkylating agents (G to A transition of the second G of a GG dinucleotide) contributed 44% (14/32) of all mutants.

In tumours originating on the right side of the colorectum, 30 mutant alleles were detected (Table 1) in 29 carcinomas (one tumour had two mutant *RAS* alleles) giving a mutation rate of 43% (30/70) which is significantly different from that found in carcinomas originating on the left of the colorectum ($P = 0.0025$, Fisher's exact test). Seventy-seven per cent of mutations (23/30) occurred in codon 12 and 23% (7/30) in codon 13. Seventy per cent (21/30) of mutations lead to Asp12, Asp13 and Val12. Mutations characteristic of alkylating agents contributed 37% (11/30) of all mutants. Examination of the mutation type with respect to disease dissemination showed no significant relationship between the nature of the dissemination and *C-KI-RAS* activation (Table 2).

Carcinomas originating on the left side of the colorectum showed a marked association between *C-KI-RAS* activation and Dukes' Stage (Table 3). There was an increase from 8% of Dukes' Stage A tumours to 40% of Dukes' Stage D carcinomas showing *C-KI-RAS* activation. In contrast, carcinomas arising on the right side of the colorectum do not show a clear increasing mutation rate with increasing Dukes' Stage, with Dukes' Stage A and Dukes' Stage C carcinomas showing high rates of *C-KI-RAS* activation, which were statistically different from the rates found on the left of the colon ($P = 0.004$ and 0.0009, respectively). Further analysis of this cohort showed that the pattern of *C-KI-RAS* activation on the right of the colorectum was at least partially due to interactions between *C-KI-RAS* activation, Dukes' Stage and sex that were not evident in carcinomas originating on the left of the colon (Table 4).

Table 1. *Mutations detected in codons* 12 *and* 13 *of the* C-KI-RAS *proto-oncogene in colorectal adenocarcinomas originating on the left or right of the colorectum*

	Left ($n = 140$)	Right ($n = 70$)*
Asp12 (GGT to GAT)	9 (6%)	5 (7%)
Val12 (GGT to GTT)	7 (5%)	10 (14%)
Cys12 (GGT to TGT)	4 (3%)	6 (9%)
Ser12 (GGT to AGT)	3 (2%)	1 (1%)
Ala12 (GGT to GCT)	2 (1%)	1 (1%)
Asp13 (GGC to GAC)	5 (4%)	6 (9%)
Cys13 (GGC to TGC)	2 (1%)	1 (1%)
Total	32 (23%)	30 (43%)

*One tumour had two mutations.

Table 2. Mutations in codons 12 and 13 of the C-KI-RAS proto-oncogene in relation to distant organ metastasis

	No distant metastasis		Metastasis* (organ)	
	Left	Right	Left	Right
Asp12	4	5	3 × Lung 2 × Liver	0
Val12	2	7	3 × Liver 2 × Other†	1 × Liver 1 × Lung/liver 1 × Other
Cys12	1	5	2 × Liver 1 × Lung/brain	1 × Other
Ser12	0	0	1 × Liver 1 × Lung 1 × Other	1 × Other
Ala12	1	1	1 × Lung/brain	0
Asp13	2	6	2 × Liver 1 × Liver/other	0
Cys13	0	1	1 × Lung/liver 1 × Liver/other	0

*Includes cases clinically detected at time of presentation or during patient follow-up. †Other sites include mesentary, omentum, cervix and pelvis. Left, tumours arising in the left side of the colorectum. Right, tumours arising in the right side of the colorectum.

Table 3. Distribution of C-KI-RAS mutations in relation to clinicopathological variables

		Left colorectum		Right colorectum	
		No mutation	Mutation (%)	No mutation	Mutation (%)
Dukes' stage	A	23	2 (8%)	3	5 (63%)
	B	25	5 (17%)	19	8 (30%)
	C	34	8 (19%)	8	13 (62%)
	D	26	17 (40%)	11	3 (21%)
Sex	F	44	18 (29%)	19	15 (44%)
	M	64	14 (18%)	22	14 (39%)
Site		108	32 (23%)	41	29 (41%)

Logistic regression analysis was used to determine which, if any, of the variables contributed independently to the frequency of *C-KI-RAS* activation. Models were constructed with the variables of patient age, sex and Dukes' Stage, as well as all possible interaction terms between these variables. *C-KI-RAS* mutation in tumours originating on the left of the colorectum was found to be associated with Dukes' Stage ($P = 0.0167$, 3 d.f. [degrees of freedom]) and the age-sex term ($P = 0.0382$, 1 d.f.). Overall significance of the model was $\chi^2 = 15.4$, $P = 0.0038$, 4 d.f. In tumours originating on the right side of the colorectum, *C-KI-RAS* mutation was found to be significantly associated with the Dukes' Stage-sex term ($P = 0.0409$, 3 d.f.). Overall significance of the model was $\chi^2 = 18.5$, $P = 0.0004$, 3 d.f. Similar modelling for the whole cohort of 210 samples, but with the inclusion of the term for tumour location (site), and all interactions, led to a model that only contained the term age–sex–site (S.E. 0.0055, $P = 0.003$; 1 d.f.). Overall significance of the model was $\chi^2 = 9.9$, $P = 0.0016$; 1 d.f.

C-KI-RAS *activation and clinicopathological parameters*

In carcinomas originating on the left of the colorectum there was a strong association with disease dissemination (Table 5). Non-disseminated, left sided tumours showed a low rate of *C-KI-RAS* mutation (13%, 7/55) compared to disseminated tumours (29%, 25/85; $P = 0.016$). There was a strong association between *C-KI-RAS* activation and the presence of distant organ metastasis at the time of surgery with 43% of patients who presented with distant organ metastasis showing *C-KI-RAS* activation compared to 17% of patients without distant organ metastasis. This association was also seen during patient follow up; only 12% of patients who did not have distant metastasis at the time of surgery or develop metastases during the follow-up period showed *C-KI-RAS* activation in the primary tumour. In contrast 39% of patients who had distant metastasis upon presentation or who developed metastases during follow-up showed *C-KI-RAS* activation in the primary tumour ($P > 0.00027$).

Carcinomas originating on the right of the colorectum showed no association between disease dissemination and *C-KI-RAS* activation (Table 5). In each case, the rate of tumours with and without *C-KI-RAS* activation was relatively constant, irrespective of the stage of dissemination. Comparatively marked differences were seen in the rate of *C-KI-RAS* activation between the left and right colon. In each case, the rate of *C-KI-RAS* activation in non-disseminated tumours on the right of the colorectum was significantly higher than the rate found in non disseminated tumours on the left of the colorectum (Table 5). The rate of *C-KI-RAS* mutation in metastatic tumours was comparable in tumours originating on the left and right of the colorectum.

C-KI-RAS *activation and prognostic significance*

Patient survival was analysed in relation of *C-KI-RAS* gene activation status (Figure 2). In colorectal carcinomas originating on the left of the colorectum, *C-KI-RAS* activation was found to be associated with a significantly poorer prognosis than those without *C-KI-RAS* activation ($P = 0.0001$ by log

Table 4. Distribution of C-KI-RAS mutations by Dukes' Stage–sex–site

	Left colorectum				Right colorectum			
	Male		Female		Male		Female	
	No mutations	Mutations (%)	No mutations	Mutations (%)	No mutations	Mutations (%)	No mutations	Mutations (%)
Dukes' A	13	1 (7)	10	1 (9)	1	2 (67)	2	3 (60)
Dukes' B	12	2 (14)	13	3 (19)	12	3 (20)	7	5 (42)
Dukes' C	20	4 (17)	14	4 (22)	2	9 (82)	6	4 (40)
Dukes' D	19	7 (27)	7	10 (59)	7	0 (0)	4	3 (43)

Table 5. Distribution of C-KI-RAS mutations in relation to disease dissemination by site

	Left colorectum		Right colorectum		
	No mutation	Mutation (%)	No mutation	Mutation (%)	*P* (Fisher's exact test)
Dissemination					
(Dukes' A + B) N	48	7 (13%)	22	13 (37%)	$P = 0.0074$
(Dukes' C + D) Y	60	25 (29%)	19	16 (46%)	P = NS
P (Fisher's exact)	$P = 0.016$		P = NS		
DM at surgery*					
N	91	19 (17%)	35	26 (43%)	$P = 0.0003$
Y	17	13 (43%)	6	3 (33%)	P = NS
P (Fisher's exact)	$P = 0.0039$		P = NS		
DM during foll†					
N	73	10 (12%)	33	24 (42%)	$P < 0.0001$
Y	35	22 (39%)	8	5 (38%)	P = NS
P (Fisher's exact)	$P = 0.00027$		P = NS		

N, not present, Y, present. *DM at surgery: Clinically detected cases of distant organ metastasis at the time of patient presentation for primary colorectal adenocarcinoma. †DM during foll: Clinically detected cases of distant organ metastasis either at the time of presentation or during subsequent patient follow-up.

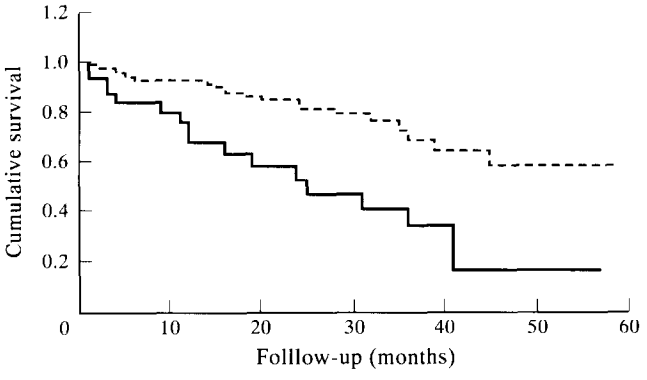

Figure 2. Kaplan–Meier analysis of *C-KI-RAS* point mutation and patient survival (carcinomas from the left of the colorectum only). Solid line: patients with *C-KI-RAS* point mutations; broken line: patients without detectable *C-KI-RAS* point mutations. The two curves were significantly different ($P = 0.0001$ by log rank analysis).

rank analysis). Activation of the *C-KI-RAS* proto-oncogene was not found to be associated with a poorer prognosis in patients with tumours originating on the right of the colorectum ($P = 0.68$ by log rank analysis; data not shown). Cox regression analysis was used to determine independent variables contributing towards patient mortality. The factors of patient age, sex, Dukes' Stage and mutation status, as well as all possible interaction terms were assessed. The final model for tumours originating on the left of the colorectum contained the variables Dukes' Stage (S.E. 0.33, $P < 0.0001$; 3 d.f.) and mutation status (S.E. 0.25, $P = 0.042$; 1 d.f.). Overall significance of the model was $\chi^2 = 46.7$, $P < 0.0001$. For tumours originating on the right of the colorectum, the only significant variable was the age-Dukes' Stage term (S.E. 0.0056, $P = 0.001$). Overall significance of the model was $\chi^2 = 12.6$, $P = 0.0004$.

DISCUSSION

Mutation of the *C-KI-RAS* proto-oncogene occurs in 20–50% of colorectal carcinomas [1–3]. The cause of such point mutations has yet to be established, but factors including bile acids and fecapentanes, such as 3-ketosteroids, have been implicated [42]. It is becoming clear that the frequency at which *C-KI-RAS* mutations are found in colorectal adenocarcinomas depends upon such factors as the patient's age, sex and the location of the tumour [32]. Indeed, on the only analysis of all tumours in this cohort, we found that logistic regression modelling produced only one significant term, that of age–sex–location, to describe the distribution of tumours with *C-KI-RAS* mutations. This is in close agreement with the result of Brevik and coworkers [32]. However, we found that, in tumours originating on the left of the colorectum, the terms for Dukes' Stage and the age–sex interaction were significant variables, whereas in tumours originating on the right of the colorectum, the only significant factor was the Dukes' Stage–sex term. The overall significance of the combined model was lower than for either the left or the right cohorts. Hence, overall modelling of *C-KI-RAS* mutation throughout the colorectum may prove misleading in attempting to elucidate factors promoting *RAS* mutation.

While we noted a marked difference in the frequency of *C-KI-RAS* activation in tumours originating on the left of the colorectum (23%) as opposed to those originating on the right of the colorectum (43%), we also noted that the spectrum of mutations in carcinomas originating on the left of the colorectum was almost identical to that in carcinomas originating on the right of the colorectum, which would indicate that similar mutagens are responsible for *C-KI-RAS* mutation throughout the length of the bowel. It is possible that the difference in frequency is due to a decreasing gradient of mutagen from the right of the bowel to the left.

In this data set, we found no evidence for the nature of the *C-KI-RAS* mutation determining metastatic potential as has been proposed by other authors [27, 33], although these studies are also contradictory. For example, Moerkerk and associates [33] proposed that tumours with G to A mutations do not progress beyond Dukes' Stage B, while Finkelstein and associates [27] proposed that this type of mutation accounts for the majority of cases associated with haematogenous dissemination. In our study, G to A transitions were found in all stages on both sides of the colon, with the exception of Dukes' Stage A on the right of the colorectum, and we found no

statistically significant association between the specific nature of the *C-KI-RAS* mutation and the target organ of distant metastasis.

It is becoming increasingly apparent that many genetic changes in colorectal carcinomas show marked differences in frequencies of occurrence when analysed in light of the site of tumour formation. Genetic changes that have been shown to occur at different frequencies in carcinomas originating in the left and right of the colorectum include DNA aneuploidy [28, 43], allelic loss of chromosomes 17, 18 and 5 [28], *C-MYC* mRNA overexpression [44], *TP53* point mutation [45] and more recently *C-KI-RAS* activation [32], a finding confirmed in this study.

The left and right side of the colorectum derive from different embryonic origins, and are served by different blood supplies. The right side of the colorectum is derived from embryonic mid gut and is served by the superior mesenteric artery, while the left side of the colon is derived from the embryonic hind gut and is served by the inferior mesenteric artery. While this might affect the target organ of blood borne metastasis, it does not seem sufficient to account for the wide range of colonic markers found differentially expressed in the colon (for a thorough review see [46]), and analysis of colonic markers and genetic changes has not yet been able to establish the basis for differences in neoplastic transformation in the colorectum.

Tumours originating on the left of the colorectum show strong associations with *C-KI-RAS* activation and disease progression, particularly dissemination of the disease to distant organs. Moreover, *C-KI-RAS* activation has a strongly predictive role in assessing patient mortality. The association of *RAS* activation with tumour progression, as exemplified by the association with Dukes' Stage, would appear to be inconsistent with proposals that *C-KI-RAS* gene activation is associated with the progression from benign adenoma to the larger more aggressive villous adenoma. On the right of the colorectum, the relatively constant rate of *C-KI-RAS* activation between non-disseminated and disseminated carcinomas (37% and 45%, respectively compared to 13% and 30% for carcinomas originating on the left of the colorectum) would seem to be consistent with a role for *C-KI-RAS* activation in adenoma progression.

Carcinomas originating on the left side of the colorectum have been described as being more genetically unstable (as typified by DNA aneuploidy and multiple allelic losses) and phenotypically more aggressive, while carcinomas on the right of the colorectum are generally diploid and less aggressive [46]. Hence *C-KI-RAS* activation does not occur in isolation, but rather is part of a matrix of genetic alterations in the tumour cell. Multiple control pathways will provide a degree of redundancy so that phenotypic expression of point mutations may be moderated. However, despite the fact that we have shown that the spectrum of *C-KI-RAS* mutations on the left and right of the colorectum are similar, the frequency, timing and biological impact of these mutations differ. The strong association of *C-KI-RAS* activation in carcinomas originating on the left of the colorectum and disease dissemination and patient mortality may have an impact on patient management. The clearly different biological behaviour of *C-KI-RAS* activation in left and right carcinomas will further strengthen calls that these neoplasms are treated as different biological entities.

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Acknowledgements—This work was supported by grants from the Lee Foundation, the Shaw Foundation and the Singapore Totalisator Board.