Kirsten ras Mutations in Patients With Colorectal Cancer: the Multicenter ''RASCAL'' Study

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For the RASCAL Group

Background: **Kirsten ras (Ki-ras) gene mutations occur early in the progression of colorectal adenoma to carcinoma. The aim of this collaborative study was to clarify the association between Ki-ras mutations, patient outcome, and tumor characteristics by use of data from colorectal cancer patients worldwide.** ***Methods:*** **Investigators who had published data on Ki-ras and colorectal cancer were invited to complete a questionnaire for each patient entered into a database. Two-sided statistical tests were used to analyze data.** ***Results:*** **Patients (n = 2721) were recruited from 22 groups in 13 countries. Mutations of Ki-ras codon 12 (wild type = GGT = glycine) or codon 13 (wild type = GGC = glycine) were detected in 37.7% of the tumors; 80.8% (584 of 723) of all the specified mutations occurred in codon 12, and 78.1% (565 of 723) of all the specified mutations were at the second base of either codon. Mutations were not associated with sex, age, tumor site, or Dukes' stage. Mutation rates seen in patients with sporadic tumors were comparable to those observed in patients with a predisposing cause for their cancer. Poorly differentiated tumors were less frequently mutated (*P *= .002). Multivariate analysis suggested that the presence of a mutation increased risk of recurrence (*P*<.001) and death (*P *= .004). In particular, any mutation of guanine (G) to thymine (T) but not to adenine (A) or to cytosine (C) increased the risk of recurrence (*P *= .006) and death (*P*<.001). When individual, specific mutations were evaluated, only valine codon 12 was found to convey an independent, increased risk of recurrence (*P *= .007) and death (*P *= .004).** ***Conclusions:*** **Ki-ras mutations are associated with increased risk of relapse and death, but some mutations are more aggressive than others. [J Natl Cancer Inst 1998;90:675–84]**

More than 75 research groups worldwide have published data on the significance of the Kirsten ras (Ki-ras) gene (also known as C-KI-RAS2) in colorectal cancer. As a result, it is widely accepted that mutations in this gene develop early in the progression from adenoma to carcinoma. However, there is little agreement on how mutations relate to other histologic and clinical factors. For example, at least nine groups *(1–9)* have suggested that the presence of a Ki-ras mutation conveys prognostic significance, but 14 groups *(10–23)* have reached the opposite conclusion. Patterns of recurrence of colorectal tumors have been attributed to specific mutations, but studies finding that a given mutation has a relatively benign influence on outcome are contradicted by other studies *(3,4,24)* suggesting that the same mutation is particularly aggressive. Even studies examining the association between the presence of Ki-ras mutations, Dukes' stage, histology, and such factors as tumor site or age or sex of the patient *(1,2,6,13,18,19,23–26)* have failed to reach a consensus.

Interpretation of published data may be complicated for a number of reasons. First, colorectal tumors have frequently been considered as a single entity, although some authors have suggested that mutations in the Ki-ras gene may differ according to the patient's geographic origin *(27)*, the type of tumor *(28)*, or whether there is an underlying predisposition for developing colorectal cancer, such as ulcerative colitis *(29–32)*. However, since the number of colorectal adenocarcinomas included within studies that have explored these issues have ranged from three to 410, some studies have been too small to reach conclusions with a high degree of certainty. Moreover, methodology may influence the frequency at which mutations are detected *(22,23,33)*.

If the role of the Ki-ras gene in patients with colorectal cancer were clearer, it might lead to a better understanding of cancer development and be helpful in determining prognosis or more appropriate use of adjuvant treatments. In addition, mutations in abnormal tissues offer tempting targets for screening or molecular treatments, and a larger pool of information may indicate whether targeting of specific mutations would be useful. Finally, the failure to coordinate data leads to a relatively unprofitable repetition of similar studies.

Few centers have sufficient patients to collect detailed information on the large numbers required to determine the impact of individual Ki-ras genotypes on outcome. Therefore, the aim of this study was to persuade researchers to share primary data on the clinical, histologic, and outcome parameters of their patients with colorectal cancer in whom the Ki-ras status was also known. The primary goal was to determine whether the presence of a mutation in the Ki-ras gene was of prognostic significance. A secondary goal was to try to identify whether specific Ki-ras mutations had prognostic significance or correlated with clinical and histologic parameters.

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See ''Notes'' following ''References.''

Patients and Methods

Patients

After a literature search, at least two invitations were sent to all investigators who had published original data in English and to researchers known to have unpublished data on the significance of the Ki-ras gene in patients with colorectal adenocarcinoma. They were invited to participate in a collaborative register collecting original clinical data from such patients.

Participants were required to complete a questionnaire divided into ''critical'' and ''secondary'' data on each patient to be entered into the database. All collaborators were asked to ensure that the critical data in particular were as complete as possible. Nine critical questions requested information in the following three areas: 1) details on the genotype of the Ki-ras gene in the primary tumor at codons 12 and 13; 2) the date, Dukes' stage, and apparent immediate outcome of any surgery for that cancer; and 3) dates of follow-up and long-term outcome. Specific causes of death and dates of disease recurrence, if relevant, were also sought. However, the Ki-ras genotype of recurrent tumors was not requested. The database was set up this way so that data could be analyzed irrespective of whether the exact type and position of a mutation were known (e.g., after sequencing of the DNA) or of whether the contributor's methods could only determine the presence of a mutation but could not necessarily determine the amino acid substitution that had occurred or whether it was on codon 12 or 13 (e.g., with some types of single-strand conformation polymorphism [SSCP] analysis).

Secondary data included questions about predisposing causes for colorectal cancer. There were further questions about the tumor: its site and histologic appearance and sites of tumor recurrence. Finally, researchers were asked to detail the methods used to determine the Ki-ras genotype. All data were coded so that the patients' identity was known only to their physicians and were entered into a database called ''RASCAL.''

Statistical Methods

To detect a reduction in survival of 10% between patients with and without mutations with 90% power (5-year survival of 55% compared with 65%), it was calculated that follow-up data on at least 1000 patients in whom there had been about 400 ''events'' would be needed.

Survival curves were generated by use of the product-limit method of Kaplan–Meier. The logrank test was used to evaluate differences in failure-free survival and overall survival curves. Failure-free survival was defined as the time to relapse or death from any cause. Overall survival was defined as the time to death from any cause. Chi-squared tests were used to compare categorical data. In view of the multiple statistical analyses performed and the large number of patients, only P values less than .01 were considered to be statistically significant. Multivariate analysis was performed by use of Cox's model for proportional hazards survival analysis. All P values were two-sided.

The survival curve stratified by Dukes' stage (Fig. 1) was generated by use of the baseline survival function from the Cox multivariate analysis. The hazard ratios (HRs) and 95% confidence intervals (CIs) were generated from individual Cox multivariate analyses for each center (Fig. 2). The box size in Fig. 2 indicates the relative number of patients from each center. When a center reported no relapses or deaths, no HR was calculated.

If an answer to the questionnaire was not available to collaborating groups, they were instructed to answer ''unknown.'' Where this is important, the number of unknowns is indicated in the text or tables. There were very few multiple mutations, compared with the size of the cohort and number of events, so these were treated as single mutations for the purposes of the analyses. Bias introduced by the findings of different centers seemed to be of little significance because the test for heterogeneity across centers evaluated to $P = .36$. In spite of this, the ''modeling of center effects'' was treated as a fixed effect by stratifying by center. Thus, patients within each center were compared only with each other. Therefore, the overall estimate of the effect was stratified by center.

Results

Collaborating Groups (Table 1)

Data on the Ki-ras genotype of some 4500 patients with colorectal cancer have been published, although some patients have been reported in more than one published paper. A realistic estimate is that the Ki-ras status of approximately 4000 such individuals is known.

In response to our proposals, 26 groups expressed an interest, and data on 2721 patients were finally received from 22 centers in 13 countries [*see* ''Appendix: Collaborating Authors'' section]. European researchers who have previously published reports on about 1550 patients were the largest contributors with 2207 patients. Researchers from Singapore, Japan, and Australia contributed 374 patients (23% of Australia's and Southeast Asia's published total). However, about half of the groups from this region were not approached because they had not published papers in English. Only one of 15 North American groups participated and provided 11% of this region's previously published data. Of the groups identified as having previously published studies reporting a positive prognostic finding for a ras mutation, two published their results after the completion of this study and three have not joined the study, but data from four have been used for our multivariate analysis. Of the 14 groups that did not identify any prognostic effect from the presence of a mutation, data from seven have been included. However, many of these groups have provided the database with more patients than they have previously published.

Only four centers that have published data on more than 100 patients were not recruited because they did not respond to multiple invitations to take part or declined to do so when they did respond (total of 590 patients). However, unpublished data have been included on approximately 850 patients.

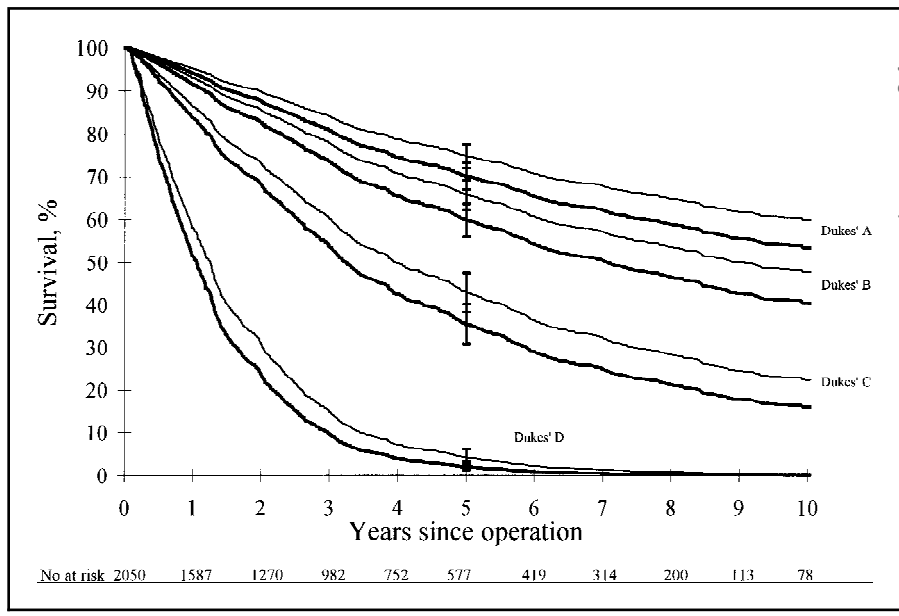

Fig. 1. Overall survival function of colorectal cancer patients in the RASCAL study stratified by Dukes' stage. Model shows the calculated impact of wild-type Kirsten ras compared with any mutation of codon 12 or 13 on overall survival (*see* ''Statistical Methods'' section). Bold line = mutations; light line = wild type.

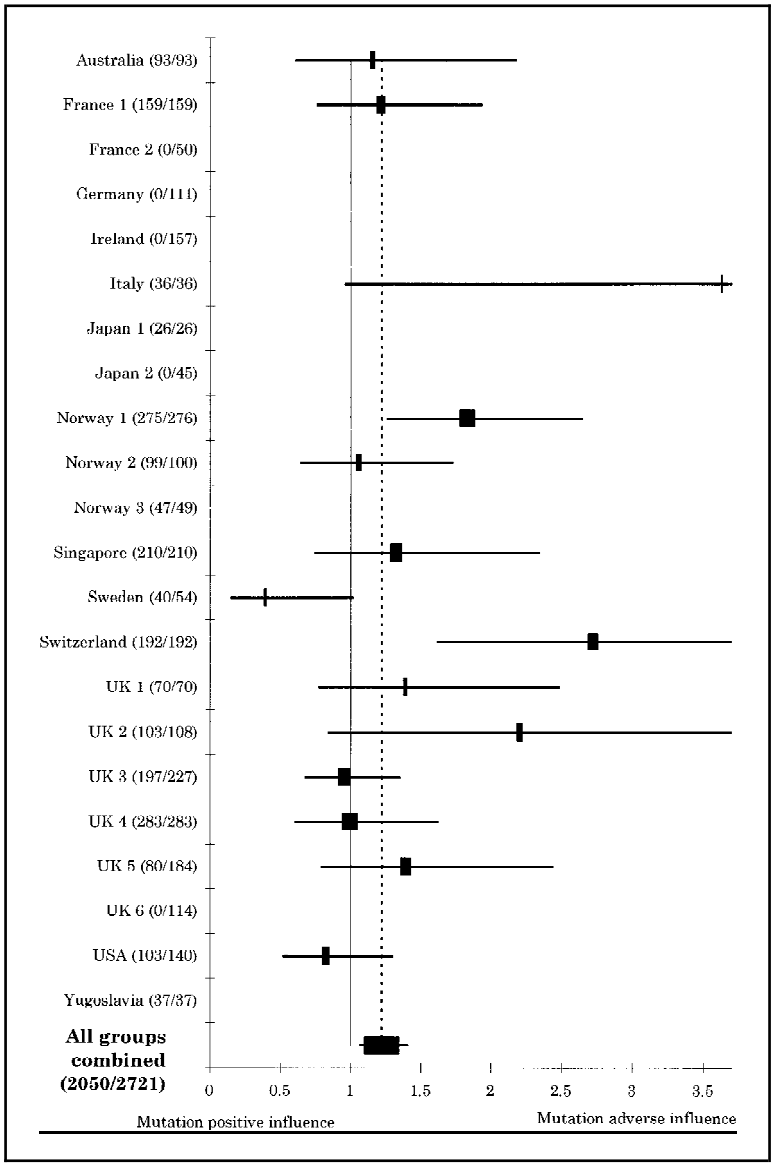

Fig. 2. Overall survival hazards ratios from data provided by different groups collaborating in the RASCAL study. Boxes show the relative size of the cohort from each center; the box for all groups combined is shown reduced to one-fourth size compared with those for individual centers. The position of the box represents the degree of hazard conveyed by the presence of any mutation in the cohort from each of those centers providing survival data. The arms on either side of the box indicate the 95% confidence intervals of the hazards ratios. Where no events occurred among the patients from a center, no box is shown on this diagram. The number of patients from each center is shown in parentheses after the name of the center (number used in the multivariate analysis/total number).

Patient Characteristics (Table 2)

The characteristics of these patients are in keeping with larger populations with colorectal cancer reported elsewhere *(34)*. Of the 2721 patients entered in the database, 1455 (54.0%) were men, 1238 (46.0%) were women, and 28 were of unspecified sex. The median age at presentation was 68 years (range, 17–103 years); there was no significant age difference between sexes.

Follow-up data were provided for 2445 patients, of whom 1216 remained well after a median of 4.7 years of follow-up (range, 0.1–17.6 years). There were 87 deaths within a month of surgery (''perioperative deaths''), 716 deaths from cancer, 184 deaths from unrelated causes, and 261 deaths from unknown causes.

At surgery, a Dukes' stage A tumor was present in 413 patients, stage B in 1173 patients, stage C in 834 patients, and stage D in 265 patients; in 36 patients, stage was not known. There was no difference in the frequency of each stage between the sexes. The primary site of the tumor was more commonly the right colon in women (24.6%) compared with men (19.3%) ($P = .001$), was similar for both groups in the transverse and left (descending) colons, but was higher among men (62.0%) than among women (56.0%) for sigmoid or rectal cancers ($P = .002$). The result of the original surgery was not stated for one quarter of all patients. In those for whom the outcome of the surgery was known, it did not vary between the sexes. A curative resection was reported to have been carried out in 1793 (86.4%) of 2075 patients, whereas 282 patients (13.6%) underwent only palliative surgery or no resection at all.

Ki-ras and Methods Used for Detection of Mutations

Methods to detect the presence of a mutation could be broadly divided into three types. In 96 patients from two centers, SSCP was used. Of these, a mutation was detected in 42 (43.8%). Polymerase chain reaction (PCR) amplification, which was followed by direct sequencing, was used in 679 samples from nine centers and detected mutations in 225 patients (33.1%). Methods involving allele-specific primers, either PCR alone or PCR followed by hybridization, were used by 14 centers in 1893 patients and detected a mutation in 619 (32.7%). The method was not stated for 53 patients from four centers. All researchers using allele-specific methods were able to detect all possible mutations on codons for which they reported data with the exception of one group. This was taken into account during the analyses. There was no statistical difference between the mutation rate detected by sequencing and allele-specific methods ($P = .83$).

Status of the Ki-ras Gene at Codons 12 and 13 (Table 3)

The status of Ki-ras codon 12 was known in all 2721 patients; 755 (27.7%) had a mutation. Codon 13 status was determined in 2214 patients; 146 (6.6%) had a mutation. Mutations were detected in an additional 39 patients, but the codon was not specified. The status of both codons 12 and 13 was known in 2214 patients; 835 (37.7%) had a mutation on one or both codons. Nine patients had mutations on both codons 12 and 13. The rate of mutation (calculated from patients with information on both codons 12 and 13) in men (36.2%; 430 of 1187) and in women (39.5%; 399 of 1009) was not significantly different ($P = .11$) when all those in whom the status of both codons was known were compared.

The most common mutation was glycine to aspartate on codon 12 (30.6%; 221 of all 723 mutations). Mutation from glycine to valine was the second most common of all specified mutations (23.4%; 169 of 723). Mutation from glycine to aspartate on codon 13 accounted for 16.7% (121 of 723) of specified mutations. Mutation from guanine (G) to adenine (A) occurred in 55.0% (398 of 723) of mutations, mutation of G to thymine (T) in 34.3% (248 of 723), and mutation of G to cytosine (C) in

Table 1. Country of origin of researchers invited to join the RASCAL study, their responses, and the number of patients entered in the study

Country of origin	No. of research groups or investigators contacted	Response			No. of patients entered
		No reply	Unable or unwilling to participate	Provided data	
Australia	1	—	—	1	93
Canada	1	1*	—	0	—
France	3	1	—	2	209
Germany	2	1	—	1	111
Greece	1	1*	—	0	—
Holland	2	—	2	—	—
Ireland	2	—	1	1	157
Italy	1	—	—	1	36
Japan	19	12 + 1*	4	2	71
Norway	3	—	—	3	425
Singapore	1	—	—	1	210
Spain	1	—	1	—	—
Sweden	1	—	—	1	54
Switzerland	2	1	—	1	192
Taiwan	1	1	—	—	—
U.K.	11	3	2	6	986
United States	15	6	7 + 1*	1	140
Yugoslavia	1	—	—	1	37

*Group expressed interest but did not provide any patient data.

Table 2. Characteristics of patients enrolled in the RASCAL study

Characteristic	Men	Women	Unknown sex	Total
Total No.	1455	1238	28	2721
Median age at diagnosis (range), y	67 (17–95)	69 (19–103)	—	68 (17–103)
Primary tumor site, No. (%)				
Right colon/hepatic flexure	281 (19.3)	304 (24.6)	7	592 (21.8)
Transverse colon	64 (4.4)	60 (4.8)	2	126 (4.6)
Splenic flexure/descending colon	109 (7.5)	94 (7.6)	3	206 (7.6)
Sigmoid or rectum	902 (62.0)	693 (56.0)	6	1601 (58.8)
Two sites	6 (0.4)	3 (0.2)	0	9 (0.3)
Not known	93 (6.4)	84 (6.8)	10	187 (6.9)
Dukes' stage, No. (%)				
A	221 (15.2)	187 (15.1)	5	413 (15.2)
B	630 (43.3)	534 (43.1)	9	1173 (43.1)
C	435 (29.9)	391 (31.6)	8	834 (30.7)
D	150 (10.3)	115 (9.3)	0	265 (9.7)
Not known	19 (1.3)	11 (0.9)	6	36 (1.3)
Outcome of initial surgery, No. (%)				
Apparently curative	967 (66.5)	820 (66.2)	6	1793 (65.9)
Palliative	163 (11.2)	119 (9.6)	0	282 (10.4)
Not known	325 (22.3)	299 (24.2)	22	646 (23.7)
No. with ulcerative colitis	34	16	12	62
No. with FAP*	19	6	3	28
No. with HNPCC†	17	12	0	29
No. with mutated Ki-ras				
Codon 12	387	357	11	755
Codon 13	81	65	0	146
Codon not specified	13	26	0	39

*FAP = familial adenomatous polyposis.
†HNPCC = hereditary nonpolyposis colorectal cancer.

10.7% (77 of 723). Mutations occurred at the first base of codon 12 or 13 in 21.1% (160 of 723) and at the second base in 78.1% (565 of 723) (two patients had two mutations).

Ki-ras and Tumor Site

The primary tumor sites were classified as either ascending, hepatic flexure, transverse, splenic flexure, descending or sigmoid colon, or rectum. The rate of mutation did not differ according to site. Moreover, there was no difference seen if tumors on the right side of the colon (ascending and transverse colons) were grouped together and compared with those on the left (splenic flexure to rectum) ($P = .46$). When specific mutations were considered, their distribution at individual sites and on the left or the right side of the bowel was also similar.

Table 3. Types and number of each Kirsten ras mutation at codons 12 and 13

Mutation	Codon 12	Codon 13	Codon 12 or 13
G → A			
GGT (glycine)—AGT (serine)	57	—	56
GGT (glycine)—GAT (aspartate)	238	—	221
GGC (glycine)—GAC (aspartate)	—	121	121
Total	295	121	398
G → T			
GGT (glycine)—TGT (cysteine)	67	—	64
GGT (glycine)—GTT (valine)	183	—	169
2 mutations, GTC/TGC	1	—	1
GGC (glycine)—TGC (cysteine)	—	10	10
GGC (glycine)—GTC (valine)	—	3	3
2 mutations, GTT/TGT	—	1	1
Total	251	14	248
G → C			
GGT (glycine)—CGT (arginine)	29	—	27
GGT (glycine)—GCT (alanine)	47	—	46
GGC (glycine)—CGC (arginine)	—	1	1
GGC (glycine)—GCC (alanine)	—	3	3
Total	76	4	77
Ki-ras mutation details unknown	133	7	82
Mutation but codon unknown			39
Total patients with mutations	755	146	835*
Total patients	2721	2214	2214

*Nine patients had mutations on codon 12 and codon 13.

Ki-ras and Histology (Table 4)

There was no statistically significant difference in the rate of Ki-ras mutation (codons 12 and 13) and histologic stage. Of the 384 patients with Dukes' stage A tumor, 130 (33.9%) had a mutation, 340 (39.8%) of the 855 Dukes' stage B tumors contained a mutation, and 284 (38.3%) of 742 patients with a Dukes' stage C tumor and 78 (35.8%) of 265 patients with Dukes' D tumor showed a mutation.

One hundred fifty-one (38.7%) of 390 well-differentiated and 447 (41.3%) of 1083 moderately differentiated tumors more frequently had a mutation than the poorly differentiated tumors (present in 84 [30.7%] of 274; $P = .002$). When the chi-squared test was then used to compare all three grades of differentiation separately with frequency of any mutation, a P value of .006 was obtained. This comparison appears to be strongly influenced by the lower mutation rate in the poorly differentiated tumors compared with the other grades. Specific mutations did not correlate with differentiation grade. However, differentiation was not stated for 546 patients, and the frequency with which poor differentiation was reported varied widely between centers (1.4%–49% of all tumors). Therefore, it may be that histopathologic grading criteria differed between centers. There was no apparent correlation between the presence of a mutation and other histologic markers of prognosis, such as vascular invasion, the presence of colloid elements, or the degree of host lymphocyte re-

Table 4. Kirsten ras mutations and tumor histology in patients enrolled in the RASCAL study

	No. of patients with a codon 12 mutation/total No. of patients (%)*	No. of patients with a codon 13 mutation/total No. of patients (%)†	No. of patients with a mutation on codon 12 or 13/total No. of patients (%)‡
Dukes' stage			
A	112/413 (27.1)	26/384 (6.8)	130/384 (33.9)
B	329/1173 (28.0)	57/855 (6.7)	340/855 (39.8)
C	228/834 (27.3)	50/742 (6.7)	284/742 (38.3)
D	78/265 (29.4)	12/218 (5.5)	78/218 (35.8)
Not known	8/36 (22.2)	1/15 (6.7)	3/15 (20.0)
Differentiation			
Well differentiated	155/480 (32.3)	17/390 (4.4)	151/390 (38.7)
Moderately differentiated	387/1335 (29.0)	84/1083 (7.8)	447/1083 (41.3)
Poorly differentiated	76/360 (21.1)	20/274 (7.3)	84/274 (30.7)
Not known	137/546 (25.1)	25/467 (5.4)	153/467 (32.8)
Type of tumor			
Flat	23/95 (24.2)	10/85 (11.8)	31/85 (36.5)
Polypoid	120/452 (26.5)	14/291 (4.8)	128/291 (44.0)
Not known	612/2174 (28.2)	122/1838 (6.6)	676/1838 (36.8)
Vascular invasion			
Present	30/168 (17.9)	3/79 (3.8)	26/79 (32.9)
Absent	112/405 (27.7)	24/316 (7.6)	125/316 (39.6)
Not known	613/2148 (28.5)	119/1819 (6.5)	684/1819 (37.6)
Lymphocyte response§			
+	77/266 (28.9)	8/247 (3.2)	79/247 (32.0)
++	39/151 (25.8)	7/135 (5.2)	42/135 (31.1)
+++	17/51 (33.3)	7/47 (14.9)	23/47 (48.9)
Not known	622/2253 (27.6)	124/1785 (6.9)	691/1785 (38.7)
Colloid elements			
Present	77/290 (26.6)	22/252 (8.7)	111/252 (44.0)
Absent	140/640 (21.9)	27/538 (5.0)	189/538 (35.1)
Not known	538/1791 (30.0)	97/1424 (6.8)	535/1424 (37.6)

*Codon 12 mutation data were available for all patients.
†Only patients with codon 13 data are included.
‡Only patients with data for both codons are included.
§+ = mild; ++ = moderate; +++ = marked.

action, but, again, much data were not available for these histologic parameters.

Ki-ras and Geographic Origin

When the geographic variation in mutation rate was examined, no consistent difference in the predominance of a single type of mutation in individual regions was seen. However, our dataset is too small to rule out such a difference with certainty. A heterogeneity test suggested that data did not differ statistically between centers. However, when the rate of mutations between centers was examined, a value of $P<.001$ was obtained, suggesting a significant association between center and mutation rate. However, geographically, this conclusion did not make sense (one Japanese group has the lowest and the other has the highest mutation rate), and some of the similar mutation rates found by groups in a given country (e.g., two of the three Norwegian collaborating groups) may have occurred because there were subtle differences in their methodology compared with other groups (e.g., a unique enriched PCR technique).

However, a codon 12 G to A mutation was found in 8.5%–13.9% of patients except in a small group (37 patients) from Yugoslavia in whom the incidence was 2.7% and in a larger group (192 patients) from Switzerland in whom the incidence was 22.9%. However, the G to A mutation rate in both of these groups on codon 13 did not appear to differ from rates in other countries. Perhaps a difference might have been expected if a single mutagen causing G to A mutation was present in Switzerland and absent in Yugoslavia. These differences need to be confirmed in other patients from these countries.

G to T change occurred at a frequency of 8.3%–16.7% except in the Yugoslav group (27%). Because numbers of patients were small, it was difficult to be certain whether this increased frequency was maintained on codon 13, but this did not appear to be the case. G to C change occurred in 1.4%–5.6% of the cohort from each country; however, it was found more commonly among the 71 Japanese patients with a frequency of 14.1%. No data on codon 13 were available from one Japanese group, and there were too few patients in the other group for a definitive answer.

Ki-ras and Predisposing Causes for Colorectal Cancer

Predisposing causes for the development of colorectal cancer were reported in 119 patients. Sixty-two patients had ulcerative colitis. Of these, 17 had codon 12 mutations and two had codon 13 mutations (30.6%). Twenty-eight patients were included with familial adenomatous polyposis; nine of these patients (32.1%) had codon 12 mutations. Of the 29 patients with hereditary nonpolyposis colorectal cancer, eight (27.6%) had codon 12 mutations and two had codon 13 mutations (34.5% with both mutations considered). None of these figures differed significantly from the overall mutation rate for patients with no predisposing cause.

Ki-ras and Pattern of Relapse (Table 5)

There are a number of factors that predispose to relapse, including the adequacy of the original surgery and the tumor site. Even with 480 relapses, the possibility of four or five patterns of relapse and 12 different mutations suggested that it was unlikely that any specific mutation could be shown to predispose to any single type of relapse. Indeed, no consistent patterns emerged to suggest that any one mutation predisposes to a specific method of tumor spread. G to A mutations were more frequent (58.3%) among patients with an anastomotic recurrence than among patients with other types of recurrence collectively (about 22%). But since this group includes only 12 patients, this finding is considered to be unreliable ($P = .02$).

Table 5. Kirsten ras genotype and the pattern and numbers of relapses seen with each mutation in patients* enrolled in the RASCAL study

Genotype		Total No. of patients with mutations	No. of patients with a mutation at each site of relapse					
			Anastomotic	Local	Lymph node	Bloodborne	Other	Unknown
Codon 12	(GGT)†							
Serine	(AGT)	57	2	4	1	10	1	3
Arginine	(CGT)	29	0	1	1	1	0	2
Cysteine	(TGT)	67	0	5	2	17	0	1
Aspartate	(GAT)	238	4	15	4	36	0	21
Alanine	(GCT)	47	0	2	0	5	0	1
Valine	(GTT)	183	0	16	1	19	2	6
2 mutations	(GTT/TGT)	1	0	0	0	0	0	0
Unspecified		133	1	4	0	0	0	5
Codon 13	(GGG)†							
Arginine	(CGC)	1	0	0	0	0	0	0
Aspartate	(GAC)	121	1	12	4	13	0	4
Cysteine	(TGC)	10	0	1	1	5	0	0
Alanine	(GCC)	3	0	0	0	0	0	1
Valine	(GTC)	3	0	0	0	1	0	0
2 mutations	(GTC/TGC)	1	0	0	0	0	0	0
Unspecified		7	0	0	0	0	0	4
Wild type for both codons		—	4	93	26	156	9	66
Total		—	12	153	40	263	12	114

*Of 2721 patients, 755 had a mutation at codon 12; of 2214 patients, 146 had a mutation at codon 13.
†Wild type for codons 12 and 13.

Ki-ras, Tumor Recurrence, and Survival (Table 6)

Multivariate analysis was performed stratified by referral center because some centers reported data from consecutively diagnosed patients and others came from skewed population groups. One group obtaining their data mainly from a perioperative death autopsy series were excluded from the survival analysis.

In the initial analysis, variables included Dukes' stage, sex, and the presence of any mutation. End points were time to failure (i.e., disease recurrence or death) and survival. Risk of failure was increased by higher Dukes' stage and by any mutation (HR = 1.25; 95% CI = 1.10–1.42; $P<.001$). Overall survival was also reduced by any mutation (HR = 1.22; 95% CI = 1.07–1.40; $P = .004$). Fig. 1 shows the impact of a mutation on predicted survival for each Dukes' stage.

If histologic tumor differentiation was added as a variable, it did not become a statistically significant independent risk factor for failure-free survival or survival, although it was indicative of

Table 6. The RASCAL study: results of the multivariate analysis*

	Patients included	No. of events	Hazard ratio	95% confidence interval	Two-sided *P*
			Any mutation		
Failure-free survival	2050	1006			
Dukes' B	799	328	1.48	1.16–1.88	.001
Dukes' C	705	423	2.80	2.21–3.54	<.001
Dukes' D	200	152	9.17	6.90–12.19	<.001
Any mutation	777	411	1.25	1.10–1.42	<.001
Poor differentiation	253	139	1.17	0.96–1.43	.07†
Sex	1094	564	1.15	1.01–1.30	.03
Overall survival	2050	903			
Dukes' B	799	293	1.48	1.14–1.92	.002
Dukes' C	705	375	2.83	2.20–3.66	<.001
Dukes' D	200	146	11.25	8.33–15.20	<.001
Any mutation	777	364	1.22	1.07–1.40	.004
Poor differentiation	253	128	1.29	1.05–1.59	.02†
Sex	1094	498	1.12	0.98–1.28	.10
Failure-free survival	1845	916			
Dukes' B	707	291	1.49	1.16–1.91	.002
Dukes' C	628	385	2.92	2.29–3.74	<.001
Dukes' D	193	145	9.22	6.87–12.37	<.001
Valine 12	150	84	1.39	1.10–1.74	.007
Serine 12	53	31	1.48	1.03–2.13	.05
Aspartate 12	203	107	1.16	0.93–1.44	.25
Alanine 12	39	19	1.27	0.80–2.01	.38
Cysteine 12	62	38	1.16	0.83–1.62	.59
Arginine 12	17	10	1.23	0.65–2.33	.64
Aspartate 13	111	47	1.10	0.82–1.50	.74
Overall survival	1845	819			
Dukes' B	707	258	1.52	1.15–2.00	.002
Dukes' C	628	341	3.03	2.32–3.96	<.001
Dukes' D	193	139	11.29	8.26–15.44	<.001
Valine 12	150	75	1.43	1.13–1.82	.004
Cysteine 12	62	38	1.42	1.01–1.98	.05
Serine 12	53	26	1.36	0.92–2.03	.14
Alanine 12	39	18	1.42	0.89–2.29	.18
Arginine 12	17	10	1.53	0.81–2.90	.24
Aspartate 12	203	86	1.06	0.84–1.35	.81
Aspartate 13	111	41	1.03	0.79–1.51	.98
			Types of mutation		
Failure-free survival	1845	916			
Dukes' B	707	291	1.49	1.16–1.91	.001
Dukes' C	628	385	2.89	2.26–3.69	<.001
Dukes' D	193	145	9.09	6.78–12.20	<.001
All G to A mutations	366	185	1.22	1.02–1.45	.03
All G to C mutations	59	31	1.35	0.94–1.95	.17
All G to T mutations	222	129	1.31	1.09–1.59	.006
Overall survival	1845	819			
Dukes' B	707	258	1.51	1.15–1.99	.002
Dukes' C	628	341	2.99	2.29–3.91	<.001
Dukes' D	193	139	11.18	8.17–15.28	<.001
All G to A mutations	366	153	1.12	0.92–1.35	.36
All G to C mutations	59	30	1.54	1.06–2.24	.03
All G to T mutations	222	119	1.44	1.18–1.75	<.001

*Initial analysis was performed with all mutations combined and other variables, e.g., death, survival time, time to relapse (equals failure time), and Dukes' stage. (†Tumor differentiation was included in a separate analysis with 467 fewer patients than for the other variables). The analysis was then rerun with all mutations combined replaced by all the individual mutations on codon 12 and also aspartate 13 and then with individual mutations replaced with type of mutation. *P* value above .01 indicates that the variable did not reach statistical significance as an independent risk factor.

doing so ($P = .02$). However, 467 patients without differentiation data were dropped from the analysis, which reduced its reliability.

In order to identify the independent effects of each mutation, the analysis was performed a second time when all codon 12 mutations and codon 13 aspartate mutations were individually entered into Cox's proportional hazards model in place of ''any mutation.'' The remaining 18 codon 13 mutations were not included because they were so few. Failure-free survival was again adversely affected by increasing Dukes' stage and by the presence of a valine codon 12 mutation (HR = 1.39; 95% CI = 1.10–1.74; $P = .007$). Overall survival was adversely affected by stage and by the presence of a valine codon 12 mutation (HR = 1.43; 95% CI = 1.13–1.82; $P = .004$).

If individual mutations were replaced by the three categories of mutation in the multivariate model, i.e., G to A or G to T or G to C change on either codon, G to T became an independent marker of both failure-free survival (HR = 1.31; 95% CI = 1.09–1.59; $P = .006$) and overall survival (HR = 1.44; 95% CI = 1.18–1.75; $P<.001$).

Discussion

The aim of this study was to determine whether Ki-ras mutations have prognostic significance. Primary data were obtained for 2721 colorectal cancer patients provided by 22 research groups from 13 countries. This study includes half of all the data previously published in this field and makes a substantial contribution with new data. The study has shown that mutations in the Ki-ras gene are associated with poorer prognosis. In particular, it is any substitution of G by T in this gene at very specific positions, rather than the more common mutation to adenosine, that is associated with increased risk of relapse and death. It is intriguing that this study has also shown that one specific mutation, a change from glycine to valine on codon 12, is particularly associated with an adverse outcome.

In addition to examining the prognostic impact of a mutation, data on tumor and patient details (although not always available in as many patients as the survival data) allow a number of other conclusions to be reached. They include the lack of correlation between mutations and sex, tumor site, or Dukes' stage. However, mutations were more commonly found in well-differentiated or moderately differentiated rather than poorly differentiated tumors. This finding is surprising, since improved differentiation endows tumors with a better prognosis, while the presence of a mutation has the opposite effect. It is possible that inconsistency in the histologic grading of differentiation between centers led to this finding. We believe that our study does not answer this question reliably in the absence of complete information on tumor differentiation in all patients.

A number of other findings cannot be considered as definitive. Although these arise from subgroups constituting a series with more tumors from different individuals than any other yet published, the cohort size is still insufficiently large. However, one conclusion contradicts frequently quoted dogma in that this study has found that sporadic tumors and those arising in patients with familial adenomatous polyposis, hereditary nonpolyposis colorectal cancer, and ulcerative colitis all seem to have the same frequency of mutation. A number of authors *(29–32)* have suggested that Ki-ras mutations are less common in these last three groups of patients compared with those with sporadic tumors. If correct, our finding is important because it challenges some of the assumptions made about the developmental pathways of ''nonsporadic'' tumors.

Potential weaknesses of this study include the failure to persuade all researchers in this field to share their data. This situation may have compromised some conclusions, particularly those related to analysis of individual mutations. Also, the data came from heterogeneous populations whose Ki-ras status was determined for different reasons and by use of different techniques. This is most evident in that the number of patients included with a Dukes' D tumor is less than might be expected if this was a consecutive series. Adjuvant or palliative chemotherapy or radiotherapy could also have skewed survival data. Of the patients in this study, however, no difference in survival was seen between the 131 patients, with or without a mutation, receiving such extra treatments (data not shown).

On the other hand, the patients who have been entered in this study appear to be similar to populations with colorectal cancer reported elsewhere in that the patient characteristics, rates of relapse, and stage-specific survival (data not shown) are in keeping with those of larger studies *(34)*. Second, this study not only has relied on published data but also has included a substantial number of previously unreported patients. Third, data trends seen from most of the participating centers in this study were consistent (Fig. 2). Finally, we have shown that, apart from SSCP, which in this study was used infrequently, no statistically significant difference exists in the number of mutations detected by different methods. For all these reasons, there are grounds to believe that the data presented here represent an accurate analysis of the significance of Ki-ras status in colorectal cancer.

In summary, since the influential early study of Vogelstein et al. *(35)*, it has been widely accepted that Ki-ras mutations are important in the development of colorectal cancer. The RASCAL data are in keeping with this hypothesis inasmuch as the frequency of mutation was found to be similar for every Dukes' stage. In contrast, many commentators have stated that the mutated ras gene is unlikely to be of importance in established colorectal cancer. The RASCAL group takes issue with such a view. This international collaborative study suggests that mutations in the Ki-ras gene are indeed important for the progression and outcome of established colorectal cancer, although some mutations are more important than others.

This collaboration continues, and we invite others to join it. Among other issues to be addressed, we hope to define those patients in whom routine mutation analysis could be of benefit. However, larger numbers of patients will be required to show whether specific mutations could have particular influence at different stages and whether the rarer mutations also convey an adverse effect. In the meantime, other research can now concentrate on why the findings revealed by this study occur. In particular, why does the valine mutation exert a particularly malign effect compared with that of other mutations? Does the early development of a Ki-ras mutation in the adenoma–carcinoma sequence really protect against poor differentiation of these tumors and, if so, how? Finally, perhaps this study offers a fresh rationale for targeting these specific abnormalities with molecu-

lar therapies that, for some patients, may one day provide a new weapon against colorectal cancer.

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Notes

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