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Original Paper

Ki-ras Mutations and Prognosis in Colorectal Cancer

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A total of 191 colorectal adenocarcinomas, obtained from consecutive patients with a median follow-up of 6 years, were studied in order to evaluate the possible association of *Ki-ras* mutations with tumour stage, tumour differentiation and survival time. Resected full-cross tumour samples were screened for *Ki-ras* mutations in codons 12 and 13 using temporal temperature gradient gel electrophoresis (TTGE). *Ki-ras* mutations were detected in 62 (32%) of the samples. The most frequent mutation, observed in 21 samples, was from GGT to GAT changing glycine to aspartic acid in codon 12. The study did not show any association between *Ki-ras* mutations and Dukes' stage or tumour differentiation. Patients with *Ki-ras* mutations had a marginally shorter survival time (median 50 months) compared with patients without (median 59 months), but the difference was not statistically significant. The results indicate that *Ki-ras* gene mutations have no relevant prognostic importance in this cohort of colorectal cancer patients.

Key words: *Ki-ras*, colorectal cancer, tumour stage, tumour differentiation, survival time
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INTRODUCTION

RECENT DATA from clinical trials have shown that peri-operative radiotherapy [1] and/or chemotherapy [2] has substantial value for selected patients with colorectal cancer. The scientific evidence indicates that radiotherapy should be given pre-operatively in order to be most dose-efficient [3]. It has also been proposed that chemotherapy should be initiated intra-operatively or immediately postoperatively in order to obtain optimal effects [4]. The collected experience from randomised trials indicate that short treatment the week after surgery may not be appreciably inferior to a prolonged postoperative course [5]. Accordingly, predictive factors may be required pre-operatively to select adequate treatment.

Colorectal tumorigenesis proceeds through a series of genetic alterations [6]. *Ki-ras* mutations, occurring in up to 50% of colorectal adenocarcinomas, are assumed to be involved early in tumorigenesis [7, 9]. Point mutations in codons 12, 13 and 61 in the *Ki-ras* gene result in amino acid alterations in the $p21^{ras}$ protein and activation of oncogenic potential [10]. $p21^{ras}$ is thought to be involved in the transduction of growth and differentiation signals from activated receptors to downstream protein kinases [7, 10]. Mutations in the *Ki-ras* gene have, in some studies, been shown to correlate with prognosis [11, 12], while these findings have not been seen by others [13–15]. Moreover, some recent studies have reported an association between overexpression of $p21^{ras}$ and poor prognosis [16, 17]. In light of the conflicting information in the literature, we considered it important to explore further the prognostic value of *Ki-ras* gene mutations in a large series of patients resected for colorectal adenocarcinoma with long follow-up.

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PATIENTS AND METHODS

Patients

Tumour samples were collected from 194 consecutive patients resected for colorectal cancer in Uppsala and Falun County from January 1988 to November 1992. One hundred and ninety-one samples were available for analysis. Patient and tumour characteristics are shown in Table 1. The ages ranged from 39 to 92 years (median 72). 167 patients (87%) were resected for cure, Dukes' stages A–C. In the other 24

Table 1. Ki-ras *mutation and its relation to age, gender, tumour stage, tumour differentiation and tumour localisation in colorectal cancer*

	Ki-ras mutations	Number of cancer related deaths *Ki-ras* mutations	
	Yes *n* (%)	Yes *n* (%)	No *n* (%)
Age (years)			
≤ 70 (*n* = 89)	29 (33)	16/29 (55)	19/60 (30)
> 70 (*n* = 102)	33 (32)	14/33 (42)	26/69 (38)
Gender			
Female (*n* = 109)	32 (29)	12/32 (38)	26/77 (34)
Male (*n* = 82)	30 (37)	18/30 (60)	18/52 (35)
Dukes' stage			
A (*n* = 29)	6 (21)	1/6 (17)	2/23 (9)
B (*n* = 98)	35 (36)	10/35 (29)	14/63 (22)
C (*n* = 40)	12 (30)	10/12 (83)	13/28 (46)
D (*n* = 24)	9 (38)	9/9 (100)	15/15 (100)
Tumour differentiation			
Good (*n* = 26)	8 (31)	0/8 (0)	2/18 (11)
Moderate (*n* = 129)	41 (32)	20/41 (49)	31/88 (35)
Poor (*n* = 36)	13 (36)	10/13 (77)	11/23 (48)
Tumour localisation			
Colon (*n* = 125)	43 (34)	22/43 (51)	28/82 (34)
Rectum (*n* = 66)	19 (29)	8/19 (42)	13/47 (28)

patients, distant metastases were detected perioperatively, and consequently underwent a palliative resection. At follow-up (median follow-up was 6 years, 2–106 months) in January 1997, 74 (39%) patients had died from cancer, or from other causes, but with known tumour burden. 28 (15%) patients had died from other causes without any indication of tumour relapse. The median survival time of the 89 living patients was 87 months (range 51–106).

Tumour biopsies

Full-cross tumour biopsies were snap frozen in dry-ice isopenthane and stored at −70°C. Routine biopsies were taken for histopathological classification. The tumours were graded according to WHO classification [18], and staged according to Dukes' classification system [19].

Ki-ras *detection*

GC-clamped polymerase chain reaction (PCR) products were analysed for *K-ras* exon 1 mutations in a DCODE electrophoresis system from BioRad using the TTGE (temporal temperature gradient gel electrophoresis) technique, which is outlined in detail elsewhere [20, 21]. TTGE is based on the same separation principle as denaturant gradient gel electrophoresis (DGGE), described by Fischer and Lermann [22] and constant denaturant gel electrophoresis (CDGE), described by Hovig and colleagues [23], where sequence determined melting characteristics of DNA are exploited to separate fragments with minute differences. In TTGE, the gels contain a uniform denaturant, determined by the theoretical melting behaviour of the PCR products, and a polyacrylamide concentration based on the length of the fragment to be analysed. During electrophoresis, the temperature of the gel increases gradually. Briefly, the PCR reactions were performed by mixing 100 ng template DNA with 25 μM of each dNTP (Perkin Elmer Norge, Etterstad, Oslo, Norway), 10 × Pfu buffer, 2 units cloned Pfu (Stratagene, St Hanshaugen, Olso, Norway), and 50 pmol of each primer (MedProbe, St Hanshaugen, Olso, Norway) 5′-ATG ACT GAA TAT AAA CTT GTG-3′, 5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCGC CCG CCC GCC TCT ATT GTT GGA TCA TAT TC-3′ in a final volume of 50 μl. Cycling parameters were: denaturation 60 sec at 94°C, annealing 60 sec at 53°C, elongation 60 sec at 72°C, for 35 cycles. The PCR products were denatured for 5 min at 94°C and incubated for 1 h at 65°C for heteroduplex formation before analysis using TTGE. The TTGE analyses were performed in 10% polyacrylamide gels with 29% denaturant (100% denaturant = 7 M urea, and 40% v/v formamide), with a temperature ramp rate of 1.7°C/h from 63 to 68°C during the 3 h electrophoresis run. After the run, the gels were stained with SYBR green in 1 × TAE (tris-acetate acid EDTA) for 3 min, followed by washing the 1 × TAE and photographed under UV light. All samples with abnormal migration bands were re-analysed together with known mutations to determine the sequence alteration. The sensitivity of the TTGE analysis has been estimated to detect a mutation in 1% of cells (data not shown).

Statistical analyses

The Cox proportional Hazards model was used in both the univariate and the multivariate survival analyses. Survival curves were constructed using the Kaplan–Meier method, and differences between curves were tested using the log-rank test. The Chi squared test was used to test for differences in distribution between groups. The statistical software Statistica (Statsoft Inc.®, version 5.0) was used for the analyses. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Ki-ras point mutations were detected in 62 (32%) of the 191 tumour samples. We found 51 tumour biopsies with mutations in codon 12, and 11 with mutations in codon 13. The most frequent mutation in codon 12, observed in 22 tumour samples, was a G to A transition changing a gly to asp. The mutations in codon 13 were all G to A transitions in the second base of the codon, also changing a gly to asp. An example of a TTGE gel with test samples and known mutants is shown in Figure 1.

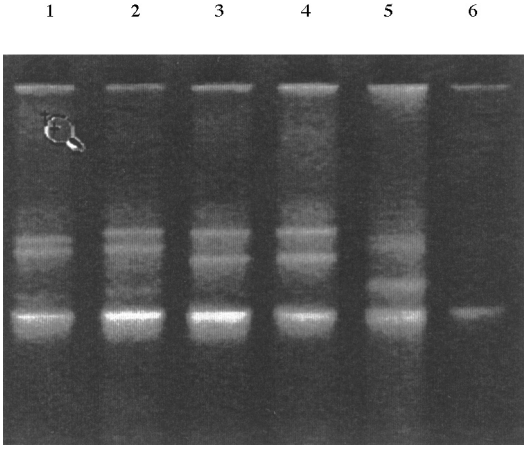

Figure 1. Temporal temperature gradient gel electrophoresis (TTGE) gel, stained with SYBR green, of different *Ki-ras* exon 1 control mutants (lanes 1–3), test samples (lanes 4, 5) and a normal control (lane 6). The known mutant in lane 1 had the sequence alteration GGC → GAC, in lane 2 GGT → GAT and in lane 3 GGT → GCT. Note that the band pattern of one of the test samples in lane 4 is identical to the mutation in lane 3.

There were no significant differences in the proportions of tumours with *Ki-ras* mutations according to age, sex, tumour site and tumour differentiation (Table 1).

Ki-ras *mutations and prognosis*

The proportions of cancer-specific deaths in different groups of patients are presented in Table 1. Overall, 22/62 (35%) of the patients with *Ki-ras* positive tumours were alive after 5 years, while the corresponding figure for patients with no mutation was 53 (41%), ($P = 0.6$). There was no statistically significant difference in survival between patients with *Ki-ras* mutations (median 50 months, log-rank, $P = 0.09$) compared with those without (median 59 months). Of 53 patients (Dukes' stages A–C) with *Ki-ras* mutated tumours, 21 (40%) had a cancer-related death compared with 29/114 (25%) patients without *Ki-ras* mutation ($P = 0.15$). Univariate analyses showed significant correlations with survival for Dukes' stage C and D and tumour differentiation, but not for *Ki-ras* mutation (Table 2). In a multivariate analysis including all patients, the Dukes' stage was the most important prognostic factor for survival ($P < 0.001$), whereas the *Ki-ras* mutation did not show any significant correlation with survival (Table 3). If the Dukes' stage, not known preoperatively, was excluded from the analysis, tumour differentiation was of most importance but no significant information was provided by the *Ki-ras* mutations (data not shown).

Table 2. Univariate analyses showing the effects of age, gender, Ki-ras, *Dukes' stage, and tumour differentiation on survival in patients potentially curatively resected for colorectal cancer*⋆

Variable	β	SEM (β)	*P* value	RH
Age	0.015	0.011	NS	1.0
Gender				
Male	0.00 (ref.)			1.0
Female	−0.323	0.233	NS	0.7
Ki-ras				
No mutation	0.00 (ref.)			1.0
Mutation	0.404	0.237	0.09	1.5
Dukes' stage				
A	0.00 (ref.)			1.0
B	0.985	0.6124	NS	2.7
C	2.067	0.6153	0.001	7.9
D	3.855	0.6271	0.0001	47.2
Tumour differentiation				
Good	0.00 (ref.)			1.0
Moderate	1.834	0.721	0.01	6.3
Poor	2.406	0.741	0.001	11.1

RH, relative hazard; SEM, standard error of the mean; ref., reference category; NS, not significant.
⋆Number of patients (cancer-specific deaths) = 191(74).

Table 3. Multivariate analysis showing the effects of age, sex, Ki-ras *mutation, Dukes' stage and tumour differentiation on survival in patients operated on for colorectal cancer*⋆

Variable	β	SEM (β)	*P* value	RH
Age	0.027	0.013	0.03	1.03
Gender				
Male	0.00 (ref.)			1.0
Female	−0.531	0.248	0.03	0.58
Ki-ras				
No mutation	0.00 (ref.)			1.0
Mutation	0.232	0.250	0.42	1.26
Dukes' stage				
A	0.00 (ref.)			1.0
B	1.060	0.617	NS	2.9
C	2.051	0.622	0.001	7.8
D	3.857	0.639	0.0001	47.3
Tumour differentiation				
Good	0.00 (ref.)			1.0
Moderate	1.614	0.724	0.03	5.0
Poor	1.846	0.750	0.01	6.5

RH, relative hazard; SEM, standard error of the mean; ref., reference category; NS, not significant.
⋆Number of patients (cancer-specific deaths) = 191(74).

DISCUSSION

In the present study, we found *Ki-ras* gene mutations in 32% of the tumour samples, a frequency consistent with other studies [8, 11, 13, 24]. Our tumour samples were collected consecutively, and only 2% of the total number of samples were excluded due to insufficient samples. No patient was lost to follow-up.

In this study, we could not find any significant correlation between *Ki-ras* mutations and prognosis, although a weak trend was seen. This was also found when follow-up data on 2667 patients from 20 research groups in 12 countries were pooled and analysed [24]. In that analysis, only a slightly higher risk of recurrence (hazards ratio (HR) 1.28, 95% confidence interval (CI) 1.23–1.46) and increased risk of death (HR 1.27, 95% CI 1.1–1.45) were seen. Previously, in some studies [11, 12] a higher proportion of *Ki-ras* mutations in tumours in patients with recurrent disease and worse prognosis have been observed, whereas this was not seen in other studies [14, 15]. Thus, taken together with the present results, it is possible that the existense of *Ki-ras* gene mutations in a tumour may indicate a slightly poorer outcome than if no mutations are found. However, this will be of no practical relevance as other much stronger prognostic parameters are available [25].

The present study did not show any correlation between *Ki-ras* gene mutations and tumour stage, tumour differentiation or tumour localisation. Similar results have been demonstrated in other studies [13, 15], while different proportions were recently reported with higher figures in the left colon and rectum [26].

It has been proposed that the *Ki-ras* gene mutation is an early event, which may cause the adenoma to increase in size and, subsequently, may progress into a malignant adenocarcinoma [9]. This implies that the *Ki-ras* mutation occurs before the conversion to malignant carcinoma. Progression from severe dysplasia to invasion can occur by pathways which are not dependent on *ras* mutations. Many factors other than mutations in the *Ki-ras* gene are important for progression of colorectal cancer. Several studies have suggested that later genetic changes, involving *p53* [27], DCC [28] and *nm23* [29], are important for tumour progression and, thus, prognosis. However, the prognostic relevance of these 'later occurring events' has also been questioned. In several studies, no correlation with advanced stage or prognosis has been detected in patients with *p53* overexpressing tumours [30], or in tumours with reduced *nm23-HI* expression [31]. Many genetic changes are involved in colorectal tumorigenesis, thereby making the assessment of the prognostic significance of each alteration difficult.

1. Swedish Rectal Cancer Trial. Improved survival with preoperative radiotherapy in resectable rectal cancer. *New Engl J Med* 1997, **336**, 980–987.
2. Moertel CG. Chemotherapy for colorectal cancer. *N Engl J Med* 1994, **330**, 1136–1142.
3. Glimelius B, Isacsson U, Jung B, Påhlman L. Radiotherapy in addition to radical surgery in rectal cancer: evidence for a dose–response effect favoring preoperative treatment. *Int J Radiat Oncol Biol Phys* 1997, **37**, 281–287.
4. Graf W, Westlin JE, Påhlman L, Glimelius B. Adjuvant intraperitoneal 5-flourouracil and intravenous leucovorin after colorectal cancer surgery: a randomized placebo-controlled phase I study. *Int J Colorect Dis* 1992, **9**, 35–39.
5. Liver Infusion Meta-analysis Group. Portal vein chemotherapy for colorectal cancer: a meta-analysis of 4000 patients in 10 studies. *J Natl Cancer Inst* 1997, **89**, 497–505.
6. Fearon E, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990, **61**, 759–767.
7. Forrester K, Almoguera C, Han K, Grizzle WE, Perucho M. Detection of high incidence of K-ras oncogenes during human colon tumorigenesis. *Nature* 1987, **327**, 298–303.
8. Capella G, Cronauer-Mitra S, Peinado M, Perucho M. Frequency and spectrum of mutations at codon 12 and 13 of the c-K-ras gene in human tumors. *Environ Health Perspect* 1991, **93**, 125–131.
9. Fearon E. K-ras gene mutation as a pathogenic and diagnostic marker in human cancer. *J Natl Cancer Inst* 1993, **85**, 1978–1981.
10. McCormick F. Signal transduction. How receptor turns ras on. *Nature* 1993, **63**, 15–16.
11. Benhattar J, Losi L, Chaubert P, Givel JC, Costa J. Prognostic significance of K-ras mutations in colorectal carcinoma. *Gastroenterology* 1993, **104**, 1044–1048.
12. Finkelstein S, Przygodski R, Pricolo V, *et al.* Prediction of biologic aggressiveness in colorectal cancer by p53/K-ras-2 topographic genotyping. *Molec Diagn* 1996, **1**, 5–28.
13. Morrin M, Kelly M, Barret N, Delaney P. Mutations of Ki-ras and p53 genes in colorectal cancer and their prognostic significance. *Gut* 1994, **35**, 1627–1631.
14. Norheim-Andersen S, Lövig T, Breivik J, *et al.* K-ras mutations and prognosis in large-bowel carcinomas. *Scand J Gastroenterol* 1997, **32**, 62–69.
15. Bell SM, Scott N, Cross D, *et al.* Prognostic value of p53 overexpression and c-Ki-ras gene mutations in colorectal cancer. *Gastroenterology* 1993, **104**, 57–64.
16. Miyahara M, Saito T, Kaketani K, *et al.* Clinical significance of ras p21 overexpression for patients with an advanced colorectal cancer. *Dis Colon Rectum* 1991, **34**, 1097–1102.
17. Sun XF, Wingren S, Carstensen JM, *et al.* Ras p21 expression in relation to DNA ploidy, S-phase fraction and prognosis in colorectal adenocarcinoma. *Eur J Cancer* 1991, **27A**, 1646–1649.
18. Morson B, Sobin L. Histological typing of intestinal tumors. *International Histological Classification of Tumours*, Vol. 15. Geneva, WHO, 1976.
19. Dukes CE, Bussey HJR. The spread of rectal cancer and its effect on prognosis. *Br J Cancer* 1958, **12**, 309–320.
20. Børresen-Dale AL, Lystad S, Langerød A. Temporal temperature gradient gel electrophoresis (TTGE) compared with denaturing gradient gel electrophoresis (DGGE) and constant denaturant gel electrophoresis (CDGE) in mutation screening. *Bioradiation US/EG Bulletin* 2133, 1997.
21. Børresen-Dale AL. In Pfeifer, ed. *Constant Denaturant Gel Electrophoresis (CDGE) in Mutation Screening in Technologies for Detection of DNA Damage and Mutation*, Vol. 20. New York, Plenum Press, 1996, 267–279.
22. Fischer SG, Lerman LS. DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels. Corresponding with melting theory. *Proc Natl Acad Sci USA* 1983, **80**, 1579–1583.
23. Hovig E, Smith-Sørensen B, Brøgger A, Børresen A-L. Constant denaturant gel electrophoresis, a modification of denaturing gradient gel electrophoresis, in mutation detection. *Mutation Res* 1991, **262**, 63–71.
24. Andreyev J, Norman A, Cunningham D, Oates J, Clarke P. Ki-ras mutations in 2,667 patients with colorectal cancer; RASCAL collaboration (abstract). *Proc ASCO* 1997, **16**, 257a.
25. Lindmark G, Gerdin B, Påhlman L, Bergström R, Glimelius B. Prognostic predictors in colorectal cancer. *Dis Colon Rectum* 1994, **37**, 1219–1227.
26. Elnatan J, Goh HS, Smith DR. C-Ki-ras activation and the biological behaviour of proximal and distal colonic adenocarcinomas. *Eur J Cancer* 1996, **32A**, 491–497.
27. Goh HS, Yao J, Smith DR. p53 point mutation and survival in colorectal cancer patients. *Cancer Res* 1995, **55**, 5217–5221.
28. Jen J, Hougen K, Piandosi S, *et al.* Allelic loss of chromosome 18q and prognosis in colorectal cancer. *N Engl J Med* 1994, **331**, 213–221.
29. Martinez JA, Prevot S, Nordlinger B, *et al.* Overexpression of nm23-H1 and nm23-H2 genes in colorectal carcinomas and loss of nm23-H1 expression in advanced tumour stages. *Gut* 1995, **37**, 712–720.
30. Kressner U, Lindmark G, Gerdin B, Påhlman L, Glimelius B. Immunohistochemical p53 staining is of limited value in the staging and prognostic prediction of colorectal cancer. *Anticancer Res* 1996, **16**, 951–958.
31. Lindmark G. Nm 23-H1 immunohistochemistry is not useful as a predictor of metastatic potential of colorectal cancer. *Br J Cancer* 1996, **74**, 1413–1418.

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