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K-ras oncogene mutations in sporadic colorectal cancer in The Netherlands Cohort Study

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Activation of *K-ras* oncogene has been implicated in colorectal carcinogenesis, being mutated in 30–60% of the adenocarcinomas. In this study, 737 incident colorectal cancer (CRC) patients, originating from 120 852 men and women (55–69 years at baseline) participating in the Netherlands Cohort Study (NLCS), were studied in order to evaluate subgroups with respect to *K-ras* mutation status. Mutation analysis of the exon 1 fragment of the *K-ras* oncogene, spanning codons 8–29, was performed on archival colorectal adenocarcinoma samples of all patients using macrodissection, nested PCR and direct sequencing of purified fragments. The method of mutation detection was validated by the confirmation of reported *K-ras* status in CRC cell lines, a good correlation between fresh-frozen and routinely fixed, paraffin-embedded tissue, a detection limit of 5% mutated DNA and a good reproducibility. Various types of *K-ras* mutations were evaluated with respect to tumour sub-localization, Dukes' stage and tumour differentiation. In 37% (271/737) of the patients, the exon 1 fragment of *K-ras* gene was found to be mutated. The predominant mutations are G>A transitions and G>T transversions, and codons 12 and 13 are the most frequently affected codons. Patients with a rectal tumour were found to have the highest frequency of G>T transversions as compared with patients with a colon or rectosigmoid tumour. This difference appeared to be confined to women with a rectal tumour harbouring G>T transversions. No significant differences were observed for Dukes' stage with respect to types of *K-ras* mutation, which does not support direct involvement of the *K-ras* oncogene in adenocarcinoma progression. The equal distribution of *K-ras* mutations among cases with or without a family history of colorectal cancer argues against an important role for this mutation in familial colorectal cancer, and could imply that *K-ras* mutations are more probably involved in environmental mechanisms of colorectal carcinogenesis.

Abbreviations: CRC, colorectal cancer; GDP, guanosine-diphosphates; GTP, guanosine-triphosphates; NLCS, Netherlands Cohort Study.

Introduction

The development of colorectal cancer (CRC) is a multi-step process characterized by the accumulation of genetic alterations (1,2). The Fearon and Vogelstein model assumes the involvement of the *APC* (Adenomatous Polyposis Coli) gene in adenoma formation and the *K-ras* oncogene in the transition from intermediate adenomas to carcinomas in sporadic CRC (1).

The *K-ras* oncogene has been found mutated in 10–15% of the screened adenomas <1 cm and in 30–60% of adenomas >1 cm. Also, 30–60% of the adenocarcinomas have a *K-ras* mutation (3–5). It was suggested, therefore, that a mutated *K-ras* gene contributes to the transition of an intermediate adenoma to a late adenoma or carcinoma (2). The *K-ras* gene product, a 21 kDa protein located at the inner plasma membrane, is involved in the transduction of mitogenic signals. The Ras protein is activated transiently as a response to extracellular signals such as growth factors, cytokines and hormones that stimulate cell surface receptors (6). The hallmark of Ras function is a switch between an inactive state, in which the proteins are bound to guanosine-diphosphates (GDP) and an active state in which conversion to guanosine-triphosphates (GTP) has occurred. This transit is governed by two types of regulatory proteins: GDP–GTP exchange factors that catalyse the GDP–GTP exchange and GTPase-activating proteins that enhance the intrinsic capacity of Ras proteins to hydrolyse GTP into GDP, thereby returning Ras to the inactive state (7).

Mutant, activated forms of Ras proteins have an impaired intrinsic GTPase activity, which renders the protein resistant to inactivation by regulatory GTPase-activating proteins (4). Approximately 90% of the activating mutations are found in codons 12 (wild-type GGT) and 13 (wild-type GGC) of exon 1 and ~5% in codon 61 (wild-type CAA) located in exon 2 (8–10). Previous studies from various countries have revealed specific point mutations in codons 12 and 13. The most frequently observed types of mutations are G>A transitions (11) and G>T transversions (12) and these alterations were found associated with gender and sub-localization of the tumour (9).

To date, frequencies and specific types of point mutations in the *K-ras* oncogene in colorectal cancer have been investigated in several studies (4,5,10,13). These reports were generally based on small numbers of selected patients. In the current study, however, the frequency of *K-ras* mutations is studied in a large series of unselected, incident colorectal cancer patients from The Netherlands identified in a prospective cohort study. Potential differences in tumour sub-localization, Dukes' stage and tumour differentiation with respect to presence and type of the *K-ras* mutations are studied.

Materials and methods

Study population

The participants in this study are incident colorectal cancer cases from the Netherlands Cohort Study (NLCS) on diet and cancer. The NLCS has been

described in detail elsewhere (14). Briefly, the prospective study was initiated in 1986 and includes 58 279 men and 62 573 women, aged between 55 and 69 years old, who completed a self-administered questionnaire on diet, family history of cancer and other risk factors for cancer at baseline. The entire cohort is being monitored for cancer occurrence by annual record linkage to the Netherlands Cancer Registry (NCR) and to PALGA, a nationwide database of pathology reports (15). From 1989 until 1994, with exclusion of the first 2.3 years of follow up due to incomplete nationwide coverage of PALGA, 819 incident cases with histologically confirmed colorectal cancer have been identified. The PALGA database was also used to identify the location of tumour tissue storage in the Dutch PA laboratories. Colorectal cancer was classified according to site as follows. Proximal colon: cecum through transverse colon (ICD-O codes: 153.0, 153.1, 153.4, 153.5, 153.6); distal colon: splenic flexure through sigmoid colon (ICD-O codes: 153.2, 153.3, 153.7); rectosigmoid (ICD-O code 154.0) and rectum (ICD-O code 154.1). Information about age at diagnosis, gender and family history of colorectal cancer (at baseline) was retrieved from the NLCS database. Information about tumour sub-localization, Dukes' stage and differentiation of the tumour was retrieved from the Netherlands Cancer Registry database.

Tissue samples

Tumour material was collected after approval by the Medical Ethical Committees (MEC) of the Maastricht University, PALGA and the NCR. Subsequently, all pathology laboratories in the Netherlands agreed to make relevant tissue samples available for this study. Tumour tissue sample collection started in August, 1999 and was completed in December, 2001. The 819 tissue samples were distributed among 54 pathology laboratories throughout The Netherlands, and only 44 (5%) tumour tissue samples could not be traced. Finally, 775 (95%) of the eligible tissue samples have been retrieved and 737 (90%) of the tissue samples contained sufficient tumour material as confirmed by a pathologist (A.d.B.) and hence were available for molecular analyses. Archival tissue sample blocks were registered and coded using a consecutive, unique identification number.

Five CRC cell lines, i.e. HT29, Colo205, CaCo2, SW480 and HCT116 [American Type Culture Collection (ATCC), Rockville, MD] were used to check the specificity of *K-ras* mutation detection. In order to validate mutation analysis on paraffin-embedded tissue, 10 fresh CRC specimens were divided into two adjacent tissue blocks, one of which was fresh-frozen, and one routinely fixed and embedded in paraffin. These specimens were obtained from patients who did not participate in the NLCS.

DNA isolations

Sections (5 μm) were cut from paraffin-embedded tumour tissue blocks and stained with haematoxylin & eosin (H&E) for histopathological examination. For DNA isolation, five 20 μm sections of tumour tissue were used. Deparaffination of the sections was performed and, using the H&E section as a reference, tumour tissue was macrodissected from the normal colonic epithelium and scraped off.

Genomic DNA was extracted from macrodissected tumour tissue using the Puregene® DNA isolation kit (Gentra Systems). Briefly, 475 μl cell lysis solution and 25 μl proteinase K stock solution (20 mg/ml, obtained from Qiagen, St Louis, MO) were added to the tissue samples and incubated overnight at 55°C. Subsequently, DNA was extracted for 72 h at 37°C, protein was removed and DNA was precipitated using 100% 2-propanol and dissolved in hydration buffer. The DNA concentration and purity was measured at 260 and 280 nm.

DNA from fresh, unfixed CRC cell lines and the fresh-frozen tissue samples was extracted as described for paraffin-embedded sections.

Mutation analysis

An exon 1 fragment of the *K-ras* gene was amplified from isolated, genomic DNA using a nested PCR approach. In the first reaction, a fragment of 179 bp was generated, using the sense primer 5′-AGG CCT GCT GAA AAT GAC TGA ATA-3′ and antisense primer 5′-CTG TAT CAA AGA ATG GTC CTG CAC-3′. The annealing temperature was 58°C. The resulting fragment was used as a template to amplify a 114 bp fragment spanning codons 8–29. This PCR is performed using the biotinylated, sense primer 5′-AAA ATG ACT GAA TAT AAA CTT GTG G-3′ and the antisense primer 5′-CTC TAT TGT TGG ATC ATA TTC GTC-3′ at an annealing temperature of 50°C. The inside products were checked for purity and size by electrophoresis on a 2% agarose gel and subsequently used for direct sequencing.

Mutations in the exon 1 fragment of the *K-ras* gene were detected by direct sequencing with a solid phase sequencing kit (Amersham Pharmacia) using the ALFexpress II DNA sequencer (Pharmacia Biotech). In brief, the biotinylated PCR product is captured on a sequencing comb coated with streptavidin. After removal of the non-biotinylated strands by alkaline denaturation, the remaining immobilized strand was used as a template for dideoxy sequencing reactions with a Cy5 labelled primer 5′-CTC TAT TGT TGG ATC ATA TTC GTC CAC-3′ and T7 DNA polymerase. The sequence profile is analysed on the ALFexpress II DNA Analysis System. Evaluation was independently performed using ALFwin software (Amersham Pharmacia Biotech) by two observers, based on the criterion that an increase of at least 5% is observed for the mutant peak as well as a decrease of at least 5% in the wild-type peak, relative to the wild-type pattern in the same sequence run. Data entry was performed blindly by two independent observers.

The mutation detection limit was determined by mixing wild-type DNA from the cell line CaCo2 and the homozygously mutated cell line SW480 in amounts varying from 0–100%.

Statistical analysis

The overall frequency of *K-ras* mutations as well as the type of mutation and affected codon was computed for all 737 cases with respect to age at diagnosis, gender, family history of colorectal cancer, tumour sub-localization, Dukes' stage and tumour differentiation. Differences in mean values of age at diagnosis as a continuous variable was evaluated using the Student's *t*-test. Differences in the categorical variables gender, family history of colorectal cancer, tumour sub-localization, Dukes' stage and tumour differentiation between patients without and with *K-ras* mutations were evaluated for significance with the χ^2-test. In addition, differences in tumour sub-localization, Dukes' stage and tumour differentiation between patients without a *K-ras* mutation and patients with one or more G>A transition or G>T transversion or patients with at least one codon 12 or codon 13 mutation were evaluated with the χ^2-test. A *P*-value of 0.05 or less was considered statistically significant. Statistical analyses were performed using the SPSS software (version 9.0).

Results

Validation of techniques

In the colorectal cancer cell lines HT29, Colo205 and CaCo2, wild-type *K-ras* was found, whereas homozygously mutated alleles in SW480 and heterozygously mutated alleles in HCT116 cells were revealed with direct sequence analysis. For the SW480 line, a GGT to GTT mutation in codon 12 was observed and for the HCT116 line, a GGC to GAC mutation in codon 13.

The effect of tissue processing was assessed by comparing an adjacent fresh-frozen and a paraffin-embedded tissue block in a series of 10 fresh colon tumour specimens. In nine specimens, the *K-ras* status in the paraffin-embedded tissue was identical to fresh, unfixed tissue, i.e. six specimens with wild-type *K-ras*, one with a G>T transversion at the second position of codon 12, one with a G>C transversion at the second position of codon 12 and one with a G>C transversion at the second position of codon 13. In one specimen, a G>C transversion at the third position of codon 19 was observed in the routinely fixed, paraffin-embedded tissue, but not in DNA extracted from the fresh tissue.

The detection limit of mutation detection was determined by mixing wild-type DNA isolated from the CaCo2 cell line with decreasing concentrations of mutated DNA which was prepared from the homozygously mutated colorectal cell line SW480. The lowest level of detection was 5% mutant DNA in a background of wild-type DNA as found in three independent experiments.

To establish the reproducibility of the mutation analysis, 32 NLCS adenocarcinoma specimens were subjected twice to the complete procedure, from tissue sectioning to DNA sequencing. In 88% (28/32) of the samples, the same *K-ras* status was observed in the duplicate experiments.

Types of mutations

In exon 1 of the *K-ras* oncogene, a total of 281 mutations were found in 271 (37%) out of 737 patients. No significant differences were observed in mean age at diagnosis (67.75 ± 4.21

versus 68.29 ± 4.32), gender (55 versus 57%) and family history of colorectal cancer (11 versus 9%) between patients without and patients with one or more *K-ras* oncogene mutations in the tumour (Table I).

For 10 patients, two different mutations were observed in exon 1. In six of these patients both mutations were found in codon 12, and the other four patients showed one mutation in codon 12 or codon 13 and one mutation in another codon. In 18 patients without aberrations in codon 12 or codon 13, a mutation was found in codons 8, 9, 10, 15, 16, 19, 20 or 25.

Table II summarizes the frequencies of genetic aberrations by type of mutation, affected codons and corresponding amino acids in the exon 1 fragment of the *K-ras* oncogene. The most frequently observed mutations in the gene are G>A transitions, G>T transversions and G>C transversions, i.e. 55 (155/281 mutations), 32 (90/281 mutations) and 9% (24/281 mutations), respectively. Of the total number of mutations in the exon 1 fragment, 72% (201/281) was observed in codon 12 (GGT) and 22% (62/281) in codon 13 (GGC). In codon 12, the GAT codon (37%) leading to aspartic acid and the GTT codon (35%) leading to valine were the most frequently observed (Table 2). In codon 13 the G>A transition at the second base, which would lead to substitution of a glycine by aspartic acid, was by far the predominant mutation (94%). Furthermore, the point mutations (17/277) observed in codons 8, 10, 15, 16, 19, 20 and 25 were all transitions. In one case, an insertion of six nucleotides was observed in codon 9, leading to one altered codon and two inserted codons, however, without a frameshift in the gene (Table II). The only alteration in the protein is the insertion of two extra amino acids.

Tumour sub-localization

Patients with rectal tumours have relatively the highest frequency of *K-ras* mutations as compared with patients with proximal or distal colon tumours or patients with a rectosigmoid tumour (42 versus 38%, 30 and 40%, respectively, $P = 0.09$) (Table III). Moreover, different tumour sub-localizations showed different frequencies of G>T transversions as well as different frequencies of codon 12 or codon 13 mutations. Patients with a rectal tumour have the highest frequency of G>T transversions (16 versus 11%, 9 and 14% for proximal, distal and rectosigmoid tumours, respectively, $P = 0.22$) and codon 12 mutations (34 versus 25%, 22 and 25%, respectively, $P = 0.07$) (Table III). It should be noted that most G>T transversions are confined to codon 12 (Table II). Patients with a tumour in the rectosigmoid, however, have the highest frequency of codon 13 mutations (14 versus 11%, 5 and 6% for proximal, distal and rectal tumour, respectively, $P = 0.03$) (Table III). Stratification by gender, in addition, reveals that women with a rectal tumour have the highest prevalence of *K-ras* mutations (53 versus 37%, 23 and 37% for proximal, distal and rectosigmoid tumour, respectively, $P = 0.001$). This difference was confined, in particular, to the G>T transversions (27% for rectal tumour versus 8%, 11 and 7% for

Table I. Characteristics of CRC cases ($n = 737$) subdivided into cases without a *K-ras* ($n = 466$) mutation and cases with at least one *K-ras* mutation ($n = 271$)

	Total cases ($n = 737$)	Cases with wild-type *K-ras* ($n = 466$)	Cases with *K-ras* mutation ($n = 271$)	*P*-value[a]
Age at diagnosis (mean ± SD)	67.95 ± 4.26	67.75 ± 4.21	68.29 ± 4.32	0.10
Gender (men)	410 (56%)	257 (55%)	153 (57%)	0.73
Family history of CRC (yes)	76 (10%)	53 (11%)	23 (9%)	0.21

[a]Comparing cases with at least one *K-ras* mutation to cases without a *K-ras* mutation.

Table II. Number and type of mutations, affected codons and corresponding altered amino acids in exon 1 of the *K-ras* gene

Codon[a]	Type of point mutation[a]	Number of point mutations	Wild-type codon[a] (amino acid[b])	Mutated codon[a] (amino acid[b])	Putative altered amino acid
8	A>G	3 (1%)	GTA (val)	GTG (val)	3
9	Insertion[c]	1 (0.4%)	GTT (val)	GTG (val)-GAG (glz)-CTT (leu)	1
10	G>A	1 (0.4%)	GGA (gly)	AGA (arg)	1
12	G>A	91 (33%)	GGT (gly)	GAT (asp)	75 (37%)
				AGT (ser)	16 (8%)
	G>T	87 (31%)		GTT (val)	70 (35%)
				TGT (cys)	17 (8%)
	G>C	23 (8%)		GCT (ala)	16 (8%)
				CGT (arg)	7 (3%)
13	G>A	58 (21%)	GGC (gly)	GAC (asp)	58 (94%)
	G>T	3 (1%)		TGC (arg)	3 (5%)
	G>C	1 (0.4%)		CGC (cys)	1 (2%)
15	G>A	1 (0.4%)	GGC (gly)	AGC (ser)	1
16	G>A	1 (0.4%)	AAG (lys)	AAA (lys)	1
19	G>A	1 (0.4%)	TTG (leu)	TTA (leu)	1
20	C>T	8 (3%)	ACG (thr)	ATG (met)	8
25	G>A	2 (0.8%)	CAG (glu)	CAA (glu)	2

[a]For 10 patients, two mutations were found and these are included in this table.
[b]gly, glycine; asp, aspartic acid; ser, serine; val, valine; cys, cysteine; ala, alanine; arg, arginine; thr, threonine; met, methionine; glu, glutamine; leu, leucine; glz, glutamic acid.
[c]The insertion is six nucleotides (TGGAGC), located after the first position of codon 9, resulting in three altered codons.

proximal and distal colon tumour and rectosigmoid tumour, respectively, $P = 0.002$) (Table IV). We also evaluated the *K-ras* mutational status for tumours in the proximal and distal colon and for tumours in the colon versus the rectum (tumours in the rectosigmoid were excluded for this analysis). It was found that patients with a proximal colon tumour had a higher frequency of G>A transitions as compared with patients with a distal tumour ($P = 0.11$) (Table III). This difference in frequencies of G>A transitions with respect to proximal and distal colon tumours was more pronounced for women ($P = 0.02$) (Table IV). Patients with a rectal tumour showed a relatively higher frequency of *K-ras* mutations ($P = 0.08$), and in particular G>T transversions ($P = 0.06$), as compared with patients with a colon tumour. Again, this difference was due to the high frequency of G>T transversions in rectal tumours observed in women ($P = 0.0003$) (Table IV).

Dukes' stage

Most tumours were staged as Dukes B (35%, 238/685), and tumours with Dukes D stage (12%, 85/685) constitute the smallest group (Table III). Patients with a Dukes A staged tumour more often have G>A transitions (26 versus 16, 20 and 19% for Dukes B, C and D, respectively; $P = 0.13$), whereas patients with a Dukes D staged tumour have relatively a higher frequency of G>T transversions (18 versus 8, 15 and 9% for Dukes A, B and C, respectively; $P = 0.04$). No clear differences were observed in the frequencies of codon 12 or

Table III. Characterization of *K-ras* mutations at gene and codon level according to tumour sub-localization, Dukes' stage and differentiation of the tumour

	Total CRC cases ($n = 737$)[d]	Wild-type *K-ras* /it> ($n = 466$)	*K-ras* mutation ($n = 271$)[d]	Point mutation			Affected codon	
				G>A transition ($n = 153$)[d,e]	G>T transversion ($n = 88$)[d,e]	G>C transversion ($n = 23$)[d,e]	Codon 12 ($n = 195$)[d,e]	Codon 13 ($n = 62$)[d,e]
Sub-localization[a]								
Proximal colon	240	149 (62%)	91 (38%)	55 (23%)	27 (11%)	7 (3%)	61 (25%)	26 (11%)
Distal colon	224	156 (70%)	68 (30%)	38 (17%)	20 (9%)	6 (3%)	50 (22%)	12 (5%)
Rectosigmoid	85	51 (60%)	34 (40%)	20 (24%)	12 (14%)	2 (2%)	21 (25%)	12 (14%)
Rectum	176	102 (58%)	74 (42%)	37 (21%)	28 (16%)	9 (5%)	60 (34%)	11 (6%)
P-value[b]			0.09	0.39	0.22	0.48	0.07	0.03
Dukes' stage[c]								
A	179	114 (64%)	65 (36%)	46 (26%)	15 (8%)	2 (1%)	46 (26%)	17 (9%)
B	238	154 (65%)	84 (35%)	39 (16%)	36 (15%)	7 (3%)	61 (26%)	18 (8%)
C	183	120 (66%)	63 (34%)	37 (20%)	17 (9%)	9 (5%)	43 (23%)	17 (9%)
D	85	47 (55%)	38 (45%)	16 (19%)	15 (18%)	4 (5%)	26 (31%)	8 (9%)
P-value[b]			0.40	0.13	0.04	0.17	0.68	0.88

[a]For eight patients without a *K-ras* mutation and four patients with at least one mutation, the site of localization in the colon could not be determined (colon, NOS).
[b]Comparing patients without a *K-ras* mutation to patients with at least one *K-ras* mutation, patients with at least one G>A transition ($n = 153$) or those with at least one G>T ($n = 88$) or G>C ($n = 23$) transversion.
[c]For 21 patients without a *K-ras* mutation and 31 patients with at least one *K-ras* mutation, information on Dukes' stage was not available.
[d]For 10 patients, two mutations were found. These patients were included in the analyses and treated as patients with at least one *K-ras* mutation, etc.
[e]The frequencies of G>A transition, G>T or G>C transversion per subsite were calculated by dividing the number of patients with G>A transitions, for example, through the total number of CRC cases.

Table IV. Stratification by gender with respect to tumour sub-localization

	No *K-ras* mutation	*K-ras* mutation[c]	G>A transition[c]	G>T transversion[c]	G>C transversion[c]
Men					
Sub-localization[a]	252	151	87	47	11
Proximal colon	71 (61%)	45 (39%)	25 (22%)	17 (15%)	0 (0%)
Distal colon	84 (64%)	47 (36%)	27 (21%)	10 (8%)	6 (5%)
Rectosigmoid	25 (57%)	19 (43%)	9 (20%)	9 (21%)	1 (2%)
Rectum	72 (64%)	40 (36%)	24 (21%)	11 (9%)	5 (4%)
P-value[b]		0.77	0.99	0.07	0.13
Women					
Sub-localization[a]	206	116	66	40	12
Proximal colon	78 (63%)	46 (37%)	30 (24%)	10 (8%)	7 (6%)
Distal colon	72 (77%)	21 (23%)	11 (12%)	10 (11%)	0 (0%)
Rectosigmoid	26 (63%)	15 (37%)	11 (27%)	3 (7%)	1 (2%)
Rectum	30 (47%)	34 (53%)	13 (20%)	17 (27%)	4 (6%)
P-value[b]		0.001	0.09	0.002	0.10

[a]For five male patients and three female patients without a *K-ras* mutation and two male patients and two female patients with at least one mutation, the site of localization in the colon could not be determined (colon, NOS)
[b]Comparisons between patients without a *K-ras* mutation and patients with at least one *K-ras* mutation, patients with at least one G>A transition, G>T or G>C transversion.
[c]The frequencies of G>A transition, G>T or G>C transversion per sub-localization and gender was calculated by dividing the number of patients with G>A transitions, for example, through the total number of CRC patients.

codon 13 mutations with respect to the Dukes' stage of the tumour (Table III).

Tumour differentiation

No differences were observed in frequencies of *K-ras* mutations, G>A transitions, G>T or G>C transversions or mutations in codon 12 or codon 13 ($P > 0.05$) with respect to tumour differentiation.

Discussion

Overall, we observed that the frequency of *K-ras* oncogene mutations in a large representative sample of CRC patients from The Netherlands (age at diagnosis between 57 and 76 years) was 37%. The frequency of *K-ras* gene mutations in CRC patients reported in the literature, ranges from 30 to 60% (3,9,11,12,16–27). This broad range of reported frequencies of *K-ras* mutations may be due to various factors, such as the sensitivity and specificity of mutation detection methods, small series of selected patients (3,12,17,20,21,23) and/or variability in analysed gene region, i.e. only codons 12 (21), 13 (12,20,22,23,28) and/or 61 (11). Also, environmental factors may be involved. Techniques used for mutation screening such as temperature gradient gel electrophoresis (3) or SSCP (17,29) and methods for mutation detection like PCR-restriction fragment length polymorphism (22,28), or PCR-based mutant allele-specific amplification (MASA) (20) may show differences in sensitivity and/or specificity of mutation detection in the *K-ras* gene. In this study, the analysis of *K-ras* mutations is based on a highly sensitive and specific detection method, i.e. direct sequencing of purified PCR fragments. This method identifies mutations in DNA samples, which contain at least 5% or more mutated DNA. The reported frequency of *K-ras* mutations could, therefore, be an underestimation. Direct sequencing was validated by mutation analysis of five CRC cell lines, and of adjacent blocks with paraffin-embedded and fresh tissue from a series of 10 CRC specimens. The mutation analysis of five CRC cell lines confirmed the reported sequences of exon 1 fragments of the *K-ras* oncogene (30). In addition, the *K-ras* status as determined in paraffin-embedded tumour tissue was also found in fresh tumour tissue in nine out of 10 specimens. In one specimen, the mutation detected in paraffin-embedded tissue was not found in fresh tissue, which may be related to heterogeneity in the tumour tissue or a lack in the reproducibility of this sample. These results indicate that tissue processing does not significantly affect the reliability of mutation analysis in archival specimens. The reproducibility of the identification technique used in this study was found to be good, as duplication of the complete analytical procedure yielded the same results for 28 out of 32 (88%) adenocarcinomas. The *K-ras* gene mutation analysis is based on a relatively large series of non-selected, incident CRC patients, which indicates that the found frequency of *K-ras* mutations is representative for colorectal cancer patients in the Dutch population.

Slattery *et al*. (31) have evaluated the association between several genetic alterations and the presence or absence of family history of colorectal cancer using incident colon cancer cases. These authors did not find an association with overall *K-ras* mutations, although patients with a G>T transversion of the second base of codon 12 were more likely to have a family history of colorectal cancer compared with those without this specific type of point mutation. In our study, no significant differences were observed in family history of CRC between patients without and with a *K-ras* mutation. Our findings suggest that these genetic alterations in sporadic colorectal tumours are not associated with family history of CRC and that therefore, diet, environment and/or lifestyle factors may contribute to the acquirement of *K-ras* gene mutations involved in the early phases of carcinogenesis. However, more studies evaluating other genetic markers and/or a different kind of population study, i.e. twin studies, may be necessary to address this issue.

Studies from various countries have analysed the frequency of the type of *K-ras* point mutation in colorectal cancer. These studies were conducted in the UK (24,25), former Yugoslavia (12), Czech Republic (21), Norway (9), Switzerland (18), Mexico (11), USA (26) and The Netherlands (17,19,20). All studies except for the study performed in former Yugoslavia (12) have identified the G>A transition as the most frequently found type of *K-ras* mutation. In the current study, the G>A transition appeared also to be the predominant mutation. The pattern of specific alterations observed, i.e. G>A transitions and G>T transversions, could be due to differences in diet and/or other lifestyle factors. *N*-nitroso compounds, for example, in red and processed meat could induce G>A transitions (25) and this is supported by previous experimental studies (32,33). Two mechanisms, which could explain the G>A transition are the formation of guanine-adducts in the DNA and the silencing of the O^6-methylguanine DNA methyltransferase (MGMT). MGMT is a DNA repair protein that removes adducts from the O^6 position of guanine (34) in DNA. Promotor hypermethylation of the *MGMT* gene, a phenomenon often seen in colon cancer cells (28,35) and which leads to silencing of the gene, results in conversions of guanine-cytosine pairs to adenine–thymine pairs. Guanine-to-thymine transversions, however, are more likely to be induced by carcinogenic agents like the polycyclic aromatic hydrocarbons found in smoked and barbecued meat (36), dietary fats (37) and cigarette smoke (38).

In the present study, codons 12 and 13 were affected in 94% of the tumours with a *K-ras* mutation in the exon 1 fragment, and the majority of mutations would result in an amino acid substitution of glycine by aspartic acid (57%) or by valine (33%). Mutations in either of these codons could lead to an activated Ras protein. Al-Mulla *et al*. (39) compared detailed crystal structures of the *K-ras* protein with these two most frequently observed mutations in codon 12, i.e. substitutions of glycine by aspartic acid or valine. The tightly bound complex of GTP in codon 12 glycine-to-valine mutant Ras protein may generate a more stable signal as compared with the codon 12 glycine-to-aspartic acid mutant Ras protein or the codon 12 wild-type Ras protein. Moreover, Andreyev *et al*. (27) have shown that the presence of the glycine to valine substitution in codon 12 leads to a decreased survival of CRC patients and suggest this alteration is important for cancer progression and also that it may predispose to a more aggressive biological behaviour in patients with advanced colorectal cancer. Span *et al*. (19) also observed an association between specific *K-ras* point mutations and cancer progression. Recently, Bazan *et al*. (40) reported biological relevance for codon 13 mutations in terms of colorectal cancer clinical outcome, such as Dukes' stage ($P < 0.05$). They also reported a possible role for codon 12 mutations in the mucinous differentiation pathway. Associations between specific *K-ras* point mutations and cancer

progression were, however, not supported by our and other studies (3,26,41).

Eighteen patients were observed with an affected codon other than codons 12 or 13. It is unknown whether mutations in these codons lead to a constituent activation of the Ras protein. Our results show that mutations in codons other than codons 12 or 13 of exon 1 are rare. The observed pattern of mutated codons, i.e. 94% of the mutations were found in codons 12 and 13, is probably due to a selective growth advantage (42).

Previous studies have presented frequencies of *K-ras* mutations based on tissue samples of selected series of patients with various distributions of Dukes' staged tumours. Some suggested an increase in the frequencies of the *K-ras* oncogene mutations with more advanced stages of Dukes' classification (12,17) whereas others (3,9,42) did not find any association between frequencies of point mutations and Dukes' stage. In the current study, all incident CRC cases were included regardless of the Dukes' stage. No significant differences in the distribution of Dukes' stage were observed between patients without a *K-ras* mutation and those with a specific *K-ras* gene mutation. It suggests that *K-ras* mutations are not involved in the progression of adenocarcinomas and that genetic aberrations occur in pathways, which do not depend on *K-ras* mutations during the more advanced stages of colorectal cancer.

Various topographical subdivisions of the large intestine have been proposed, according to different criteria (22,43–45). The so-called rectosigmoid can be considered as a rather more clinically applied term than an anatomically defined transitional zone between the colon and rectum. Although the rectum is considered to comprise the last 12 cm of the intestine proximal of the anal verge, clinicopathological data are inconsistent as to whether the tumours in this region can be assigned to the colon or rectum (46). In our study, the NCR database contains the clinicopathological data, including the sub-localization of the tumour, as supplied by the clinicians. Therefore, in the current multi-centre study, for so-called rectosigmoid tumours it was not possible to definitively assign a tumour to either the colon or rectum. Consequently, the rectosigmoid was excluded from analysis when comparing colonic versus rectal adenocarcinomas. Among similar lines, another topographical issue, which we addressed, was the comparison of proximal versus distal colon tumours. Differences could be expected between these two with respect to faecal content and composition, microbial flora and activity, and the local variations in distribution of intestinal epithelial cell types. Owing to the mentioned unclear definition of the term rectosigmoid tumour, analyses were performed with both the ex- and inclusion of rectosigmoid tumours within the group of distal colon tumours. The resulting asymmetry between proximal and distal colon tumours was maintained, regardless of these differences in type of analysis.

Patients with a rectal tumour have a relatively higher frequency of G>T transversions as compared with patients with a distal colon tumour and this difference appeared to be most pronounced for female patients. This was not found in other studies (3,21,26,44). However, Breivik *et al*. (9) reported that *K-ras* mutations were not found in tumours located proximal to the descending colon of men under the age of 70, whereas rare mutations such as G>C transversions were almost exclusively observed in tumours of the rectum of women. Again, results based on a smaller number of samples (123 men and 125 women, stratified by age) could lead to biased, inconsistent associations. In the current study, G>C transversions also constitute a small group of specific point mutations, but were mainly observed in men with tumours in the distal colon and in women with tumours in the proximal colon. Plausible explanations for differences in tumour site could be the role of diet with respect to bowel transit time and bacterial fermentation of carbohydrates (47), production of volatile fatty acids (9) or exposure of colonic epithelium to potential dietary carcinogens (48). The relatively high frequency of G>T transversions in the rectum of women might be related to gender differences in faecal concentration and transit time. Both bowel transit time and frequency of constipation have been reported to be substantially higher in women than in men under similar conditions (49). The G>T transversions and also the generally higher frequency of *K-ras* mutations in women might be related to the time of contact with, and the concentration of, particular carcinogens. More aetiological insight in the underlying mechanisms is required to clarify this issue.

The *K-ras* mutational status was evaluated for differences in frequencies with respect to tumour differentiation. Most tumours were classified as 'moderately' differentiated. Generally, the distinction between good or moderately differentiated tumours is often ambiguous. Therefore, the classification of tumour differentiation could be biased. Our findings do not support a role of the *K-ras* oncogene in tumour differentiation.

In conclusion, we observed a frequency of 37% for mutations in exon 1 fragment of the *K-ras* oncogene, predominantly in codons 12 and 13. The G>A transition and the G>T transversion are the most frequently observed mutations, with the G>T transversion primarily confined to codon 12. Patients with a rectal tumour have a relatively higher frequency of G>T transversion as compared to patients with a left colon tumour and this is confined to female patients. The pattern of *K-ras* point mutations observed in the several sub-localizations of the colorectal tract is suggestive for the involvement of dietary factors.

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