Pergamon

*European Journal of Cancer*, Vol. 34, No. 13, pp. 2053–2057, 1998

Printed in Great Britain
0959-8049/98/$—see front matter

PII: S0959-8049(98)00283-4

## Original Paper

# K-*ras* Mutations in Colorectal Adenocarcinomas and Neighbouring Transitional Mucosa

H. Zhang,[1] B. Nordenskjöld,[2] M. Dufmats,[2] P. Söderkvist[1] and X.-F. Sun[2]

[1]Divisions of Cell Biology; and [2]Oncology, Department of Biomedicine and Surgery, Linköping University, S-58185 Linköping, Sweden

**The K-*ras* gene in codons 12 and 13 was investigated using allele-specific polymerase chain reaction in matched normal mucosa ($n = 106$), transitional mucosa ($n = 69$) and tumours ($n = 149$) from 149 patients with colorectal adenocarcinomas. K-*ras* mutations in codon 12 were detected in 41/149 (28%) of tumours and 4/69 (6%) of transitional mucosa samples, but not in the normal mucosa. Further, mutation rates were increased in younger patients ($P = 0.001$) and in mucinous carcinomas (50%) compared with well differentiated (17%), moderately differentiated (26%) or poorly differentiated (24%) tumours. Our findings indicate that mucinous carcinoma may represent a distinct genetic entity.** © **1998 Elsevier Science Ltd. All rights reserved.**

**Key words: K-*ras*, transitional mucosa, mucinous carcinomas, colorectal cancer, allele-specific PCR**
*Eur J Cancer*, Vol. 34, No. 13, pp. 2053–2057, 1998

## INTRODUCTION

K-*ras* GENE MUTATION is an early event in colorectal tumorigenesis. The mutations are more common in large adenomas than small ones, suggesting that the mutations are required for adenoma progression [1, 2]. Moreover, recent studies report high frequencies of *ras* mutations in aberrant crypt foci, the earliest putative precursor of the colorectal cancer [3, 4]. There is, however, little information about K-*ras* mutations in the mucosa adjacent to the tumour, e.g. transitional mucosa from colorectal cancer patients.

The ras proteins are involved in the process of signal transduction across the cellular membrane and are implicated in cellular proliferation and terminal differentiations [5]. Many groups have investigated the relationship of the K-*ras* mutations with clinicopathological factors and prognosis, but there is little consistency [6–13].

Glutathione S-transferase M1 (GSTM1) and T1 (GSTT1) are enzymes responsible for the metabolism of a broad range of carcinogens [14] and are polymorphic in humans with various null genotypes. A significant excess of homozygous null *GSTM1* and *GSTT1* genotypes have been demonstrated among individuals with certain types of cancers including colorectal cancer [15–19]. This suggests that *GSTM1* and *GSTT1* may play a role in tumour susceptibility.

In this study, we used allele-specific polymerase chain reaction (allele-specific PCR) to examine K-*ras* mutations in codons 12 and 13 in genomic DNA from patients' normal mucosa, transitional mucosa and tumour tissue. The data of K-*ras* mutations were compared with clinicopathological parameters, *GSTM1* and *GSTT1* genotypes to investigate possible correlations.

## PATIENTS AND METHODS

*Patients and clinicopathological subjects*

Samples from 149 tumours were collected from patients with primary colorectal adenocarcinomas diagnosed at the Departments of Pathology, Linköping University Hospital and Norrköping Central Hospital, Sweden, between 1982 and 1997. There were 91 males and 58 females. Fifty-four tumours were localised in the proximal colon (six in the caecum, 32 in the ascending colon and 16 in the transverse colon), 27 in the distal colon (five in the descending and 22 in the sigmoid colon) and 62 in the rectum (six unknown). Seventeen tumours were Dukes' A stage, 50 Dukes' B, 39 Dukes' C and 13 Dukes' D (30 unknown). The grade of differentiation was reconfirmed by two of the authors. The tumours were considered as mucinous carcinomas if the mucin lakes represented more than 50% of the tissue. Six tumours were graded as well differentiated, 98 as moderately differentiated, 17 as poorly differentiated and 22 as mucinous carcinomas (six unknown). DNA ploidy and S-phase fraction

Correspondence to H. Zhang.
Received 26 Mar. 1998; revised 26 Jun. 1998; accepted 15 Jul. 1998.

(SPF) were measured using flow cytometry in a limited number of cases [20]. Twenty-two cases were diploid and 21 non-diploid. In 12 cases SPF was less than 5% and in 25 cases SPF $\geq$ 5. Sixty-nine corresponding samples from transitional mucosa were available (samples were taken from the non-malignant tissue adjacent to the adenocarcinoma by the pathologists and surgeons in Linköping and Norrköping Hospitals, Sweden and the transitional mucosa showed morphologically premalignant changes). One-hundred and six normal samples from the distant resection margin which was histologically free from tumour (> 10 cm from the tumour) were also included. The patients were followed until the end of October 1997 and 26 deaths due to cancer were registered.

*DNA extraction from normal, transitional and tumour tissues*

Genomic DNA was extracted from frozen normal mucosa, transitional mucosa and tumour tissues. The tissue (approximately 200 mg) was sliced into small pieces and dissolved in tissue lysis solution (40 µl of 20% sodium dodecyl sulphate (SDS), 40 µl of 10 mg/ml proteinase K, 400 µl TEN buffer, pH 8.5). The tissue samples were incubated in a shaking water bath at 55°C for 24 h. The process was repeated by the addition of half the volume of the above lysis solution until the sample solution turned completely clear. DNA was isolated with phenol, phenol/chloroform and chloroform extraction, precipitated with ice-cold absolute ethanol, washed with ice-cold 70% ethanol, and redissolved in double distilled water.

*Allele-specific PCR*

There are 12 possible K-*ras* point mutations in codons 12 and 13 which can result in amino acid substitutions in the K-ras protein. Primers used in this study to detect these mutations are listed in Table 1 [8]. The 5′PCR primers contain at the 3′ end one base mutated specifically for each point mutation. The 3′ PCR primer for the complementary strand was: 5′-GATGGATCCTGCTCCTGCACCAGTAAT-3′. As an internal positive control for successful amplifications, β-globin gene was amplified with the following primers: 5′-CAA CTT CAT CCA CGT TCA CC-3′ and 5′-GAA GAG CCA AGG ACA GTT AC-3′. PCRs were performed in 20 µl of solution containing PCR buffer (1.5 mM $MgCl_2$, 50 µM KCl, 10 µM Tris–HCl, pH 8.4), 0.2 µM each of dATP, dCTP, dGTP and dTTP (Pharmacia Biotech, Uppsala, Sweden) and 0.5 unit Taq DNA polymerase (Sigma Chemical, St. Louis, Missouri, U.S.A.), and amplifications were carried out in a Peltier Thermal Cycler PTC-200 (MJ Research, Inc., Watertown, U.S.A.) as follows: 3 min of denaturation at 94°C, then 35 cycles consisting of denaturation at 94°C for 90 sec, annealing at 58°C for 30 sec and extension at 72°C for 30 sec. A negative control was included in each set of PCRs. The PCR products were separated by electrophoresis in 1.5% agarose gel with 0.2 µg/ml ethidium bromide. After completion of the electrophoresis, the gels were photographed using a TM-20 UV dual-intensity transilluminator (UVP, Upland, U.S.A.). The fragments amplified were 141–145 bp for the K-*ras* gene and 268 bp for the β-globin gene. The *ras* mutation was not scored if the PCR product from the internal reference (β-globin gene) was not evident. All PCR processes were repeated twice for confirmation and to ensure reproducibility. DNA direct sequencing was performed in some samples which were detected to have the mutation from GGT to GAT in codon 12 by the allele-specific PCR method in order to confirm the mutations.

*Analysis of GSTT1 and GSTM1 genotype*

PCR was performed to detect the presence or absence of *GSTT1* and *GSTM1* genes. Primer sequences for *GSTT1* were 5′-TTC CTT ACT GGG TCC TCA CAT CTC-3′ and 5′-TCA CCG GAT CAT GGG CCA GCA-3′, and for *GSTM1* 5′-CGC CAT CTT GTG CTA CAT TGC CCG-3′ and 5′-TTC TGG ATT GTA CAG ATC A-3′. The β-globin gene (internal control) was amplified with the primers: 5′-CAA CTT CAT CCA CGT TCA CC-3′ and 5′-GAA GAG CCA AGG ACA GTT AC-3′. PCRs were run in 20 µl of solution containing PCR buffer (1.5 mM $MgCl_2$, 50 µM KCl, 10 µM Tris–HCl, pH 8.4), 0.2 µM each of dATP, dCTP, dGTP and dTTP (Pharmacia Biotech) and 0.5 unit Taq DNA polymerase (Sigma), and amplifications were carried out in a Peltier Thermal Cycler PTC-200 (MJ Research) as follows: 3 min of denaturation at 94°C, then 35 cycles consisting of denaturation at 94°C for 90 sec, annealing at 58°C for 30 sec and extension at 72°C for 30 sec. A negative control was included in each run of PCRs. The PCR products were separated by electrophoresis in 1.5% agarose gels. The genotypes of *GSTT1* and *GSTM1* were scored when the β-globin gene was evident.

*Statistical analysis*

The chi-square method was used to test the association of K-*ras* mutations with clinicopathological variables [21]. A proportional hazards model was used to estimate and test prognostic significance [22]. All *P* values cited are two-sided and *P* values less than 0.05 were considered statistically significant.

*Table* 1. *The primer oligonucleotides for the detection of K-*ras *mutations*

| Primers | Codons | Mutations | Sequences |
|---|---|---|---|
| 1 | 12 | GGT→AGT | 5′-TTGTGGTAGTTGGAGCTA-3′ |
| 2 | 12 | GGT→CGT | 5′-TTGTGGTAGTTGGAGCTC-3′ |
| 3 | 12 | GGT→TGT | 5′-TTGTGGTAGTTGGAGCTT-3′ |
| 4 | 12 | GGT→GAT | 5′-TGTGGTAGTTGGAGCTGA-3′ |
| 5 | 12 | GGT→GCT | 5′-GTGGTAGTTGGAGCTGC-3′ |
| 6 | 12 | GGT→GTT | 5′-TGTGGTAGTTGGAGCTGT-3′ |
| 7 | 13* | GGC→AGC | 5′-TGGTAGTTGGAGCTGGTA-3′ |
| 8 | 13* | GGC→CGC | 5′-GGTAGTTGGAGCTGGTC-3′ |
| 9 | 13* | GGC→GAC | 5′-GTAGTTGGAGCTGGTGA-3′ |

*These three possible K-*ras* mutations in codon 13 were the most common ones and the only ones that have been reported [8].

## RESULTS

*The distribution of K-*ras *mutations in normal mucosa, transitional mucosa and tumour tissue*

K-*ras* mutations were examined with allele-specific PCR in matched normal mucosa, transitional mucosa and tumours from the 149 patients with colorectal adenocarcinomas. No mutation was detected in normal mucosa from 106 patients, whereas the mutations were identified in four (6%) of the 69 transitional mucosa samples and in 41 (28%) of the 149 tumours (Figure 1). All K-*ras* mutations were found in codon 12. 4 patients had GGT to GAT mutations in their transitional mucosa and showed the same type of mutations in the corresponding tumours. Of the 41 tumours with mutations, 34 (83%) were GGT to GAT (glycine to aspartic acid), six (15%) were GGT to GTT (glycine to valine) and one (2%) was GGT to GCT (glycine to alanine). 1 patient had two mutations with both GGT to GAT and GGT to GTT.

*The relationships of K-*ras *mutations with clinicopathological and other factors*

The relationships of K-*ras* mutations with clinicopathological factors including DNA ploidy and SPF are summarised in Table 2. The frequency of K-*ras* mutations was significantly decreased as patients' age increased ($P = 0.001$). Apparent differences in the frequency of K-*ras* mutation were seen with different degrees of differentiation ($P = 0.03$). The frequency of K-*ras* mutations was highest in mucinous carcinomas (50%), and lowest in well differentiated tumours (17%). There was no significant relationship between K-*ras* mutations and patients' gender, tumour location, Dukes' stage, DNA ploidy and SPF ($P > 0.05$).

Univariate survival analysis was undertaken in 131 patients with available follow-up data. The results revealed that 28% of 32 patients with *ras* mutations died, whilst the death rate in 99 patients without the *ras* mutations was 17%. The difference did not reach statistical significance ($P > 0.05$).

The K-*ras* mutations were not significantly associated with *GSTM1* or *GSTT1* deletions in normal tissue, transitional mucosa and tumours (Table 3, $P > 0.05$).

We did not find relationships between specific mutations of GGT to GAT, GTT or GAT and clinicopathological variables including gender, site, stage, grade, DNA ploidy, SPF, GSTs and prognosis ($P > 0.05$).

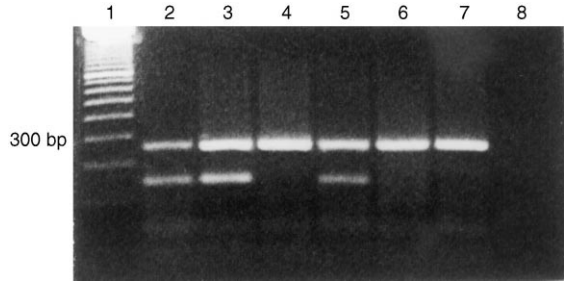


**Figure 1. Allele-specific polymerase chain reaction (PCR) detection of K-*ras* mutations in colorectal cancer. The K-*ras* mutation is indicated by the 145 base pairs (bp) band. Lane 1 is a DNA size marker (100 bp ladder). Lanes 2, 3 and 5 show the mutations of K-*ras*; lanes 4, 6 and 7 only show the presence of the internal control gene, β-globin; lane 8 is a negative control, a PCR with no addition of DNA. β-globin (268 bp) indicates the presence of coamplified DNA in all the samples.**

*Table 2. K-*ras *mutations in relation to gender, age, site, Dukes' stage, grade of differentiation, DNA ploidy and S-phase fraction (SPF) in colorectal adenocarcinomas*

| Variables | No. of patients | K-*ras* mutations | *P* value |
|---|---|---|---|
| Gender | | | |
| Male | 91 | 22 (24%) | |
| Female | 58 | 19 (33%) | 0.25 |
| Age (years) | | | |
| < 60 | 24 | 14 (58%) | |
| 60–69 | 50 | 13 (26%) | |
| 70–79 | 59 | 12 (20%) | |
| > 80 | 14 | 2 (14%) | 0.001 |
| Unknown | 2 | | |
| Site | | | |
| Colon | 81 | 18 (22%) | |
| Rectum | 62 | 20 (32%) | 0.18 |
| Unknown | 6 | | |
| Dukes' stage | | | |
| A | 17 | 9 (53%) | |
| B | 50 | 13 (26%) | |
| C | 39 | 13 (33%) | |
| D | 13 | 5 (38%) | 0.63 |
| Unknown | 30 | | |
| Differentiation | | | |
| Well | 6 | 1 (17%) | |
| Moderate | 98 | 25 (26%) | |
| Poor | 17 | 4 (24%) | |
| Mucinous | 22 | 11 (50%) | 0.03 |
| Unknown | 6 | | |
| DNA ploidy ($n = 43$) | | | |
| Diploid | 22 | 10 (45%) | |
| Non-diploid | 21 | 11 (52%) | 0.65 |
| SPF ($n = 37$) | | | |
| < 5% | 12 | 4 (33%) | |
| ≥ 5% | 25 | 14 (56%) | 0.20 |

*Table 3. Tumour K-*ras *mutations in relation to* GSTM1 *and* GSTT1 *genotypes in patients with colorectal adenocarcinomas*

| Variables | No. of patients | K-*ras* mutations | *P* value |
|---|---|---|---|
| *GSTM1* in normal tissue | | | |
| Absent | 45 | 6 (13%) | |
| Present | 61 | 14 (23%) | 0.21 |
| *GSTM1* in transitional mucosa | | | |
| Absent | 39 | 6 (15%) | |
| Present | 29 | 6 (21%) | 0.93 |
| *GSTM1* in tumour | | | |
| Absent | 55 | 8 (15%) | |
| Present | 51 | 12 (24%) | 0.24 |
| *GSTT1* in normal tissue | | | |
| Absent | 59 | 9 (15%) | |
| Present | 47 | 11 (23%) | 0.29 |
| *GSTT1* in transitional tissue | | | |
| Absent | 46 | 7 (15%) | |
| Present | 22 | 5 (23%) | 0.93 |
| *GSTT1* in tumour | | | |
| Absent | 71 | 11 (15%) | |
| Present | 35 | 9 (26%) | 0.21 |

## DISCUSSION

Transitional mucosa is morphologically characterised by an increase in mucosal thickness, lengthening of the crypts and goblet cell hyperplasia. Histochemical changes are increased sialomucin content of the goblet cells with decreased or absent sulphomucin [23, 24]. The results are controversial concerning genetic changes in transitional mucosa. Shibata and colleagues [25] and Thomas and associates [13] did not find any K-*ras* mutation in the histological transition between normal and tumour tissue, whilst Burmer and Loeb [6] and Zhu and colleagues [26] did. Further, they observed that most cases have the same mutations in normal mucosa and tumour. In the present series of paired transitional mucosa and tumours, we found identical mutations in both transitional and tumour tissues in 4 patients. This genetic alteration in the region indicated that the transitional mucosa may be a preneoplastic lesion. K-*ras* mutations may thus be a marker for the identification of patients at increased risk of developing colorectal cancer. In most cases, the mutation is detected only in tumour tissue but not in transitional mucosa. In these cases, other genetic alterations in the transitional mucosa may provide a selective growth advantage required for tumorigenesis. Also, as we analysed only one sample from each patient, we may have missed some cases with *ras* mutations in a small population of cells. Finally, it is possible that in some cases the transitional mucosa may represent non-specific changes in response to the tumour [24]. Clearly this area needs to be investigated further.

In the present study, K-*ras* mutations were present in 28% of 149 colorectal tumours. All tumours appeared to have only one K-*ras* mutation, except one which harboured two mutations. This suggests that a homogeneous clone resulted in the tumour. Our study further showed that all the mutations occurred at the second base of codon 12. GGT to GAT was more frequent (83%) than the GGT to GTT mutation (15%). Other studies have identified a wide range of frequencies of K-*ras* mutations from 7 to 71% in colorectal cancers [6–13]. The most common types of K-*ras* mutations vary in different studies, with GGT to GAT [8, 13] or GGT to GTT [11]. This difference might be due to variations in populations in which patients have been exposed to different mutagens. For instance, alkylating agents may cause GGT to GAT transitions, whilst the GGT to GTT transition is characteristic for heterocyclic amines and polycyclic aromatic hydrocarbons [5].

Approximately 15% of colorectal adenocarcinomas are mucinous [27]. It has been reported that the frequency of K-*ras* mutations is increased whilst p53 abnormality is decreased in mucinous tumours [7, 28]. We confirmed the increased frequency of K-*ras* mutations in mucinous carcinomas. Thus, mucinous carcinoma might be considered as a distinct genetic entity.

We found that individuals with *GSTT1* null but not *GSTM1* null genotype may be predisposed to developing colorectal cancer. In the present analysis, however, K-*ras* mutations were not associated with *GSTM1* and *GSTT1* genotypes. Therefore, our findings and similar results reported in colorectal [29] and pituitary tumours [18] suggest that K-*ras* mutations and GSTs might represent different pathways in promoting tumorigenesis.


1. Vogelstein B, Fearon ER, Hamilton SR, *et al.* Genetic alterations during colorectal-tumor development. *N Engl J Med* 1988, **319**, 525–532.
2. Vogelstein B. Genetic alterations in colorectal tumors. *Adv Oncol.* 1991, **7**, 3–6.
3. Smith AJ, Stern HS, Penner M, *et al.* Somatic APC and K-ras codon 12 mutations in aberrant crypt foci from human colons. *Cancer Res* 1994, **54**, 5527–5530.
4. Losi L, Roncucci L, Gregorio CD, Leon MPD, Benhattar J. K-ras and p53 mutations in human colorectal aberrant crypt foci. *J Pathol* 1996, **178**, 259–263.
5. Barbacid M. ras genes. *Annu Rev Biochem* 1987, **56**, 779–827.
6. Burmer GC, Loeb LA. Mutations in the KRAS2 oncogene during progressive stages of human colon carcinoma. *Proc Natl Acad Sci USA* 1989, **86**, 2403–2407.
7. Laurent-Puig P, Olschwang S, Delattre O, *et al.* Association of Ki-ras mutation with differentiation and tumor-formation pathways in colorectal carcinoma. *Int J Cancer* 1991, **49**, 220–223.
8. Losi L, Benhattar J, Costa J. Stability of K-ras mutations throughout the natural history of human colorectal cancer. *Eur J Cancer* 1992, **28**, 1115–1120.
9. Breivik J, Meling GI, Spurkland A, Rognum TO, Gaudernack G. K-ras mutation in colorectal cancer: relations to patient age, sex and tumour location. *Br J Cancer* 1994, **69**, 367–371.
10. Morrin M, Kelly M, Barrett N, Delaney P. Mutations of Ki-ras and p53 genes in colorectal cancer and their prognostic significance. *Gut* 1994, **35**, 1627–1631.
11. Span M, Moerkerk PTM, Goeij AFPMD, Arends JW. A detailed analysis of K-ras point mutations in relation to tumor progression and survival in colorectal cancer patients. *Int J Cancer* 1996, **69**, 241–245.
12. Pauly M, Schmitz M, Kayser I, *et al.* Ki-ras oncogene and p53 tumour suppressor gene mutations in colorectal carcinomas from the European Saar-Luxembourg region are less frequent than predicted by the classic adenoma-carcinoma sequence model. *Eur J Cancer* 1997, **33**, 2265–2272.
13. Thomas RJS, Liu TS, Clair FS, Norris PM, Valentine R, Phillips WA. Frequency and clinico-pathological associations of ras mutations in colorectal cancer in the viktorian population. *Aust NZ J Surg* 1997, **67**, 233–238.
14. Mannervik B, Awasthi YC, Board PG, *et al.* Nomenclature for human glutathione transferase. *Biochem J* 1992, **282**, 305–308.
15. Strange RC, Matharoo B, Faulder GC, *et al.* The human glutathione S-transferases: a case-control study of the incidence of the GST1 0 phenotype in patients with adenocarcinoma. *Carcinogenesis* 1991, **12**, 25–28.
16. Hirvonen A, Husgafvel-Pursiainen K, Anttila S, Vainio H. The GSTM1 null genotype as a potential risk modifier for squamous cell carcinoma of the lung. *Carcinogenesis* 1993, **14**, 1479–1481.
17. Zhong S, Wyllie AH, Barnes D, Wolf CR, Spurr NK. Relationship between the GSTM1 genetic polymorphism and susceptibility to bladder, breast and colon cancer. *Carcinogenesis* 1993, **14**, 1821–1824.
18. Perrett CW, Clayton RN, Pistorello MP, *et al.* GSTM1 and CYP2D6 genotype frequencies in patients with pituitary tumours: effects on p53, ras and gsp. *Carcinogenesis* 1995, **16**, 1643–1645.
19. Deakin M, Elder J, Hendrickse C, *et al.* Glutathione S-transferase GSTT1 genotypes and susceptibility to cancer: studies of interactions with GSTM1 in lung, oral, gastric and colorectal cancers. *Carcinogenesis* 1996, **17**, 881–884.
20. 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, **27**, 1647–1649.
21. Armitage P, Berry G. *Statistical Methods in Medical Research*. Oxford, Blackwell Scientific, 1994.
22. Cox DR. Regression models and life tables. *J R Stat Soc B* 1972, **34**, 187–220.
23. Robey-Cafferty SS, Ro JY, Ordonez NG, Cleary KR. Transitional mucosa of colon. A morphological, histochemical, and immunohistochemical study. *Arch Pathol Lab Med* 1990, **114**, 72–75.
24. Sawady J, Friedman MI, Katzin WE, Mendesohn G. Role of the transitional mucosa of the colon in differentiating primary adenocarcinoma from carcinomas metastatic to the colon. An immunohistochemical study. *Am J Surg Pathol* 1991, **15**, 136–144.
25. Shibata D, Schaeffer J, Li ZH, Capella G, Perucho M. Genetic heterogeneity of the c-K-ras locus in colorectal adenomas but not in adenocarcinomas. *J Natl Cancer Inst* 1993, **85**, 1058–1063.

26. Zhu D, Keohavong P, Finkelstein SD, *et al.* K-ras gene mutations in normal colorectal tissues from K-ras mutation-positive colorectal cancer patients. *Cancer Res* 1997, **57**, 2485–2492.
27. Morson BC. *Colour Altas of Gastrointestinal Pathology*. Oxford, Oxford University Press, 1988.
28. Mulder JW, Baas IO, Polak MM, Goodman SN, Offerhaus GJ. Evaluation of p53 protein expression as a marker for long-term prognosis in colorectal carcinoma. *Br J Cancer* 1995, **71**, 1257–1262.
29. Chenevix-Trench G, Young J, Coggan M, Board P. Glutathione S-transferase M1 and T1 polymorphisms: susceptibility to colon cancer and age of onset. *Carcinogenesis* 1995, **16**, 1655–1657.

**Acknowledgements**—This work was supported by grants from the Swedish Cancer Society, FORSS, Linköping, Nurse Siv Olssons Research Foundation, Linköping, Medical Research and Development in Landstinget in Östergötland and Doctor Society Research Foundation, Linköping, Sweden.