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Cigarette smoking and genetic alterations in sporadic colon carcinomas

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Cigarette smoking has been inconsistently associated with colon cancer risk. To evaluate the hypothesis that smoking is primarily linked to a specific colon tumor subgroup(s), we assessed associations between smoking and the occurrence of mutations in the *APC*, K-*ras* and *p53* genes, p53 overexpression, and microsatellite instability (MSI) in a Dutch population-based case–control study on sporadic colon carcinomas. The study population consisted of 176 cases and 249 controls. Smoking status (never, ever), number of cigarettes smoked per day (never, <15, ≥15), total years of smoking (never, ≤30, >30), and years since first started smoking (never, ≤35, >35) were all evaluated. Cigarette smoking status was significantly differently related to p53 overexpression-positive ($p53^{pos}$) tumors compared with p53 overexpression-negative ($p53^{neg}$) tumors ($p53^{pos}$ versus $p53^{neg}$, OR 0.4, 95% CI 0.2–0.9), as well as to tumors with transversion mutations in *APC*, K-*ras* or *p53* ($transv^{+}$) compared with tumors without transversion mutations in one of these genes ($transv^{-}$) ($transv^{+}$ versus $transv^{-}$, OR 2.5, 95% CI 1.0–5.9). Positive associations were observed with $p53^{neg}$ tumors and $transv^{+}$ tumors when compared with the population-based controls (ever versus $transv^{-}$, OR 1.5, 95% CI 0.9–2.8 and OR 2.2, 95% CI 0.9–5.6, respectively), inverse associations with $p53^{pos}$ tumors and $transv^{-}$ tumors (ever versus never, OR 0.5, 95% CI 0.3–1.0 and OR 0.8, 95% CI 0.5–1.3, respectively). Similar patterns of association were observed for the other smoking variables evaluated. In addition, although statistically non-significant, smoking was more notably positively associated with tumors that exhibit K-*ras* mutations, especially K-*ras* transversion mutations, than with tumors without K-*ras* mutations. An inverse relationship between smoking and the occurrence of *APC* mutations was suggested, whereas no clear associations were observed with MSI. Our data suggest that smoking-related colon cancers develop through a $p53^{neg}$ pathway and that smoking particularly results in colon carcinomas with transversion mutations.

Introduction

Cigarette smoking has been consistently associated with colorectal adenomas, the precursor lesions of most colon carcinomas. Most studies observed a 2- to 3-fold increased risk of adenomas for long-term, heavy cigarette smoking (1). The association of smoking with colon cancer risk has, however, not been consistent (1). A possible explanation for this discrepancy is that cigarette smoking is only involved in the production of certain types of mutations in specific genes and, thus, primarily affects, and results in, the development of a particular subset(s) of colon carcinomas.

Mutations in the adenomatous polyposis coli (*APC*), K-*ras* and *p53* genes as well as microsatellite instability (MSI) are commonly observed genetic alterations in colon cancer. Mutations in *APC* (i.e. those mutations resulting in loss of APC function) are believed to be a key initiating event in colon cancer (2–4). K-*ras* mutations are thought to accompany the conversion of small to larger adenomas and have been reported to occur in 30–40% of sporadic colon tumors (5,6), whereas mutation of *p53* seems to be especially important in the later stages of colon tumorigenesis (7). MSI occurs in most colon tumors associated with the hereditary non-polyposis colorectal cancer syndrome and in ~10–20% of sporadic colon tumors (8–11).

Interestingly, significant inverse relationships have been observed between the presence of MSI and mutations in *APC*, K-*ras* and *p53* in sporadic colon tumors (12,13). This suggests different molecular pathways to colon cancer which in turn may reflect different environmental exposures. Supporting this idea, Bardelli *et al.* (14) recently demonstrated that exposure to specific carcinogens can indeed select for colon tumor cells with distinct forms of genetic alterations. Regarding smoking, K-*ras* (15,16) and *p53* mutations (17,18), G→T transversions in particular, are significantly more prevalent in lung cancers from smokers than in lung cancers from non-smokers, suggesting an etiological link between exposure to tobacco smoke carcinogens and these genetic alterations.

Few studies have examined associations between smoking and genetic alterations in colon cancer. Those that have (6,19–21) generally limited their analyses to alterations in one specific gene. Intriguingly, Freedman *et al.* (19), who used p53 overexpression as an indicator of *p53* mutations, observed an increased risk of p53 overexpression-negative ($p53^{neg}$) colon tumors for smokers. Slattery *et al.* (20) and Yang *et al*. (21) both reported a positive association between cigarette smoking and sporadic colon tumors with MSI. To (further) explore the hypothesis that cigarette smoking is primarily associated with a specific colon tumor subgroup(s), in this study we assess the associations between smoking and the occurrence of (specific) mutations in the *APC*, K-*ras* and *p53* genes, p53 overexpression and MSI in sporadic colon carcinomas.

Material and methods

Study population

A population-based case–control study on diet and colon cancer was conducted in The Netherlands between 1989 and 1993. Details were described previously (22,23). Briefly, cases (n = 204) were women and men newly diagnosed with histologically confirmed, first primary incident colon carcinoma. They were

Abbreviations: APC, adenomatous polyposis coli; CI, confidence interval; MCR, mutation cluster region; MSI, microsatellite instability; OR, odds ratio; $p53^{neg}$, p53 overexpression-negative; $p53^{pos}$, p53 overexpression-positive; SSCP, single-strand conformation polymorphism.

recruited in regional hospitals located in the eastern and central regions of The Netherlands and invited to participate by their medical specialists within 3 months of diagnosis. Cancer registries were used to check for completeness. Of all eligible cases diagnosed in the cooperating hospitals, 47% were invited to participate. Sixty percent of those invited agreed to participate. Controls (n = 259), frequency matched to the cases by age (5 year intervals), sex, region and degree of urbanization, were randomly recruited by the general practitioners of the cases. Of the controls invited, 57% agreed to participate. All subjects were Dutch speaking, Caucasian, up to 75 years old at time of diagnosis, mentally competent to complete the interview and had no known personal history of cancer, familial adenomatous polyposis, hereditary non-polyposis colorectal cancer, ulcerative colitis or Crohn's disease. Except for a more favorable Dukes' stage among cases, participants did not differ importantly from non-participants. Cigar and/or pipe only smokers (n = 18) and two subjects with missing data on smoking status were excluded from the analyses. Colon tumor tissue could not be obtained from 18 cases due to administrative reasons. In total, 176 cases and 249 controls were included in the analyses here presented.

Data collection

Participants were interviewed in their own homes by trained dieticians using a structured questionnaire. The interval between diagnosis and the interview was, for cases, 3–6 months. Cigarette smoking status (never, ever, ex, current) was determined. Participants who had stopped smoking 1 year or more prior to the date of the interview were classified as ex-smokers; participants who had stopped <1 year prior to the interview date or were still smoking were classified as current smokers. Information about the number of years smoked and the number of cigarettes usually smoked per day (categorized in four categories: 1–<5, 5–<15, 15–<25 and ≥25 cigarettes) was obtained. Years since first started smoking was calculated from information on duration of smoking and, if applicable, time since stopped smoking. The interview also consisted of a dietary history part in which information on the frequency and amounts of foods consumed in the year prior to the interview (for cases, the year preceding diagnosis or complaints) was collected. Information on aspirin and non-steroidal anti-inflammatory drug use, family history of colorectal cancer and medical history was also obtained during the interview.

DNA extraction

Both tumor and normal DNA were extracted from formalin-fixed, paraffin-embedded colon tissue, collected before chemo- or radiotherapy started, as described elsewhere (24). Microdissection was performed and for tumor DNA only those areas containing >60% tumor cells were used. Corresponding normal DNA was isolated from tumor-free colon tissue.

APC mutation detection

Single-strand conformation polymorphism (SSCP) analysis was used to screen the *APC* gene for mutations. The majority of the somatic mutations in *APC* seem to cluster within a small region in exon 15 (codons 1286–1513), the so-called mutation cluster region (MCR) (25–27). Our analysis covered codons 1286–1585 (extended MCR) of *APC*. The region was divided into five ~220 bp long overlapping fragments (codons 1286–1358, 1337–1404, 1387–1455, 1437–1526 and 1509–1585, respectively), which were separately amplified in two consecutive PCRs using the following primer sets (primer sequence 5′→3′). Fragment 1: 1.1 forward CAGACTTATTGTGTAGAAG, reverse CGCTCCTGAAGAAAATTCAAG (codons 1260–1358); 1.2 forward GAAATAGGATGTAATCAGACG, reverse CGCTCCTGAAGAAAATTCAAC (codons 1286–1358). Fragment 2: 2.1 forward ACTGCAGGGTTCTAGTTTATC, reverse TCTGCTTGGTGGCATGGTTT (codons 1337–1436); 2.2 forward ACTGCAGGGTTCTAGTTTATC, reverse GAGCTGGCAATCGAACGACT (codons 1337–1404). Fragment 3: 3.1 forward CTCAGACACCCAAAAGTCC, reverse ATTTTTAGGTACTTCTCGCTTG (codons 1366–1455); 3.2 forward TACTTCTGTCAGTTCACTTGATA, reverse ATTTTTAGGTACTTCTCGCTTG (codons 1387–1455). Fragment 4: 4.1 forward AAACACCTCCACCACCTCC, reverse TCATTCCCATTGTCATTTTCC (codons 1437–1536); 4.2 forward AAACACCTCCACCACCTCC, reverse GCATTATTCTTAATTCCACATC (codons 1437–1526). Fragment 5: 5.1 forward ACTCCAGATGGATTTTTCTTG, reverse GGCTGGCTTTTTGCTTTAC (codons 1497–1596); 5.2 forward GAGCCTCGATGAGCCATTTA, reverse TGTTGGCATGGCAGAAATAA (codons 1509–1585).

PCR reaction mixtures (total volume 50 µl) contained 50 ng DNA (or 2 µl of the 1:100 diluted product of the first PCR), 0.2 µM both primers, 0.2 mM dNTPs, 10 mM Tris–HCl, pH 9.0, 1.5–2.5 mM $MgCl_2$, 50 mM KCl, 0.01% Tween, 10% glycerol and 0.3 U *Taq* DNA polymerase. Reaction conditions were: first PCR, 25 cycles of 30 s at 94°C, 45 s at 55°C (53°C for primer set 1.1; 57°C for 4.1), 1 min at 72°C, followed by 5 min at 72°C; second PCR, 30 cycles of 30 s at 94°C, 45 s at 52°C (56°C for primer sets 3.2 and 4.2; 57°C for 5.2), 1 min at 72°C, followed by 5 min at 72°C. Products were checked using an ethidium bromide stained 2% agarose gel. SSCP was performed as described earlier (24) with electrophoresis at 10 and 18°C. The original PCR products from the samples that displayed an abnormal pattern in the SSCP were subjected to sequencing in both directions using the same primers as in the second PCR. Sequencing was performed as described previously (24). Mutation analysis started in all samples with fragment 1 and only if no truncating mutations (i.e. nonsense or frameshift mutations) were detected was fragment 2 screened for mutations, and so on. We focused on truncating mutations because these mutations indisputably result in function loss of the APC protein whereas the biological significance of missense mutations in *APC* is uncertain. Carcinomas were classified as APC^+ (with a truncating mutation in the extended MCR of *APC*) or APC^- (without a truncating mutation in the extended MCR of *APC* and all five fragments completely analyzed for mutations).

K-ras mutation detection

Codons 12 and 13 of the K-*ras* gene were examined for mutations (i.e. those resulting in an amino acid change) by mutant allele-specific amplification as described earlier (28).

p53 mutation detection

To enable the evaluation of (specific) *p53* mutations, SSCP analysis was used to screen exons 5–8 of the *p53* gene for mutations as described previously (24). We focused on exons 5–8 as it has been observed that most *p53* mutations occur in this region of the gene (29). The original PCR products from the samples that displayed an abnormal pattern in the SSCP were subjected to sequencing in both directions. Samples in which a mutation (i.e. one that resulted in an amino acid change or truncation of the protein) was detected were excluded from analysis of the subsequent exons. The exons were screened in the following order: 7, 8, 5 and 6. Carcinomas were classified as $p53^+$ (with a mutation in codons 5–8 of *p53*) or $p53^-$ (without a mutation in codons 5–8 of *p53* and all four codons completely analyzed for mutations).

p53 immunohistochemistry

Overexpression of p53 was determined using a mixture of two antibodies (DO-7, which recognizes both mutant and wild-type forms of p53, and PAb 240, which recognizes only mutant forms of p53) as published earlier (24). Stained sections were scored independently by two investigators (A.A.van Kraats and G.N.P.van Muijen). Tumors were scored as $p53^{neg}$ if <20% of the cells displayed nuclear positivity and as p53 overexpression-positive ($p53^{pos}$) if otherwise, as in Freedman *et al*. (19).

Microsatellite instability

For MSI analysis, paired tumor and normal DNA were investigated with the five Bethesda reference panel markers (30): *BAT25*, *BAT26*, *D5S346*, *D2S123* and *D17S250*. PCR reaction mixtures (total volume 25 µl) contained 100 ng DNA, 10 pmol forward (fluorescent labeled) and reverse primers, 2.0 mM $MgCl_2$ (2.5 mM $MgCl_2$ for *BAT26*), 0.2 mM dNTPs, 75 mM Tris–HCl, pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.01% Tween, 0.3 U Thermoperfect DNA polymerase (Integro). PCR reaction conditions were: 35 cycles of 30 s at 92°C, 45 s at 50°C, 1 min at 72°C, followed by 30 min at 72°C. Products were checked using an ethidium bromide stained 2% agarose gel. An aliquot of 1 µl of (diluted) PCR product was added to 10 µl of formamide and 0.5 µl of ROX-500 (size standard), denatured at 95°C for 5 min, chilled on ice and loaded on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). Genotyper® ABI PRISM version 3.5 NT (Perkin Elmer) was used to analyze the data. MSI at a specific marker was defined as the presence of a novel length allele in tumor tissue when compared with corresponding normal tissue. When matching normal DNA was not available (n = 22), only the mononucleotide repeat markers *BAT25* and *BAT26* were checked for instability. Tumors were classified as MSI-H if two or more markers showed instability, MSI-L if one marker showed instability and MSS if none of the markers examined showed instability (30).

Data analysis

The distribution of *APC*, K-*ras* and *p53* mutations, p53 overexpression and MSI in the colon carcinomas was determined. Differences in (tumor) characteristics between 'never smoked' and 'ever smoked' cases and between MSI-H and MSS tumors were assessed using *t*-tests for continuous and χ^2 tests for categorical variables; P values <0.05 were considered significant. Case–case comparisons were conducted to evaluate heterogeneity in risk factors for the different tumor subsets. In addition, case–control comparisons, separately comparing cases with and cases without specific alterations with the population-based controls, were conducted to estimate the relative risk of developing carcinomas respectively with and without this particular status. The risk factors evaluated were: cigarette smoking status (never, ever), number of cigarettes usually smoked per day (never, <15, ≥15), total years of smoking (never, ≤30, >30) and years since first started smoking (never, ≤35, >35). Odds ratios (ORs) and the corresponding 95% confidence intervals (95% CIs) were calculated using multiple logistic regression models. 'Never'

Table I. Characteristics of the colon cancer cases by cigarette smoking status

	All cases (n = 176)	Never smokers (n = 55)	Ever smokers (n = 121)
Age (years, mean ± SD)	61.7 ± 10.3	62.6 ± 9.9	61.3 ± 10.2
Sex (% women)	47.2	69.1	37.2[a]
Body mass index (kg/m^2, mean ± SD)	25.9 ± 4.4	25.0 ± 4.4	26.3 ± 4.4
Smoking variables (%)			
Current smokers			26.4
⩾15 cigarettes/day			51.2
⩾25 cigarettes/day			32.3
>30 years of smoking			49.2
>35 years since first smoked			72.2
Dietary factors (mean ± SD)			
Total energy intake (kJ/day)	10373.7 ± 3241.6	9491.4 ± 2767.7	10774.8 ± 3370.2[a]
Vegetables (g/day)	170.5 ± 81.7	153.8 ± 74.9	178.5 ± 83.7
Fruit (g/day)	213.5 ± 155.7	242.6 ± 166.8	200.2 ± 149.3
Red meat (g/day)	79.7 ± 35.4	73.9 ± 34.9	82.3 ± 35.5
Alcohol[b] (g/day)	17.1 ± 25.4	9.8 ± 17.6	20.4 ± 27.6[a]

[a]Never smokers versus ever smokers $P < 0.05$, t-test for continuous variables and χ^2 test for categorical variables.
[b]Adjusted for total energy intake by regression analysis, for women and men separately.

was always the referent category. All analyses were adjusted for age, sex, total energy and alcohol intake. Additional adjustment for Dukes' stage, tumor location, body mass index and the consumption of vegetables, fruit and red meat did not change the estimates importantly (i.e. not more than 10%). All analyses were performed with the use of the SAS® statistical software package (SAS v.6.12; SAS Institute, Cary, NC).

Results

Characteristics of the cases in this study population, categorized by cigarette smoking status, are given in Table I. There were significantly more women among the never smokers. Total energy and alcohol intake were higher among the ever smokers whereas age and body mass index did not differ between the two groups. Current smokers smoked more cigarettes per day and had smoked for more years than ex-smokers (data not shown).

Tumor characteristics, categorized by cigarette smoking status of the cases, are described in Table II. Although Dukes' stage CD and proximal tumors (cecum, ascending colon, hepatic flexure and transverse colon) were both more common among the never smokers, the difference from ever smokers was not statistically significant. There were significantly more carcinomas with truncating *APC* mutations among the never smokers. p53 overexpression was observed at a higher frequency in the carcinomas of never smokers, whereas K-*ras* mutations, and especially codon 12 transversion mutations, were more common in the carcinomas of ever smokers than of never smokers (both not statistically significant). Transversion mutations, G→T in particular, were, overall, more common among ever smokers. Tumor MSI status did not differ between never and ever smokers. Regarding MSI analysis of the tumors for which no matching normal DNA was available (n = 22), in 10 of these tumors both *BAT25* and *BAT26* were unstable, whereas in the other 12 tumors both markers were stable. Fifty-five percent of all p53^{pos} tumors exhibited a *p53* mutation. Regarding the genetic alterations examined, frequencies observed among women were comparable with those observed among men (data not shown).

MSI-H tumors exhibited a significantly lower frequency of *APC*, K-*ras* and *p53* mutations and were less often p53^{pos} than MSS tumors (MSI-H tumors, 21% *APC*, 21% K-*ras*, 8% *p53*, 15% p53^{pos}; MSS tumors, 38% *APC*, 41% K-*ras*, 36% *p53*, 51% p53^{pos}; not in Table II). They were also significantly more often located in the proximal part of the colon. In most (54%) MSI-H tumors none of the examined genes were mutated and p53 was not overexpressed (data not shown).

Ever smoking was not associated with increased overall colon cancer risk in this study population (Table III). Table III also shows the adjusted ORs and 95% CIs of the case–case and case–control comparisons for cigarette smoking status and the occurrence of the various genetic alterations examined in this study. No significant associations were observed between cigarette smoking status and tumor *APC* mutation status. Of the other cigarette smoking variables evaluated (i.e. usual number of cigarettes smoked per day, total years of smoking and years since first started), only first starting smoking ⩽35 years ago was significantly differently associated with APC$^+$ tumors compared to APC$^-$ tumors (APC$^+$ versus APC$^-$, OR 0.2, 95% CI 0.1–0.7; not in Table III). In the case–control comparisons, first starting smoking ⩽35 years ago was found to be significantly inversely associated with APC$^+$ tumors only (APC$^+$ versus controls, OR 0.2, 95% CI 0.1–0.8; APC$^-$ versus controls, OR 1.2, 95% CI 0.6–2.3; not in Table III). No associations were observed between first starting smoking >35 years ago and tumor *APC* mutation status.

Ever smoking, though not statistically significantly, seems more notably positively associated with tumors that exhibit K-*ras* mutations, especially K-*ras* transversion mutations, than with tumors without K-*ras* mutations (Table III). The other smoking variables evaluated were, although again not statistically significantly, also positively associated with tumors with K-*ras* mutations, more pronounced with K-*ras* transversion mutations, and negatively with tumors without K-*ras* mutations (data not shown).

Ever smoking was significantly differently associated with p53^{pos} tumors compared to p53^{neg} tumors (Table III). The case–control comparisons showed that ever smoking was inversely associated with p53^{pos} tumors and positively, although not statistically significant, associated with p53^{neg} tumors. Evaluation of the other cigarette smoking variables provided additional support that, in this population, cigarette smoking is inversely associated with p53^{pos} tumors. Smoking ⩾15 cigarettes/day, smoking >30 years and first starting smoking >35 years ago were significantly differently associated with p53^{pos} tumors compared to p53^{neg} tumors (p53^{pos} versus p53^{neg},

Table II. Tumor characteristics, categorized by cigarette smoking status of the cases

	All cases (n = 176)	Never smokers (n = 55)	Ever smokers (n = 121)
Dukes' stage (% CD)	36.8	44.4	33.6
Tumor location (% proximal)	45.7	54.2	42.2
Mutation distribution [n (%)]			
APC			
Tumors with mutation	60 (34.1)	25 (45.5)	35 (28.9)[a]
Transversions (see *p53*)	8 (13.3) [4× G→T]	1 (4.0) [0× G→T]	7 (20.0)
Transitions	9 (15.0) [all C→T]	3 (12.0)	6 (17.1)
Insertions/deletions	43 (71.7)	21 (84.0)	22 (62.9)
K-*ras*			
Tumors with mutation(s)[b]	64 (36.4)	16 (29.1)	48 (39.7)
Codon 12 mutations	53 (81.5)	13 (81.3)	40 (81.6)
Transversions	30 (56.6) [21× G→T]	6 (46.2) [5× G→T]	24 (60.0)
Transitions	23 (43.4) [all G→A]	7 (53.8)	16 (40.0)
Codon 13 mutations	12 (18.5)	3 (18.7)	9 (18.4)
Transversions	1 (8.3) [G→T]	0 (0)	1 (11.1)
Transitions	11 (91.7) [all G→A]	3 (100)	8 (88.9)
p53			
Overexpression-positive	78 (44.3)	29 (52.7)	49 (40.5)
Tumors with mutation	54 (30.7)	16 (29.1)	38 (31.4)
Transversions	13 (24.1) [5× G→T]	2 (12.5) [0× G→T]	11 (28.9)
Transitions	35 (64.8) [20× G→A]	13 (81.3) [7× G→A]	22 (57.9)
Insertions/deletions	6 (11.1)	1 (6.3)	5 (13.2)
MSI[c]			
MSI-H	39 (22.2)	13 (23.6)	26 (21.5)
MSI-L	20 (11.4)	7 (12.7)	13 (10.7)
MSS	117 (66.5)	35 (63.6)	82 (67.8)

[a]χ^2 test, never smokers versus ever smokers, $P < 0.05$.
[b]One tumor exhibited a missense mutation in codon 12 as well as in codon 13.
[c]MSI-H, ⩾2 markers unstable; MSI-L, 1 marker unstable; MSS, 0 markers unstable.

OR 0.4, 95% CI 0.2–1.0, OR 0.3, 95% CI 0.1–0.7 and OR 0.4, 95% CI 0.2–1.0, respectively; not in Table III). Additionally, all were inversely associated with $p53^{pos}$ tumors and positively with $p53^{neg}$ tumors when compared to the population-based controls ($p53^{pos}$ versus controls, OR 0.5, 95% CI 0.2–1.0, OR 0.4, 95% CI 0.2–0.9 and OR 0.6, 95% CI 0.3–1.3, respectively; $p53^{neg}$ versus controls, OR, 1.4, 95% CI 0.7–2.8, OR 1.6, 95% CI 0.8–3.1 and OR 1.7, 95% CI 0.9–3.2, respectively; not in Table III). Interestingly, no clear associations were observed between cigarette smoking and tumor *p53* mutation status.

To evaluate whether cigarette smoking was specifically associated with the presence of transversion mutations, we additionally assessed the associations between smoking and carcinomas with transversion mutations in *APC*, K-*ras* or *p53* ($transv^+$ tumors). The majority of the $transv^+$ tumors harbored, at least, a transversion mutation in K-*ras*. Ever smoking was significantly differently associated with tumors with transversion mutations compared to tumors without transversion mutations (Table III). The case–control comparisons showed that ever smoking was positively, although not statistically significant, associated with tumors with transversion mutations and negatively, again not statistically significantly, with tumors without transversion mutations. Similar patterns were observed for the other smoking variables evaluated (data not shown). No clear associations were observed between smoking and tumors with transition mutations in *APC*, K-*ras* or *p53* (Table III).

Regarding MSI, no clear associations were observed between the smoking variables examined and MSI status (Table III). In addition, no clear associations were observed when the analyses were repeated with the subset MSI-L/MSS instead of MSS (data not shown).

Discussion

In this study, associations between cigarette smoking and various genetic alterations in sporadic colon carcinomas were assessed to evaluate the hypothesis that cigarette smoking is primarily linked to a specific colon tumor subset(s). Cigarette smoking was significantly differently related to $p53^{pos}$ tumors than to $p53^{neg}$ tumors. Consistent inverse associations were observed with $p53^{pos}$ tumors while positive associations were found with $p53^{neg}$ tumors. Smoking was also significantly differently related to tumors with transversion mutations in *APC*, K-*ras* or *p53* than to tumors without transversion mutations in one of these genes. Positive associations were observed with tumors with transversion mutations but not with tumors without transversion mutations. In addition, smoking seemed to especially increase the risk of developing tumors with K-*ras* mutations, in particular K-*ras* transversion mutations. No clear associations were observed with MSI status.

Truncating *APC* mutations were identified in 34.1%, K-*ras* mutations in 36.4%, *p53* mutations in 30.7% and p53 overexpression in 44.3% of the carcinomas in this study. Twenty-two percent of the carcinomas were MSI-H and the occurrence of MSI was significantly inversely related to the presence of genetic alterations in the *APC*, K-*ras* and *p53* genes and to p53 overexpression. Microdissection was performed and the observed frequencies and characteristics of the mutations identified were consistent with those in comparable populations previously reported by others (*APC* database, http://perso.curie.fr/Thierry.Soussi/APC/html; IARC *p53* database, http://www.iarc.fr/p53/index.html; 4–6,13,19,20,25,26). However, it remains possible that, due to contaminating normal DNA,

Table III. Cigarette smoking status and mutations in sporadic colon carcinomas: case–case and case–control comparisons

	Odds ratios (95% confidence intervals)[a]	
	Never smoked	Ever smoked
Overall		
No. cases/controls	55/79	121/170
Cases versus controls	1.0	1.0 (0.6–1.5)
APC[b]		
No. APC^+/APC^-/controls	25/30/79	35/86/170
APC^+ versus APC^-	1.0	0.6 (0.3–1.2)
APC^+ versus controls	1.0	0.7 (0.4–1.4)
APC^- versus controls	1.0	1.2 (0.7–2.1)
K-*ras*[c]		
No. K-ras^+/K-ras^-/controls	16/39/79	48/73/170
K-ras^+ versus K-ras^-	1.0	1.7 (0.8–3.4)
K-ras^+ versus controls	1.0	1.4 (0.7–2.8)
K-ras^- versus controls	1.0	0.8 (0.5–1.4)
No. K-ras^{transv}/K-ras^{trans}/K-ras^-/controls	6/10/39/79	25/23/73/170
K-ras^{transv} versus K-ras^-	1.0	2.2 (0.8–6.2)
K-ras^{transv} versus controls	1.0	2.2 (0.7–6.3)
K-ras^{trans} versus K-ras^-	1.0	1.4 (0.6–3.4)
K-ras^{trans} versus controls	1.0	1.0 (0.4–2.5)
p53 (mutations)[d]		
No. $p53^+$/$p53^-$/controls	16/39/79	38/83/170
$p53^+$ versus $p53^-$	1.0	1.1 (0.5–2.2)
$p53^+$ versus controls	1.0	0.9 (0.4–1.9)
$p53^-$ versus controls	1.0	1.0 (0.6–1.7)
Transversion mutations[e]		
No. $transv^+$/$transv^-$/controls	8/47/79	38/83/170
$transv^+$ versus $transv^-$	1.0	2.5 (1.0–5.9)
$transv^+$ versus controls	1.0	2.2 (0.9–5.6)
$transv^-$ versus controls	1.0	0.8 (0.5–1.3)
Transition mutations[f]		
No. $trans^+$/$trans^-$/controls	21/34/79	46/75/170
$trans^+$ versus $trans^-$	1.0	1.0 (0.5–2.0)
$trans^+$ versus controls	1.0	0.9 (0.5–1.7)
$trans^-$ versus controls	1.0	1.0 (0.6–1.8)
p53 (overexpression)[g]		
No. $p53^{pos}$/$p53^{neg}$/controls	29/26/79	49/72/170
$p53^{pos}$ versus $p53^{neg}$	1.0	0.4 (0.2–0.9)
$p53^{pos}$ versus controls	1.0	0.5 (0.3–1.0)
$p53^{neg}$ versus controls	1.0	1.5 (0.9–2.8)
MSI[h]		
No. MSI-H/MSS/controls	13/35/79	26/82/170
MSI-H versus MSS	1.0	0.8 (0.3–1.7)
MSI-H versus controls	1.0	0.8 (0.3–1.8)
MSS versus controls	1.0	1.1 (0.6–1.8)

[a]Adjusted for age, sex, total energy and alcohol intake.
[b]APC^+, tumors with truncating *APC* mutation; APC^-, tumors without truncating *APC* mutation.
[c]K-ras^+, tumors with mutation in K-*ras*; K-ras^-, tumors without mutation in K-*ras*; K-ras^{transv}, tumors with transversion mutation in K-*ras*; K-ras^{trans}, tumors with transition mutation in K-*ras*.
[d]$p53^+$, tumors with mutation in *p53*; $p53^-$, tumors without mutation in *p53*.
[e]$transv^+$, tumors with transversion mutations in *APC*, K-*ras* or *p53*; $transv^-$, tumors without transversion mutations in one of these genes.
[f]$trans^+$, tumors with transition mutations in *APC*, K-*ras* or *p53*; $trans^-$, tumors without transition mutations in one of these genes.
[g]$p53^{pos}$, tumors in which ⩾20% of the cells displayed nuclear positivity; $p53^{neg}$, tumors in which <20% of the cells displayed nuclear positivity.
[h]MSI-H, tumors in which two or more markers showed instability; MSS, tumors with no unstable markers.

alterations were missed, eventually resulting in misclassifications, which in turn may have attenuated some of our results.

In this study the MSI status of 22 tumors was determined using *BAT25* and *BAT26* tumor results only as for these tumors matching normal DNA was not available. The markers were either both unstable or both stable. Recently, polymorphisms have been identified in *BAT25* as well as in *BAT26*, disputing the earlier suggested quasimonomorphic allelic profile of these two loci and warranting caution in the interpretation of MSI data based on *BAT25* and *BAT26* tumor results only (31,32). However, the polymorphisms appear to be population dependent and to occur significantly more frequently in African-Americans than in Caucasians (31,32). In the latter, they truly seem to be uncommon, and being polymorphic at both loci will most likely be even more uncommon. Therefore, we do not expect that our decision to use *BAT25* and *BAT26* tumor results only, when matching normal DNA was not available, has resulted in extensive misclassification or led to serious misinterpretation of our data.

As in any retrospective study, an important concern is the possibility of information and selection bias. The smoking habits of our controls were comparable to those of the general Dutch population at the time of interview (33). Since the cases were unaware of the molecular profile of their tumors, systematic errors in recall are less likely to bias results from case–case comparisons. Recall of (smoking) habits, however, can also be influenced by tumor stage or treatments. Our cases were relatively healthy, i.e. the frequency of Dukes' A and B tumors among the cases was relatively high, 63%, compared with the 51% reported by the Dutch Cancer Registry (34). Adjusting the case–case comparisons for Dukes' stage did not change the estimates significantly. A long time lag between smoking exposure and occurrence of colon carcinomas has previously been suggested as a possible explanation for smoking being a risk factor for adenomas but not for colon cancer (35,36). In our study population 72% of the ever smokers among the cases first started smoking ⩾35 years ago.

This is, to our knowledge, the first study that has evaluated associations between cigarette smoking and alterations in the *APC* gene in sporadic colon carcinomas. If anything, our data suggest a slight inverse association between the two; it seems that most sporadic colon cancers related to smoking are not initiated via alterations in the *APC* gene. This is not entirely unexpected considering that the frequency of *APC* mutations observed in sporadic adenomas is similar to that in sporadic carcinomas (4) and that APC appears to also play a role in the later stages of tumor development (37,38).

A few studies have previously looked at cigarette smoking and other genetic alterations in colon tumors. In a large population-based case–control study on sporadic colon cancer, Slattery *et al.* (6) observed a slight increased risk of K-ras^- tumors when smoking ⩾20 cigarettes/day (K-ras^- versus controls, OR 1.3, 95% CI 1.1–1.6). However, the risk of K-ras^+ tumors increased as well (K-ras^+ versus controls, OR 1.2, 95% CI 0.9–1.5). Additionally, smoking >20 cigarettes/day was associated with an increased risk of overall colon cancer in their study population (39). In line with our results for K-*ras*, Martinez *et al.* (40) reported a slight positive, but non-significant, association with K-*ras* mutations in 0.5 cm or larger sporadic colorectal adenomas for current versus never smokers.

In the study population of Slattery *et al.*, cigarette smoking was found to be positively associated with microsatellite unstable tumors (20). Yang *et al.* (21), who purposely enriched their study for MSI-H cases, also reported a positive association between cigarette smoking and MSI-H tumors. We observed

no clear associations between smoking and MSI-H tumors and, hence, did not confirm their results. Both Slattery *et al*. and Yang *et al*. did not use the Bethesda reference panel (30) to assess MSI, which may explain the difference in results. Additionally, it is possible that we observed no associations due to the size of our study population and/or because the frequency of heavy smokers among our smokers was lower. Similarly to our results, Slattery *et al*. reported an inverse relation between K-*ras* mutations and MSI (13,20).

We observed no clear associations with *p53* mutations but our results for p53 overexpression suggest that most colon cancers related to cigarette smoking develop through a $p53^{neg}$ pathway (which is not necessarily also *p53* mutation-negative; see below). Consistent with our findings for p53 overexpression, Freedman *et al.* (19) observed an increased risk of $p53^{neg}$ colon tumors for current and former smokers. They did not evaluate *p53* mutations. Although p53 overexpression is often used as an indicator of *p53* mutations, not all *p53* mutations (e.g. nonsense and frameshift mutations) result in the accumulation of inactive p53 protein and can be detected by immunohistochemical analysis (41). In our population for instance, 20% of the *p53* mutations resulted in $p53^{neg}$ tumors. Under normal circumstances, wild-type p53 is activated and accumulates in response to DNA damage and various other types of stress, resulting in either growth arrest or apoptosis (42). Cancer cells need to somehow circumvent this checkpoint to be able to proliferate. A possible explanation for the observed association with a $p53^{neg}$ pathway is that cigarette smoking can somehow (e.g. by preventing post-translational modifications to occur) suppress the induction or stabilization of wild-type p53 (resulting in $p53^{neg}$ cells), allowing cells to proliferate in conditions where cells with intact p53 function are eliminated.

Interestingly, we observed a positive association between cigarette smoking and tumors with transversion mutations in *APC*, K-*ras* or *p53*. This suggests that cigarette smoking is particularly involved in the production of transversion mutations in colon cells. However, it should be noted that genetic alterations in tumors not only represent the interactions of carcinogens with DNA repair processes but also reflect the, possibly tissue-specific, selection of those mutations that provide pre-malignant and malignant cells with a clonal growth advantage. Consistent with our results, transversion mutations in *p53* and K-*ras* are also commonly found in smoking-related lung cancers (15–18). Additionally, Conway *et al.* (43) reported recently that *p53* transversion mutations, and especially G→T transversions, were significantly more prevalent in breast tumors from current smokers than in breast tumors from never smokers.

To conclude, our data suggest that smoking-related colon cancers develop through a $p53^{neg}$ pathway and that cigarette smoking particularly results in colon tumor cells with transversion mutations. Regarding the latter, it appears that cigarette smoking especially results in colon tumors with K-*ras* transversion mutations. This may be due to hypersensitivity of codons 12 and 13 of K-*ras* for exposure to tobacco smoke carcinogens or to a higher selective advantage for colon tumor formation exerted by these mutations in K-*ras* than in one of the other genes examined. Our results, if confirmed in other studies, provide support for the hypothesis that cigarette smoking is primarily associated with specific colon tumor subgroups.

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