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The relationship between smoking exposure and p53 overexpression in colorectal cancer

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Summary Although epidemiological studies of the relationship between cigarette smoking and colorectal cancer risk have been equivocal, a positive association is consistently found for colorectal adenoma development. We performed an epidemiological study to determine whether p53 protein overexpression, in tumours obtained at the time of resection, is associated with cigarette exposure in colorectal cancer. A total of 163 colorectal cancer cases and 326 healthy controls responded to a standardised questionnaire on colorectal cancer risk factors including detailed information on their history of cigarette smoking. All patients' tumours were analysed immunohistochemically for p53 overexpression using an avidin-biotin immunoperoxidase procedure and polyclonal anti-p53 antibody CM1. Comparison of colorectal cases with controls revealed an elevated risk for ex-smokers (OR = 1.34, 95% CI 0.85-2.12) and current smokers (OR = 1.13, 95% CI 0.63-2.02) when compared with non-smokers. No dose-response relationship was found for total pack-years of smoking (trend test: $P = 0.19$). However, a trend for total pack-years of smoking was found when p53-positive cases were compared with p53-negative cases suggesting aetiological, heterogeneity (trend test: $P = 0.06$). Estimating the individual relative risk of developing a p53-positive tumour relative to controls showed no associations for smoking status or total pack-years of smoking. However, when p53-negative cases were compared with controls, an elevated risk was found for ex-smokers (OR = 1.84, 95% CI 1.00-3.37) and current smokers (OR = 1.78, 95% CI 0.88-3.61). A significant trend of increasing risk was also found for total pack-years of smoking (trend test: $P = 0.03$). Colorectal tumours developing through p53-positive dependent pathways were not associated with smoking exposure. A significant increase in risk was observed for the p53-negative independent pathway with smoking. p53 overexpression appears to be associated with smoking exposure in colorectal cancer.

Keywords: colorectal cancer; neoplasms; p53; smoking

Cigarette smoking is known to contribute to the development of many cancers. Results from epidemiological studies investigating its role in colorectal cancer have been inconsistent. Whereas some case-control studies have demonstrated an increased risk (Martinez *et al.*, 1981; Dales *et al.*, 1979; Vobecky *et al.*, 1983; Kabat *et al.*, 1986; Jarebinski *et al.*, 1988, 1989; Slattery *et al.*, 1990; Kune *et al.*, 1992*a*), others find no association (Wynder *et al.*, 1969; Haenszel *et al.*, 1973; Williams and Horm, 1977; Graham *et al.*, 1978; Jain *et al.*, 1980; Tuyns *et al.*, 1982; Ferraroni *et al.*, 1989; Olsen and Kronberg, 1993), or a reduction in risk (Higginson, 1966; Wynder and Shigematsu, 1967; Stazewski, 1969; Haenszel *et al.*, 1980; Papdimitriou *et al.*, 1984; Tajima and Tominaga, 1985; Peters *et al.*, 1989; Choi and Kahyo, 1991). In contrast, studies of the relationship between smoking and colorectal adenomas have consistently observed a positive relationship (Olsen and Kronberg, 1993; Zahm *et al.*, 1991; Honjo *et al.*, 1992; Kikendall *et al.*, 1991; Hoff *et al.*, 1987; Monnet *et al.*, 1991; Lee *et al.*, 1993; Sandler *et al.*, 1993; Kune *et al.*, 1992*b*; Demers *et al.*, 1988; Cope *et al.*, 1991; Martinez *et al.*, 1995; Drexler, 1971). To explore these divergent findings, data from two recent cohort studies were examined (Giovannucci *et al.*, 1994*a* and *b*). Results from both studies were similar and suggest that smoking may act as an initiator of colorectal carcinogenesis. Previous studies may have yielded equivocal results because they failed to allow for the presumably long induction period between smoking onset and colorectal neoplasia.

Carcinogens present in cigarette smoke cause DNA damage and may produce specific mutations. Alterations of TP53 is the most frequent molecular abnormality found in human cancer and is a susceptible target for many exogenous carcinogens and endogenous mutagens in a variety of tumours (Hollstein *et al.*, 1991*a*; Harris, 1991; Bennett *et al.*, 1991). Mutation of TP53 induces loss of tumour-suppressor functions and usually results in the overexpression of mutant p53 protein (Lane, 1990). Overexpression of p53 protein can be detected by immunohistochemistry and correlates well with TP53 mutation (Cordon-Cardo *et al.*, 1994; Umekita *et al.*, 1994). Mutations and overexpression of p53 have been associated with a history of cigarette exposure in cancers where p53 alterations are an early occurrence in carcinogenesis such as lung (Suzuki *et al.*, 1992; Miller *et al.*, 1992), head and neck (Field *et al.*, 1991, 1994), oesophageal (Hollstein *et al.*, 1991*b*), and bladder cancer (Spruck *et al.*, 1993; Zhang *et al.*, 1994*a*).

Colorectal carcinogenesis is a multistep process involving adenoma formation and progression to carcinoma due to mutational activation of oncogenes and inactivation of tumour suppressor genes (Fearon and Vogelstein, 1990). Alteration of TP53 genes is a late event in the adenoma-carcinoma sequence and occurs before the transition of large adenomas to carcinomas (Fearon, 1993).

Given that colorectal adenomas are regarded as precursors of cancer, the repeatedly observed positive association between smoking and adenomas, but not cancer, is puzzling. The possibility exists that tumours acquiring genetic alterations late in the adenoma-carcinoma sequence

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may be unrelated to smoking exposure and may distort results of studies examining the relationship between smoking and colorectal cancer risk. To examine the hypothesis that tumour formation via the p53 (p53 negative) independent pathway is more strongly associated with smoking than p53 (p53 positive) dependent pathways, we analysed smoking exposure among 163 colorectal cancer patients with respect to p53 overexpression.

Materials and methods

Patient population

Patients diagnosed with a first primary sporadic colorectal cancer at Roswell Park Cancer Institute and Buffalo General Hospital in Buffalo, NY, USA between 1982 and 1993 were asked to complete an extensive questionnaire soliciting information on family history of cancer, tobacco use and other lifestyle behaviours. Patients who completed the questionnaire and had paraffin-embedded tumour specimens available for p53 immunohistochemical analysis were eligible for study. Using these criteria, 163 colorectal cancer patients qualified for evaluation.

Control population

Between 1982 and 1987, over 2500 patients admitted to Roswell Park Cancer Institute with non-malignant diseases or visiting the screening clinic, were also routinely asked to complete an epidemiological questionnaire. For this study a total of 326 controls were used, with the majority (256/326) made up of healthy screening clinic visitors. All colorectal cancer controls were matched to cases by gender and age within five years.

Exposure

Subjects were categorised as current smokers, never smokers, or ex-smokers (if they stopped five or more years before). To determine total lifetime smoking, a cumulative cigarette pack-year history was calculated for each subject. One pack-year of smoking is equivalent to having smoked one pack (20 cigarettes) per day for an entire year.

Immunohistochemical method

Formalin-fixed, paraffin-embedded tissue sections from the tumours were analysed immunohistochemically for altered patterns of p53 expression, using a standard avidin-biotin technique. Sections (4 μm thick) were deparaffinised in xylene, rehydrated in a graded ethanol series and incubated in 3% hydrogen peroxide for 20 min. After rinsing in phosphate-buffered saline (PBS) pH 7.4, sections were incubated for 15 min in boiling distilled water to promote antigen retrieval of masked antigens (Shi *et al.*, 1991). Tissue sections were blocked in 2% normal goat serum and then incubated overnight at 4°C with NCL-p53-CM1 antibody (Novacastra Laboratories, UK) at a dilution of 1:1000. This antibody is a rabbit polyclonal antibody that detects both wild-type and mutant forms of p53. Sections were then incubated with biotinylated goat anti-rabbit antiserum (Vector Laboratories, Burlingame, CA) for 45 min, then with ABC reagent (avidin-biotin peroxidase complex, Vectastain Elite Kit, Vector Laboratories) for 30 min. Immunoreaction was developed for 6 min using a solution containing 0.5 mg ml^{-1} 3,3'-diaminobenzidine tetrahydrochloride and 0.03% hydrogen peroxide. Tissue sections were counterstained with light haematoxylin, dehydrated with ethanol, cleared with Histo-Clear (National Diagnostics, Manville, NJ), and mounted under a coverslip. Sections known to stain positively were included in each run, receiving either primary anti-p53 antibody or PBS as positive and negative controls, respectively.

The slides were scored independently by two pathologists (JEA, SS) without any clinical or pathological information. Tumours were classified as p53 negative if 0-19% of cells displayed nuclear positivity and p53 positive if greater than or equal to 20% of cells were positive for nuclear p53. Staging of all tumours was performed (NJP) according to the TNM pathological staging system.

Statistical analysis

Associations between disease and cigarette smoking were measured using odds ratios (OR) and 95% confidence intervals (CI). Unconditional logistic regression analysis was used to obtain maximum likelihood estimates of odds ratios and their 95% confidence intervals after controlling for gender, age (<55, 55-64, 65-74, $\geqslant 75$ years), family history of colorectal cancer, body mass index and alcohol, cruciferous vegetable and meat consumption (Breslow and Day, 1980). To assess dose-response relationship, smoking exposure was classified by total pack-years of smoking in four categories: (1) non-smoking, (2) 1-19 pack-years, (3) 20-39 pack-years, (4) $\geqslant 40$ pack-years. Trend tests were performed by assigning the score j to the jth exposure level of a categorical variable, and treating it as a continuous variable in the logistic model.

To examine aetiologic heterogeneity, odds ratios were calculated for the association between p53 nuclear overexpression and smoking. This odds ratio is the odds of smoking exposure in the p53-positive group divided by the odds of smoking exposure in the p53-negative group. Each of these odds ratios represents the ratio of the relative risk of smoking for p53-positive tumours to the relative risk of smoking for p53-negative tumours. Aetiological heterogeneity is indicated by departures from the value of 1 (Begg and Zhang, 1994).

With healthy controls used as the referent group, three additional comparisons were made. First, standard analysis was conducted comparing all colorectal cases with controls. To estimate the risk of developing a colorectal tumour through a p53 (positive) dependent pathway, only colorectal patients with $p53^+$ overexpression were compared with controls. To estimate the risk of developing a colorectal tumour through a p53 (negative) independent pathway, colorectal patients with $p53^-$ overexpression were compared with controls.

Results

Characteristics of patients

Nineteen per cent of the patients were less than 55 years of age, 29% were between the ages of 55 and 64 years, 34% were between 65 and 74 years old and 17% were 75 or older. Fifty-six per cent (91/163) of the subjects were males and 96% (157/163) were white. Forty-seven per cent (77/163) of patients' tumours were located in the rectum or rectosigmoid regions of the large bowel. Patient tumour distribution by TNM stage consisted of 26% stage 0/I, 23% stage II, 34% stage III, and 16.5% stage IV.

p53 nuclear overexpression

Nuclear overexpression of p53 protein in 20% or more of the cells was found in 44.8% (73/163) of colorectal tumours. None of the mucosa adjacent to tumour showed any detectable nuclear reactivity. Neither gender, age nor tumour stage differed with respect to p53 overexpression (Table I). Patients with a positive family history of colorectal cancer were more likely to have $p53^-$ tumours ($P<0.05$). Of the 31 patients reporting a positive family history, only nine displayed p53 nuclear reactivity in their tumours, compared with 64 of 128 patients without a family history of colorectal cancer.

Case-control comparison

Among the 163 patients, 162 (99%) provided a complete smoking history. All 326 controls reported their use of

tobacco. Odds ratios for smoking status and total pack-years of smoking adjusted for gender, age, family history of colorectal cancer, body mass index and alcohol, cruciferous vegetables and meat consumption are presented in Table II. Ex-smokers (OR = 1.34, 95% CI 0.85-2.12) and current smokers (OR = 1.13, 95% CI 0.63-2.02) were observed to be at increased risk when compared with non-smokers. A significant dose-response relationship was observed for total pack-years of smoking after controlling for age, gender and family history (trend test: $P = 0.05$). However, after adjusting for dietary risk factors, the dose-response relationship no longer remained statistically significant (trend test: $P = 0.19$).

Smoking exposure and p53 overexpression

Nuclear overexpression of the p53 protein was observed in 29% (33/59) current smokers, 42.3% (30/71) ex-smokers and 55.9% (33/59) of non-smokers. The association between smoking status and p53 overexpression resulted in an odds ratio of 0.60 (95% CI 0.28-1.31) for ex-smokers and 0.35 (95% CI 0.12-0.98) for current smokers when compared with non-smokers (Table III). A dose-response relationship was observed with categories of total pack-years of smoking after controlling for potential confounders (trend test: $P = 0.06$). No significant associations or trends were observed for smoking status or total pack-years of smoking when $p53^+$ cases were compared with controls (Table IV).

Table I Distribution of colorectal cancer patients' tumours for p53 overexpression according to gender, age, TNM stage and family history of colorectal cancer

Variable	*%p53+ overexpression*
Gender	
Male	42.9 (39/91)
Female	47.2 (34/72)
Age (years)	
<55	44.4 (12/27)
55-64	45.8 (22/48)
65-74	50.0 (28/56)
⩾75	39.3 (11/28)
TNM Stage	
O/I	47.5 (19/40)
II	28.6 (10/35)
III	50.0 (26/52)
IV	56.0 (14/25)
Family history of colorectal cancer[a]	
Positive	29.0 (9/31)
Negative	50.0 (64/128)

[a]$P < 0.05$.

However, markedly different results were found when $p53^-$ cases were compared with controls. Elevated risks were observed for ex-smokers (OR = 1.84, 95% CI 1.00-3.37) and current smokers (OR = 1.78, 95% CI 0.88-3.61) when compared with non-smokers. Moreover, a significant trend of increasing risk was observed for total pack-years of smoking (trend test: $P = 0.03$). An odds ratio of 2.72 (95% CI 1.38-5.36) for 20-39 pack-years of smoking and an odds ratio of 1.68 (95% CI 0.83-3.39) for ⩾40 pack-years was observed when compared with non-smokers.

Discussion

Epidemiological studies have not found a consistent association between colorectal cancer risk and tobacco use. Both case control and cohort studies have reported conflicting findings (Martinez *et al.*, 1981; Dales *et al.*, 1979; Vobecky *et al.*, 1983; Kabat *et al.*, 1986; Jarebinski *et al.*, 1988, 1989; Slattery *et al.*, 1990; Kune *et al.*, 1992*a*; Wynder *et al.*, 1969; Haenszel *et al.*, 1973, 1980; Williams and Horm, 1977; Graham *et al.*, 1978; Jain *et al.*, 1980; Tuyns *et al.*, 1982; Ferraroni *et al.*, 1989; Olsen and Kronberg, 1993; Higginson, 1966; Wynder and Shigematsu, 1967; Stazewski, 1969; Papdimitriou *et al.*, 1984; Tajima and Tominaga, 1985; Peters *et al.*, 1989; Choi and Kahyo, 1991; Giovannucci *et al.*, 1994*a* and *b*; Cartensen *et al.*, 1987; Chute *et al.*, 1991; Doll *et al.*, 1980; Doll and Peto, 1976; Garland *et al.*, 1985; Hammond, 1966; Hammond and Horn, 1958; Hirayama, 1975; Kahn, 1966; Klatsky *et al.*, 1988; Kono *et al.*, 1987; Rogot and Murray, 1980; Sandler *et al.*, 1988; Tverdal *et al.*, 1993; Weir and Dunn, 1970; Williams *et al.*, 1981; Wu *et al.*, 1987; Heineman *et al.*, 1995). Results from our case control comparison reveal elevated risks for former (OR = 1.34, 95% CI 0.85-2.12) and current smokers (OR = 1.13, 95% CI 0.63-2.02), and a non-significant dose-response relationship for total pack-years of smoking (trend test: $P = 0.19$).

Unlike the case of colorectal cancer risk, positive associations between tobaccco use and adenomatous polyp development have been reported in 14 of 15 studies (Olsen and Kronberg, 1993; Zahm *et al.*, 1991; Honjo *et al.*, 1992; Kikendall *et al.*, 1991; Hoff *et al.*, 1987; Monnet *et al.*, 1991; Lee *et al.*, 1993; Sandler *et al.*, 1993; Kune *et al.*, 1992*b*; Demers *et al.*, 1988; Cope *et al.*, 1991; Martinez *et al.*, 1995; Drexler, 1971; Giovannucci *et al.*, 1994*a* and *b*). This incongruity is surprising since adenomas are thought to be precursors of cancer. To clarify this issue, Giovannucci and colleagues examined the association between cigarette smoking with both colorectal adenoma and carcinoma in two separate cohort studies encompassing over 35 years of follow-up (Giovannucci *et al.*, 1994*a* and *b*). The authors report that small adenomas may be associated with less than 20 years of smoking, larger adenomas may require 20 years of smoking exposure and colorectal cancers are associated

Table II Colorectal cancer risk for smoking status and pack-years of smoking

	Cases	*Controls*	*OR (95% CI)*[a]	*OR (95% CI)*[b]
Smoking status				
Non-smoker	59	145	1.00	1.00
Former smoker (⩾5 years)	71	124	1.51 (0.96-2.36)	1.34 (0.85-2.12)
Smoker	32	57	1.22 (0.70-2.14)	1.13 (0.63-2.02)
Total pack-years of smoking				
0	59	145	1.00	1.00
1-19	24	62	1.04 (0.59-1.85)	1.00 (0.56-1.80)
20-39	36	53	1.68 (0.98-2.88)	1.56 (0.89-2.72)
⩾40	43	66	1.53 (0.91-2.59)	1.28 (0.74-2.22)
P value for trend			0.05	0.19

[a]Adjusted for age, gender and family history of colorectal cancer. [b]Adjusted for age, gender, family history of colorectal cancer, body mass index and alcohol, cruciferous vegetables and meat consumption.

Table III Association between p53 overexpression and smoking in colorectal cancer

	p53+ cases	*p53− cases*	*OR (95% CI)*[a]	*OR (95% CI)*[b]
Smoking status				
Non-smoker	33	26	1.00	1.00
Former smoker (≥5 years)	30	41	0.61 (0.29–1.30)	0.60 (0.28–1.31)
Smoker	9	23	0.35 (0.35–0.95)	0.35 (0.12–0.98)
Total pack–years of smoking				
0	33	26	1.00	1.00
1–19	12	12	0.87 (0.32–2.38)	0.92 (0.33–2.61)
20–39	10	26	0.30 (0.11–0.77)	0.30 (0.11–0.78)
≥40	17	26	0.58 (0.24–1.37)	0.55 (0.22–1.37)
P value for trend			0.07	0.06

[a]Adjusted for age, gender, family history of colorectal cancer and hospital of diagnosis. [b]Adjusted for age, gender, family history of colorectal cancer, hospital of diagnosis, body mass index and alcohol, cruciferous vegetables and meat consumption.

Table IV Risk of developing a colorectal tumour for smoking status and total pack–years of smoking by p53 overexpression

				p53+ tumour		*p53− tumour*	
	p53+ cases	*p53− cases*	*Controls*	*OR (95% CI)*[a]	*OR (95% CI)*[b]	*OR (95% CI)*[a]	*OR (95% CI)*[b]
Smoking status							
Non-smoker	33	26	145	1.00	1.00	1.00	1.00
Former smoker (≥5 years)	30	41	124	1.09 (0.61–1.95)	0.95 (0.52–1.73)	2.07 (1.15–3.74)	1.84 (1.00–3.37)
Smoker	9	23	57	0.68 (0.30–1.54)	0.63 (0.27–1.46)	1.94 (0.97–3.85)	1.78 (0.88–3.61)
Total pack–years of smoking							
0	33	26	145	1.00	1.00	1.00	1.00
1–19	12	12	62	0.92 (0.44–1.92)	0.87 (0.41–1.84)	1.21 (0.56–2.61)	1.17 (0.53–2.57)
20–39	10	26	53	0.81 (0.37–1.79)	0.72 (0.32–1.64)	2.87 (1.49–5.56)	2.72 (1.38–5.36)
≥40	17	26	66	1.10 (0.55–2.18)	0.92 (0.45–1.92)	2.09 (1.07–4.10)	1.68 (0.83–3.39)
P-value for trend				0.94	0.68	0.005	0.03

[a]Adjusted for age, gender and family history of colorectal cancer. [b]Adjusted for age, gender, family history of colorectal cancer, body mass index and alcohol, cruciferous vegetables and meat consumption.

with at least 35 years of smoking. These results suggest that earlier studies of colorectal cancer risk and smoking are inconsistent because they failed to allow for an adequate induction period. In addition, these results suggest that smoking may act as an initiator of colorectal carcinogenesis, causing mutations in genes that occur early in the adenoma–carcinoma sequence (e.g. *apc*, *ras*). The fact that individuals born with a mutated *apc* gene (familial adenomatous polyposis) require an average of 35 years for their adenomas to convert to carcinoma supports this hypothesis. Moreover, since smoking within the past 35 years was related to adenoma formation but not to cancer risk, smoking is unlikely to directly influence mutations that occur late in the progression from adenoma to carcinoma. These findings imply that studies of colorectal cancer risk and smoking may be biased by the inclusion of cases whose tumours develop gene mutations late in the adenoma–carcinoma sequence and may not be directly associated with smoking.

The p53 tumour-suppressor gene is the most common genetic abnormality found in colorectal cancer (Fearon, 1993). Mutation and overexpression of p53 occurs late in colorectal carcinogenesis, before the transition of an adenoma to a carcinoma (Fearon, 1993). Carcinogens present in tobacco smoke cause DNA damage and may influence TP53 pathways in many human cancers. Associations between p53 mutation/overexpression and tobacco smoking have been observed in tumour sites where this mutation is an early occurrence in the progression to carcinoma such as lung (Suzuki *et al.*, 1992; Miller *et al.*, 1992), head and neck (Field *et al.*, 1991, 1994), oesophagus (Hollstein *et al.*, 1991*b*) and bladder (Spruck *et al.*, 1993; Zhang *et al.*, 1994*a*), but not those where p53 is a late occurrence such as stomach (Zhang *et al.*, 1995*a*) and prostate (Zhang *et al.*, 1994*b*). Zhang *et al.* (1995*b*) recently reported no obvious association between tobacco smoking and p53 nuclear expression in colorectal cancer. However, the study had a relatively small sample size and included only patients with Duke's C stage neoplasms.

To investigate the relationship between p53 overexpression and smoking exposure in colorectal cancer, we analysed tumour specimens for p53 protein and categorised them according to the patient's smoking exposure. Case series analysis suggested aetiological heterogeneity for p53 overexpression and smoking exposure and may indicate the presence of distinct causal mechanisms for $p53^+$ cases and $p53^-$ cases. To estimate the individual relative risks of developing a $p53^+$ versus a $p53^-$ tumour we used a healthy control group. We observed that tumours developing through a $p53^+$-dependent pathway were unrelated to smoking exposures. However, those tumours developing through a p53 independent pathway were significantly associated with both smoking status and total pack–years of smoking (trend test: $P=0.03$). These data may suggest that smoking may initiate tumour mutations early in the adenoma–carcinoma sequence, perhaps in *apc* or *ras* genes (Fearon and Vogelstein, 1990), that do not require p53 mutation/overexpression to progress from an adenoma to a carcinoma. This also may explain conflicting results between risk of colorectal adenomas and carcinoma relative to smoking exposure.

The p53 gene is considered a common target in human carcinogenesis and mutations of this gene may inactivate their encoded mutant proteins. Many studies have observed a high correlation between p53 gene missense mutation and immunohistochemical detection of p53 overexpression (Cordon-Cardo *et al.*, 1994; Umekita *et al.*, 1994).

However, the absence of p53 overexpression does not necessarily imply the absence of p53 mutations, since tumours with frameshift or null mutations may have undetectable levels of p53 protein, although only 8% of all colorectal tumours display this type of alteration (Greenblatt *et al.*, 1994). Therefore, misclassification of p53 protein overexpression status as it relates to the underlying mutation is likely to be small in colorectal tumours.

Jones *et al.* (1991) suggested that there are two general patterns of p53 mutations: mutations induced by exogenous carcinogens, and those occurring spontaneously due to endogenous mutagens. Carcinogen-induced mutations are caused by direct interaction of carcinogens with DNA, leading to specific point mutations, predominantly transversions. For example, a study of p53 mutations in non-small cell lung carcinomas found that most mutations were transversions at G and C residues (Chiba *et al.*, 1990). This suggests that lung cancer results from the direct interaction of carcinogens in cigarette smoke with DNA.

Spontaneous mutations arise at CpG dinucleotides, which are thought to result from the frequent methylation of the cytosine residue in CpG sites with a high frequency of transitions. These spontaneous mutations are assumed to result from an endogenous process, as no exogenous factors have been identified. In colorectal cancer, most p53 mutations are spontaneous with transitions occurring at CpG sites. However, a small percentage of p53 mutations in colorectal cancer are transversions or occur at non-CpG sites, and may result from an exogenous carcinogen. If we assume that p53 overexpression indicates mutation, it is possible that although the majority of our $p53^+$ cases may be unrelated to smoking (CpG transitions), a small percentage of $p53^+$ tumours (transversions) may be associated with tobacco use. Ideally, future studies should employ immunohistochemical detection of p53 overexpression along with sequence analysis to examine these associations.

The exact mechanism by which cigarette smoking influences colorectal carcinogenesis is unclear. Smoking increases the risk at several cancer sites not directly in contact with smoke including pancreas, stomach, kidney and bladder (US Department of Health, Education, and Welfare, 1979). Carcinogens in smoke could affect the mucosa of the large bowel through direct ingestion or through the circulatory system. Polycyclic aromatic hydrocarbons (PAHs) and heterocyclic amines are two potent carcinogens found in cigarette smoke and are also observed in well-done meat, another risk factor for colorectal cancer (Laden *et al.*, 1995).

The possibility exists that cigarette smokers consume a diet consisting of fewer protective and more numerous carcinogenic agents. Smokers generally have lower intakes of fruits and vegetables and higher intakes of fats than non-smokers (Subar and Harlan, 1993). Therefore, the association between p53 (negative) independent tumours and smoking may be confounded by dietary factors associated with this pathway. However in our analysis, a statistically significant dose–response relationship for the association between p53 negative patients and total pack–years of smoking existed (trend test: $P=0.03$), even after adjustment for alcohol, meat, cruciferous vegetables and body mass index. Therefore, it is unlikely that confounding by diet completely accounts for the association between cigarette smoking and the p53 independent pathway.

The use of hospital/screening clinic controls in a case–control comparison may lead to bias since controls may overrepresent healthier lifestyles. However, the odds ratios observed in our case–control comparison are similar to those reported in three recent population-based cohort studies (Giovannucci *et al.*, 1994*a* and *b*; Heineman *et al.*, 1995). Additionally, the primary use of this control group is to assist in the estimation of the individual relative risks for p53 positive and p53 negative tumour development separately, for smoking exposure variables. This bias is of less concern in our case series analysis since patients are unaware of their p53 status.

The positive consistent finding that smoking increases the risk of colorectal adenoma formation but not cancer has been problematic. Our results suggest that results of studies of colorectal cancer risk and smoking may be affected by inclusion of patients whose tumours harbour p53 genetic alterations that occur late in the adenoma–carcinoma sequence, that are unrelated to smoking history. In addition, smoking was significantly associated in tumours developing through a p53 independent pathway, perhaps the result of initiation of mutations in genes early in the carcinogenic process that do not need a p53 alteration to progress from an adenoma to a carcinoma.

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