BIOMARKERS, 2000, VOL. 5, NO. 3, 225–234

Expression of p27^{Kip1} and bcl-2, cigarette smoking, and colorectal cancer risk

NAOKO ISHIBE[1]*, ANDREW N. FREEDMAN[2], ARTHUR M. MICHALEK[3], CHRISTINE IACOBUZIO-DONAHUE[4], CURTIS J. METTLIN[5], NICHOLAS J. PETRELLI[6], JOHN E. ASIRWATHAM[7] and STANLEY R. HAMILTON[4, 8]

[1] Genetic Epidemiology Branch, National Cancer Institute, Bethesda, MD, USA

[2] Applied Research Program, National Cancer Institute, Bethesda, MD, USA

[3] Department of Educational Affairs, Roswell Park Cancer Institute, Buffalo, NY, USA

[4] Division of GI-Liver Pathology, The Johns Hopkins Medical Institutions, Baltimore, MD, USA

[5] Cancer Control and Epidemiology, Roswell Park Cancer Institute, Buffalo, NY, USA

[6] Department of Surgical Oncology, Roswell Park Cancer Institute, Buffalo, NY, USA

[7] Sisters Hospital, Buffalo, NY, USA

[8] Division of Pathology and Laboratory Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Received 20 October 1999, revised form accepted 7 January 2000

Although a positive association between cigarette smoking and colorectal adenoma development is consistently found, the association with colorectal cancer remains controversial. We evaluated the potential roles of p27^{Kip1} and bcl-2 protein expressions in conjunction with cigarette smoking exposure and colorectal cancer risk in a hospital-based case-control study. A total of 163 colorectal cancer patients from Roswell Park Cancer Institute and Buffalo General Hospital and 326 healthy controls responded to a standardized questionnaire on colorectal cancer risk factors including detailed information on their history of cigarette smoking; 110 of the patients' tumours were available for immunohistochemical analysis of p27^{Kip1} and bcl-2 protein overexpression. An avidin–biotin immunoperoxidase procedure was used to determine expression after incubation with mouse monoclonal p27^{Kip1} and mouse monoclonal bcl-2 antibodies, respectively. A statistically significant trend for total pack-years of smoking was found when p27^{Kip1} positive cases were compared with p27^{Kip1} negative cases (trend test, $p = 0.007$). Although a weak inverse association was observed with smoking exposure among p27^{Kip1} negative tumour cases in comparison to controls, a significant dose–response association was seen with p27^{Kip1} positive tumours. The relative risk of developing a p27^{Kip1} positive tumour was estimated to be 1.17 (95% CI 0.54–2.54) for those with less than 20 pack-years, 1.95 (95% CI 0.95–3.97) for those with 20–39 pack-years, and 2.25 (95% CI 1.14–4.45) for those with greater than 39 pack-years of smoking exposure (trend test, $p = 0.009$) when compared with controls. When cases with bcl-2 expression were compared with cases without bcl-2 expression, suggestion of a trend was also observed with pack-years smoked (trend test, $p = 0.09$). In our study of 110 patients with sporadic colorectal cancer and 326 controls, we observed differences in associations between cigarette smoking and expressions in p27^{Kip1} and bcl-2. Our data suggest that bcl-2 overexpression (or a bcl-2 dependent pathway) is associated with cigarette smoking in the development of colorectal cancer, whereas a loss of p27^{Kip1} expression is not. These associations indicate that there is aetiological heterogeneity in colorectal cancer development, and that they can indirectly allude to where these changes in protein expression occur in the adenoma–carcinoma sequence (i.e. early versus late events).

Keywords: p27^{Kip1}, bcl-2, smoking, colorectal cancer.

* Corresponding author: Naoko Ishibe, Genetic Epidemiology Branch, National Cancer Institute, 6120 Executive Blvd, MSC 7236, Bethesda, MD 20852, USA.

Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online © 2000 Taylo
http://www.tandf.co.uk/journals

Introduction

Cigarette smoking is associated with the development of many cancers. Results from epidemiological studies investigating its role in colorectal cancer have been equivocal Haenszel *et al*. 1973, 1980, Horm 1977, Graham *et al*. 1978, Dales *et al*. 1979, Jain *et al*. 1980, Tuyns *et al*. 1982, Vobecky *et al*. 1983, Papadimitriou *et al*. 1984, Tajima and Tominaga 1985, Kabat *et al*. 1986, Jarebinski *et al*. 1988, 1989, Ferraroni *et al*. 1989, Peters *et al*. 1989, Slattery *et al*. 1990, Choi and Kahyo 1991, Kune *et al*. 1992, Olsen and Kronberg 1993), in contrast to the consistently positive relationship observed between smoking and adenomas (Hoff *et al*. 1987, Demers *et al*. 1988, Cope *et al*. 1991, Kikendall *et al*. 1991, Monnet *et al*. 1991, Zahm *et al*. 1991, Honjo *et al*. 1992, Lee *et al*. 1993, Olsen and Kronberg 1993, Sandler *et al*. 1993, Martinez *et al*. 1995). While some case-control studies have demonstrated an increase in colorectal cancer risk with cigarette smoking (Graham *et al*. 1978, 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), others have found no association (Wynder *et al*. 1969, Haenszel *et al*. 1973, Williams and Horm 1977, Jain *et al*. 1980, Tuyns *et al*. 1982, Ferraroni *et al*. 1989, Olsen and Kronberg 1993), or even a reduction in risk (Haenszel *et al*. 1980, Papadimitriou *et al*. 1984, Tajima and Tominaga 1985, Peters *et al*. 1989, Choi and Kahyo 1991). Results from two recent prospective studies (Giovannucci *et al*. 1994a, b) 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.

There is molecular evidence to strengthen the hypothesis that cigarette smoke may be an initiator in colorectal carcinogenesis. We recently reported that colorectal tumour formation via the p53 independent pathway (without p53 overexpression) was more strongly associated with smoking than p53 dependent pathways (with p53 overexpression) (Freedman *et al*. 1996). This is consistent with the association observed between p53 mutation/overexpression and cigarette smoking in tumours where this aberration is an early molecular event (e.g. lung and bladder; Miller *et al*. 1992, Suzuki *et al*. 1992), but not in those where it is a late event (e.g. stomach and prostate; Spruck *et al*. 1993, Zhang *et al*. 1994). In addition, p53 mutations and gene product overexpression are more often observed with advanced colorectal cancer (Fearon 1993).

Another protein that is involved in cell-cycle control and may play an important role in colorectal cancer development is the cyclin-dependent kinase inhibitor (CDKI) $p27^{Kip1}$. Although mutations in the $p27^{Kip1}$ gene are rarely observed (Kawamata *et al*. 1995, Ponce-Castaneda 1995) and its protein expression is mainly regulated at post-transcriptional levels through its translation and degradation (Pagano *et al*. 1995, Hengst and Reed 1996), various studies have amassed evidence for its importance in controlling cell growth. In $p27^{Kip1}$ knockout mice, multiple organ hyperplasia and the development of pituitary tumours are observed (Fero *et al*. 1996, Kiyokawa *et al*. 1996, Nakayama *et al*. 1996). Furthermore, in human cell lines, an inverse correlation between the expression of $p27^{Kip1}$ and the degree of tumour malignancy has been observed in colorectal tumours (Fredersdorf *et al*. 1997). A similar inverse relationship has also been observed in molecular epidemiological studies where down-regulation of $p27^{Kip1}$ expression has been associated with tumour progression and reduced survival (Loda *et al*. 1997, Thomas *et al*. 1998, Palmqvist *et al*. 1999).

The bcl-2 gene product is thought to inhibit apoptosis (Hockenbery *et al*. 1990, Korsmeyer 1992) and has been shown to have a role in the biology of normal epithelial tissues. In the normal large bowel, bcl-2 expression is seen in the proliferative compartments at the base of the crypts but is lost in the epithelium of the upper crypts and luminal surface (Hockenbery *et al*. 1991). Overexpression of this protein has been reported in a high proportion of both adenomas and carcinomas, suggesting that it may be an early event in colorectal carcinogenesis (Hague *et al*. 1994, Bronner *et al*. 1995).

In this study, we evaluated the possible role of $p27^{Kip1}$ and bcl-2 expression in relation to cigarette smoking exposure and colorectal cancer risk in a hospital-based case-control study.

Material and methods

Study population

The cases and controls in this report are from a hospital-based case-control study of colorectal cancer described previously (Freedman *et al*. 1996). Briefly, patients diagnosed with a first primary sporadic colorectal cancer at Roswell Park Cancer Institute and Buffalo General Hospital in Buffalo, NY, between 1982 and 1993 were asked to complete an extensive questionnaire soliciting information on family history of cancer, tobacco use, and other lifestyle behaviour. Staging of all tumours was performed (NJP) according to the TNM pathological staging system. Patients who completed the questionnaire and had paraffin-embedded tumour specimens available for $p27^{Kip1}$ and bcl-2 immunohistochemical analysis were eligible for the current study. Controls were identified from among 2870 patients admitted to Roswell Park Cancer Institute between 1982 and 1987 with non-malignant diseases or among those who visited the screening clinic. These patients were routinely asked to complete an epidemiological questionnaire and were matched to cases by gender and age within 5 years. The following results were based on 110 colorectal cases and 326 controls.

Exposure

Subjects were categorized for cigarette smoking exposure as never, current, or former, where smokers were classified as former if they had quit five or more years before baseline. 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 methods

Tissue sections were processed from archival formalin-fixed paraffin-embedded tumour specimens using a microtome and mounted on clean polylysine-coated glass slides. The sections were then dewaxed in xylene and hydrated in graded concentrations of ethanol, followed by a rinse in deionized water. For antigen retrieval enhancement, slides were transferred to 1 × sodium citrate buffer (diluted from 10 × heat-induced epitope retrieval buffer, Ventana BioTek), followed by steaming at 80°C for 20 min. After treatment, slides were cooled for 5 min before staining.

Staining was performed on a BioTek-Tech Mate 1000 automated stainer (Ventana-BioTek Solutions, Inc., Tucson, AZ). Briefly, slides were washed in phosphate buffer followed by blocking for 7 min in blocking serum. Slides were then incubated overnight with primary antibody (1:800 dilution of $p27^{Kip1}$ mouse monoclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA; and 1:25 dilution of bcl-2 mouse monoclonal antibody, Dako Corporation, Carpentria, CA), followed by incubation with anti-mouse secondary antibody for 30 min. Endogenous peroxidase was blocked with hydrogen peroxide and colour developed using an avidin–biotin immunoperoxidase procedure for 30 min, followed by diaminobenzidine chromagen for 20 min. Haematoxylin was used for light counterstain of the slides. Slides were coverslipped using Baxter Accu-Mount permanent mounting medium.

Scoring method

The slides were scored independently by one of the co-authors (CID) without reference to any clinical or pathological information. Tumours were classified as $p27^{Kip1}$ negative (i.e. low expression) if less than or equal to 25% of cells displayed nuclear positivity and $p27^{Kip1}$ positive (i.e. high expression) if greater than 25% of cells were positive (Yasui *et al*. 1997). Tumours were classified as bcl-2 positive if staining was observed. The most common pattern of staining was approximately 5% of the tumour cells

accumulated nuclear bcl-2. Lymphocytes were used as the positive internal control for both classifications.

Statistical analysis

Associations between disease and cigarette smoking were measured using odds ratios and 95% confidence intervals. 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, ≥ 75 years), family history of colorectal cancer, body mass index (quartiles based on control distribution), and consumption of alcohol, cruciferous vegetables and meat (Breslow and Day 1980). To assess dose–response relationship, smoking exposure was classified as follows: (1) non-smoker, (2) 1–19 pack-years, (3) 20–39 pack-years, (4) ≥ 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 cigarette smoking and each of the immunohistochemical measures (i.e. $p27^{Kip1}$, bcl-2) separately. For example, this odds ratio is the odds of smoking exposure in the $p27^{Kip1}$ positive group divided by the odds of smoking exposure in the $p27^{Kip1}$ negative group. Each of these odds ratios represents the ratio of the relative risk of smoking for $p27^{Kip1}$ positive tumours to the relative risk of smoking for $p27^{Kip1}$ negative tumours. Aetiological heterogeneity is indicated by departures from the value of one (Begg and Zhang 1994).

Three additional comparisons were made, using healthy controls as the referent group. First, standard analysis was conducted comparing all colorectal cancer cases with controls. To estimate the risk of developing a colorectal tumour through a $p27^{Kip1}$ dependent pathway (i.e. loss of normal expression), only colorectal patients with $p27^{Kip1}$ negative expression were compared with controls. Likewise, to estimate the risk of developing a colorectal tumour through a $p27^{Kip1}$-independent pathway, colorectal patients with $p27^{Kip1}$ positive expression were compared with controls. Similar analyses were conducted for bcl-2 expression.

All tests of statistical significance were two-sided. All analysis was performed with the software package SAS Release 6.12 (SAS Institute Inc., Cary NC).

Results

Patient characteristics

Among the 110 colorectal cases and 326 controls, 45% of the subjects were male. The mean age for both cases and controls was approximately 63 years. As expected, family history of colorectal cancer was strongly associated with disease status. With the exception of obesity as measured by body mass index, none of the other colorectal cancer risk factors reached statistical significance. Table 1 summarizes the demographics and lifestyle characteristics of the subjects.

Approximately 72% of patients' tumours were located distal to the splenic flexure. Patient tumour distribution by TNM stage consisted of 28% stage I, 22% stage II, 32% stage III, and 18% stage IV.

$p27^{Kip1}$ expression and smoking in colorectal cancer

Nuclear expression of $p27^{Kip1}$ protein in less than or equal to 25% of cells was found in 23% of colorectal tumours. $p27^{Kip1}$ expression was not modified by gender, family history, stage, or tumour site (data not shown). Significant differences in pack-years of smoking were observed when cancers with $p27^{Kip1}$ expression were compared with cancer cases who lacked $p27^{Kip1}$ expression. A dose–response relationship was observed with categories of total pack-years of smoking after controlling for potential confounders (trend test: $p = 0.007$). Tumours of patients who reported greater smoking exposure tended to maintain $p27^{Kip1}$ expression; among 'heavy' smokers (i.e. greater than 40 pack-years); 29 of 31 (93.5%) patients displayed $p27^{Kip1}$ nuclear expression compared with only 24 of 38 (63.2%) non-smokers.

Table 1. Descriptive characteristics of colorectal cases ($n = 110$) and controls ($n = 326$).

Variables	Cases	Controls	p-value
Age	63.2 (±11.5)	63.7 (±11.5)	0.41
BMI	28.1 (±4.7)	26.6 (±3.9)	0.003
Gender			
Male	53 (48.2%)	182 (55.8%)	
Female	57 (51.8%)	144 (44.2%)	0.47
Family history[a]			
Yes	22 (20.6%)	24 (7.5%)	
No	85 (79.4%)	297 (92.5%)	0.001
Meat intake			
Quartile 1	22 (20.0%)	82 (25.2%)	
Quartile 2	32 (29.1%)	87 (26.7%)	
Quartile 3	31 (28.2%)	78 (23.9%)	
Quartile 4	25 (22.7%)	79 (24.2%)	0.62
Smoking status[b]			
Never	39 (35.5%)	145 (44.5%)	
Former	46 (41.8%)	124 (38.0%)	
Current	25 (22.7%)	57 (17.5%)	0.21
Pack-years			
0	39 (35.4%)	145 (44.5%)	
1–19	17 (15.5%)	62 (19.0%)	
20–39	24 (21.8%)	53 (16.3%)	
≥40	30 (27.3%)	66 (20.2%)	0.14
Cruciferous vegetable intake			
Quartile 1	37 (33.6%)	87 (26.7%)	
Quartile 2	30 (27.3%)	80 (24.5%)	
Quartile 3	22 (20.0%)	79 (24.2%)	
Quartile 4	21 (19.1%)	80 (24.5%)	0.34

[a] One case was missing information on smoking history.
[b] There are three cases and five controls for whom 'family history' information is missing.

Estimating the individual relative risk of developing a $p27^{Kip1}$ negative tumour compared with controls showed a weak inverse association with pack-years smoked. However, markedly different results were found when $p27^{Kip1}$ positive cases were compared with controls. The relative risks of developing a $p27^{Kip1}$ positive tumour were estimated to be 1.17 (95% CI 0.54–2.54) for those with less than 20 pack-years, 1.95 (95% CI 0.95–3.97) for those with 20–39 pack-years, and 2.25 (95% CI 1.14–4.45) for those with greater than 39 pack-years of smoking exposure (trend test, $p = 0.007$) when compared with controls (table 2). Moreover, a significant trend of increasing risk was observed for total pack-years of smoking (adjusted trend test: $p = 0.009$).

Bcl-2 expression in colorectal cancer

Bcl-2 immunohistochemical staining was performed on 110 available colorectal tumours; 48% of patients' tumours demonstrated bcl-2 expression. Moderate differences were observed when cases with bcl-2 overexpression were compared with cases without bcl-2 expression for family history of colorectal cancer (OR = 2.90; 95% CI 0.98–8.81), and pack-years smoked (adjusted trend test, $p = 0.09$).

When cases with bcl-2 overexpression were compared with controls, the adjusted relative risks were estimated to be 1.60 (95% CI 0.63–4.07) for those with less than 20 pack-years, 2.14 (95% CI 0.87–5.27) for those with 20–39 pack-years,

Table 2. Odds ratios and 95% confidence intervals between $p27^{Kip1}$ expression and colorectal cancer risk by cigarette smoking.

				p27 expression	
				≤25%	>25%
	≤25%	>25%	Controls	OR (95% CI)[a]	OR (95% CI)[a]
Smoking status					
Never	15	24	145	1.0 (ref.)	1.0 (ref.)
Former	7	40	124	0.51 (0.19–1.40)	2.10 (1.13–3.89)
Current	3	21	57	0.46 (0.12–1.77)	1.86 (0.89–3.88)
Trend, *p*				0.156	0.036
Cigarette smoking in pack-years					
0	14	24	145	1.0 (ref.)	1.0 (ref.)
1–19	6	12	62	1.03 (0.35–3.01)	1.17 (0.54–2.54)
20–39	3	19	53	0.59 (0.15–2.28)	1.95 (0.95–3.97)
≥40	2	29	66	0.30 (0.06–1.43)	2.25 (1.14–4.45)
Trend, *p*				0.106	0.009

[a] Adjusted for age, gender, family history of colorectal cancer, body mass index (BMI), and alcohol, cruciferous vegetable and meat consumption.

and 2.31 (95% CI 0.98–5.46) for those with greater than 39 pack-years of smoking exposure (trend test, $p = 0.03$) (table 3). Colorectal tumours developing without overexpression of bcl-2 were not associated with smoking exposure.

Discussion

Colorectal cancer is thought to develop through a sequence of genetic events that correspond to the histopathological progression from morphologically normal colonic epithelium to adenoma to adenocarcinoma. This sequence has been studied extensively since Vogelstein and Fearon first hypothesized these steps (Fearon and Vogelstein 1990). Colorectal tumours, however, do not exhibit a uniform and orderly accumulation of the genetic alterations associated with the Vogelstein model. This suggests aetiological heterogeneity where more than one pathway exists by which neoplastic progression can occur. Moreover, clinical behaviour also is heterogeneous among patients, even if they have identical classifications (e.g. size, stage, etc.).

There is evidence that different agents act at different points in this continuum of morphological and genetic abnormalities. Because of the consistently observed finding that smoking increases the risk of colorectal adenoma, but not cancer, cigarette smoking is believed to act early in this sequence (Giovannucci *et al*. 1994a, b), serving as an initiator of colorectal carcinogenesis. Similarly, there is evidence that bcl-2 overexpression occurs early in this process: overexpression of the protein has been observed more often in adenomas than in carcinomas (Hague *et al*. 1994, Baretton *et al*. 1996, Ilyas *et al*. 1996, Hawkins *et al*. 1997), suggesting that this change may give these adenomas a survival advantage in progressing to tumour.

In contrast to bcl-2, loss of $p27^{Kip1}$ expression (as well as p53 gene product overexpression of the type associated with mutation) is believed to be a late event in the adenoma–carcinoma sequence. Loss of $p27^{Kip1}$ expression has been observed to be a powerful negative prognostic marker in various epithelial carcinomas, including colorectal and breast tumours (Fredersdorf *et al*. 1997). Furthermore, it

Table 3. Odds ratios and 95% confidence intervals between bcl-2 expression and colorectal cancer risk by cigarette smoking.

				Bcl-2 expression	
				No	Yes
Variable	No	Yes	Controls	OR (95% CI)	OR (95% CI)[a]
Smoking status					
Never	26	13	145	1.0 (ref.)	1.0 (ref.)
Former	17	29	124	0.77 (0.38–1.55)	2.22 (1.08–4.59)
Current	14	11	57	1.38 (0.64–2.96)	1.82 (0.75–4.42)
Trend, *p*				0.570	0.081
Cigarette smoking in pack-years					
0	26	13	145	1.0 (ref.)	1.0 (ref.)
1–19	8	9	62	0.72 (0.30–1.75)	1.60 (0.63–4.07)
20–39	12	12	53	1.22 (0.55–2.70)	2.14 (0.87–5.27)
≥40	11	19	66	0.91 (0.39–2.09)	2.31 (0.98–5.46)
Trend, *p*				0.968	0.031

* Adjusted for age, gender, family history of colorectal cancer, body mass index (BMI), and alcohol, cruciferous vegetable and meat consumption.

has been reported that less than 10% of gastric adenomas show a loss of $p27^{Kip1}$ expression compared with 74% of gastric carcinomas (Yasui *et al*. 1997).

In our study of 110 patients with sporadic colorectal cancer and 326 controls, we observed differences in associations between cigarette smoking and expressions in $p27^{Kip1}$ and bcl-2. Our data suggest that bcl-2 overexpression (or a bcl-2 dependent pathway) is associated with cigarette smoking in the development of colorectal cancer, whereas a loss of $p27^{Kip1}$ expression is not. Given that tobacco smoke most likely acts as an initiating event, the association observed with bcl-2 overexpression is further evidence that this change in protein expression may occur early in the cascade (Hague *et al*. 1994, Baretton *et al*. 1996, Ilyas *et al*. 1996, Hawkins *et al*. 1997). The stronger association with bcl-2 overexpression observed among former smokers (adjusted OR = 2.22; 95% CI 1.08–4.59) than in current smokers (adjusted OR = 1.82; 95% CI 0.75–4.42), further supports this observation. In contrast, the lack of association with loss of $p27^{Kip1}$ expression may suggest that it is a late event, similar to that observed with p53 overexpression (Freedman *et al*. 1996).

Results from retrospective studies of disease aetiology have inherent limitations and those reported here should be interpreted with caution. One possible reason for the statistically significant effects observed may be due to the multiple comparisons made between these protein expressions, smoking characteristics and tumour site. Another potential source of bias is in the use of hospital and screening clinic controls. The latter may over-represent 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*. 1994a, b, Heineman *et al*. 1995) and may, therefore, attest to the suitability of our control population. Additionally, the primary use of this control group is to assist in the estimation of the individual relative risks for $p27^{Kip1}$ positive and $p27^{Kip1}$ negative cancer, as well as differences in bcl-2 expression in tumour development, for smoking exposure variables. This bias is of less concern in our case-series analysis,

since patients are unaware of their protein expression status and thus have no effect of recall.

The data from this study of bcl-2 expression and $p27^{Kip1}$ expression suggest that distinct causal mechanisms in the development of colorectal cancer may exist. If cells can use different groups of molecular alterations to overcome the same growth controls, understanding of how each piece fits in the multiple sequence of events may lead to the development of better forms of treatment and/or prevention to reflect the effects of these mutations. Clearly, future molecular epidemiological studies are warranted to further elucidate the exact relationship between exogenous exposures, genetic alteration and colorectal cancer risk.

References

Baretton, G. B., Diebold, J., Christoforis , G., Vogt, M., Muller, C., Dopfer, K., Schneiderbanger , K., Schmidt, M. and Lohrs, U. 1996, Apoptosis and imunohistochemical bcl-2 expression in colorectal adenomas and carcinomas. *Cancer*, **77**, 255–264.

Begg, C. B. and Zhang, Z. F. 1994, Statistical analysis of molecular epidemiology studies employing case-series. *Cancer Epidemiology, Biomarkers & Prevention*, **3**, 173–175.

Breslow, N. E. and Day, N. E 1980, The analysis of case-control studies. In *Statistical Methods in Cancer Research, IARC Scientific Publication,* Vol. 32, Walter Davis, ed. (Lyon: IARC), pp. 122–159.

Bronner, M. P., Culin, C., Reed, J. C. and Furth, E. E. 1995, The bcl-2 proto-oncogene and the gastrointestinal epithelial tumor progression model. *American Journal of Pathology*, **146**, 20–26.

Choi, W. Y. and Kahyo, H. 1991, Effect of cigarette smoking and alcohol consumption in the etiology of cancers of the digestive tract. *International Journal of Cancer*, **49**, 381–386.

Cope, G. F., Wyatt, J. I., Pinder, I. F., Lee, P. N., Heatley, R. V. and Kelleher, J. 1991, Alcohol consumption in patients with colorectal adenomatous polyps. *Gut*, **32**, 70–72.

Dales, L. F., Friedman, G. D., Ury, H. K., Grossman, S. and Williams, S. R. 1979, A case-control study of relationships of diet and other traits to colorectal cancer in American blacks. *American Journal of Epidemiology*, **109**,132–144.

Demers, R.Y., Neale, A.V., Demers, P., Deighton, K., Scott, R. O., Dupuis, M. H. and Herman, S. 1988, Serum cholesterol and colorectal polyps. *Journal of Clinical Epidemiology*, **41**, 9–13.

Fearon E. R. 1993, Molecular genetic studies of the adenoma-carcinoma sequence. *Advances in Internal Medicine*, **39**, 123–147.

Fearon, E. R. and Vogelstein, B. 1990, A genetic model for colorectal tumorigenesis. *Cell*, **61**, 759–767.

Fero, M. L., Rivkin, M., Tasch, M., Porter, P., Carcow, C. E., Firpo, E., Polyak, K., Tsai, L. H., Broudy, V., Perlmutter, R. M., Kaushansky, K. and Roberts, J. M. 1996, A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in $p27^{Kip1}$-deficient mice. *Cell*, **85**, 733–744.

Ferraroni, M., Negri, E., La Vecchia, C., D'Avanzo, B. and Franceschi, S. 1989, Socio-economic indicators, tobacco and alcohol in the aetiology of digestive tract neoplasm. *International Journal of Epidemiology*, **18**, 556–562.

Fredersdorf, S., Burns, J., Milne, A. M., Packham, G., Fallis, L., Gillett, C.E., Royds, J.A., Peston, D., Hall, P. A., Hanby, A. M., Barnes, D. M., Shousha, S., O'Hare, J. J. and Lu, X. 1997, High level expression of $p27^{Kip1}$ and cyclin D1 in some human breast cancer cells: Inverse correlation between the expression of $p27^{Kip1}$ and degree of malignancy in the human breast and colorectal cancers. *Proceedings of the National Academy of Sciences, USA*, **94**, 6380–6385.

Freedman, A. N., Michalek, A. M., Marshall, J. R., Mettlin, C. J., Petrelli, N. J., Zhang, Z.F., Black, J. D., Satchidanand, S. and Asirwathan, J. E. 1996, The relationship between smoking exposure and p53 overexpression in colorectal cancer. *British Journal of Cancer*, **73**, 902–908.

Giovannucci, E., Colditz, G. A., Stampfer, M. J., Hunter, D., Rosner, B. A., Willett, W. C. and Speizer, F. E. 1994a, A prospective study of cigarette smoking and risk of colorectal adenoma and colorectal cancer in U.S. women. *Journal of the National Cancer Institute*, **86**, 192–199.

Giovannucci, E., Rimm, E. B., Stampfer, M. J., Colditz, G. A., Ascherio, A., Kearney, J. and Willett, W. C. 1994b, A prospective study of cigarette smoking and risk of colorectal adenoma and colorectal cancer in U.S. men. *Journal of the National Cancer Institute*, **86**, 183–191.

Graham, S., Dayal, H., Swanson, M., Mittelman, A. and Wilkinson, G. 1978, Diet in the epidemiology of cancer of the colon and rectum. *Journal of the National Cancer Institute*, **61**, 709–714.

HAENSZEL, W., BERG, J. W., SEGI, M., KURIHARA, M. and LOCKE, F. B. 1973, Large bowel cancer in Hawaiian Japanese. *Journal of the National Cancer Institute*, **51**, 1765–1779.

HAENSZEL, W., LOCKE, F. B. and SEGI, M. 1980, A case-control study of large bowel cancer in Japan. *Journal of National Cancer Institute*, **64**, 17–22.

HAGUE, A., MOORGHEN, M., HICKS, D., CHAPMAN, M. and PARASKEVA, C. 1994, Bcl-2 expression in human colorectal adenomas and carcinomas. *Oncogene*, **9**, 3367–3370.

HAWKINS, N., LEES, J., HARGRAVE, R., O'CONNOR, T., MEAGHER, A. and WARD, R. 1997, Pathological and genetic correlates of apoptosis in the progression of colorectal neoplasia. *Tumor Biology*, **18**, 146–156.

HEINEMAN, E. F., HOAR ZAHM, S., MCLAUGHLIN, J. K. and VAUGHT, J. B. 1995, Increased risk of colorectal cancer among smokers: results of a 26-year follow-up of U.S. veterans and a review. *International Journal of Cancer*, **59**, 728–738.

HENGST, L. and REED, S. I. 1996, Translational control of $p27^{Kip1}$ accumulation during the cell cycle. *Science*, **271**, 1861–1864.

HOCKENBERY, D., NUNEZ, G., MILLIMAN, C., SCHREIBER, R. D. and KORSMEYER, S. J. 1990, Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature*, **348**, 334–336.

HOCKENBERY, D. M., ZUTTER, M., HICKEY, W., NAHM, M. and KORSMEYER, S. J. 1991, Bcl2 protein is topographically restricted in tissues characterized by apoptotic cell death. *Proceedings of the National Academy of Sciences USA*, **88**, 6961–6966.

HOFF, G., VATN, M. H. and LARSEN, S. 1987, Relationship between tobacco smoking and colorectal polyps. *Scandinavian Journal of Gastroenterology*, **22**,13–16.

HONJO, S., KONO, S., SHINCHI, K., IMANISHI, K. and HIROHATA, T. 1992, Cigarette smoking, alcohol use and adenomatous polyps of the sigmoid colon. *Japanese Journal of Cancer Research*, **83**, 806–811.

ILYAS, M., TOMLINSON, I. P. M., HANBY, A. M., YAO, T., BODMER, W. F. and TALBOT, I. C. 1996, Bcl-2 expression in colorectal tumors: evidence of different pathways in sporadic and ulcerative-colitis-associated carcinomas. *American Journal of Pathology*, **149**, 1719–1726.

JAIN, M., COOK, G. M., DAVIS, F. G., GRACE, M. G., HOWE, G. R. and MILLER, A. B. 1980, A case-control study of diet and colorectal cancer. *International Journal of Cancer*, **26**, 757–768.

JAREBINSKI, M., ADANJA, B. and VLAJINAC, H. 1988, Bisocial and other characteristics of the large bowel cancer patients in Belgrade (Yugoslavia). *Archives of Geschwulstforsch*, **58**, 411–417.

JAREBINSKI, M., ADANJA, B. and VLAJINAC, H. 1989, Case-control study of relationship of some bisocial correlates to rectal cancer patients in Belgrade Yugoslavia. *Neoplasma*, **36**, 369–374.

KABAT, G. C., HOWSON, C. P. and WYNDER, E. L. 1986, Beer consumption and rectal cancer. *International Journal of Epidemiology*, **15**, 494–501.

KAWAMATA, N., MOROSETTI, R., MILLER, C. W., PARK, D., SPIRIN, K. S., NAKAMICHI, T., TAKEUCHI, S., HATTA, Y., SIMPSON, J., WILCYZNSKI, S., LEE, Y. Y., BARTRAM, C. R. and KOEFFLER, H. P. 1995, Molecular analysis of the cyclin-dependent kinase inhibitor gene $p27^{Kip1}$ in human malignancies. *Cancer Research*, **55**, 2266–2269.

KIKENDALL, J. W., BOWEN, P. E., BURGESS, M. B., MAGNETTI, C., WOODWARD, J. and LANGENBERG, P. 1991, Cigarettes and alcohol as independent risk factors for colonic adenomas. *Gasteroenterology*, **26**, 758–762.

KIYOKAWA, H., KINEMAN, R. D., MANOVA-TODOROVA, K. O., SOARES, V. C., HOFFMAN, E. S., ONO, M., KHANAM, D., HAYDAY, A.C., FROHMAN, L.A. and KOFF, A. 1996, Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of $p27^{Kip1}$. *Cell*, **85**, 721–732.

KORSMEYER S. J. 1992, Bcl-2 initiates a new category of oncogenes: regulators of cell death. *Blood*, **80**, 879–886.

KUNE, G. A., KUNE, S., VITETTA, L. and WATSON, L. F. 1992, Smoking and colorectal cancer risk: data from the Melbourne Colorectal Cancer Study and brief review of literature. *International Journal of Cancer*, **50**, 369–372.

LEE, W. C., NEUGUT, A. I., GARBOWSKI, G. C., FORDE, K. A., TREAT, M. R., WAYE, J. D. and FENOGLIO-PREISER, C. 1993, Cigarettes, alcohol, coffee and caffeine as risk factors for colorectal adenomatous polyps. *Annals of Epidemiology*, **3**, 239–244.

LODA, M., CUKOR, B., TAM, S. W., LAVIN, P., FIORENTINO, M., DRAETTA, G. F., JESSUP, J. M. and PAGANO, M. 1997, Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ in aggressive colorectal carcinomas. *Nature Medicine*, **3**, 231–234.

MARTINEZ, M. E., MCPHERSON, R. S., ANNEGERS, J. F. and LEVIN, B. 1995, Cigarette smoking and alcohol consumption as risk factors for colorectal adenomatous polyps. *Journal of the National Cancer Institute*, **87**, 274–279.

MILLER, C. W., SIMON, K., ASLO, A., KOK, K., YOKOTA, J., BUYS, C. H., TERADA, M. and KOEFFLER, H.P. 1992, p53 mutations in human lung tumors. *Cancer Research*, **52**, 1695–1698.

MONNET, E., ALLEMand, H., FARINA, H. and CARAYON, P. 1991, Cigarette smoking and the risk of colorectal adenoma in men. *Scandinavian Journal of Gastroenterology*, **26**, 758–762.

NAKAYAMA, K., ISHIDA, N., SHIRANE, M., INOMATA, A., INOUE, T., SHISHIDO, N., HORII, I., LOH,

D. Y. and NAKAYAMA, K. 1996, Mice lacking p27^{Kip1} display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell*, **85**, 707–720.

OLSEN, J. and KRONBERG, O. 1993, Coffee, tobacco, and alcohol as risk factors for cancer and adenoma of the large intestine. *International Journal of Epidemiology*, **22**, 398–402.

PAGANO, M., TAM, S. W., THEODORAS, A. M., BEER-ROMERO, P., DEL SAL, G., CHAU, V., YEW, P. R., DRAETTA, G. F. and ROLFE, M. 1995, Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science*, **269**, 682–685.

PALMQVIST, R., STENLING, R., OBERG, A. and LANDBERG, G. 1999, Prognostic significance of p27^{Kip1} expression in colorectal cancer: a clinico-pathological characterization. *Journal of Pathology*, **188**, 18–23.

PAPADIMITRIOU , C., DAY, N., TZONOU, A., GEROVASSILIS , F., MANOUSOS, O. and TRICHOPOULOS , D. 1984, Bisocial correlates of colorectal cancer in Greece. *International Journal of Epidemiology*, **13**, 155–159.

PETERS, R. K., GARABRANT, D. H., YU, M. C. and MACK, T. M. 1989, A case-control study of occupational and dietary factors in colorectal cancer in young men by subsite. *Cancer Research*, **4**, 5459–5468.

PONCE-CASTANEDA, M. V., LEE, M. H., LATRES, E., POLYAK, K., LACOMBE, L., MONTGOMERY, K., MATHEW, S., KRAVTER, K., SHEINFELD, J., MASSAGUE, J. and CORDON-CARDO, C. 1995, p27^{Kip1}: chromosomal mapping to 12p12–12p13.1 and absence of mutations in human tumors. *Cancer Research*, **55**, 1211–1214.

SANDLER, R. S., LYLES, C. M., MCAULIFFE, C., WOOSLEY, J. T. and KUPPER, L. L. 1993, Cigarette smoking, alcohol and the risk of colorectal adenomas. *Gastroenterology*, **104**, 1445–1451.

SLATTERY, M. L., WEST, D. W., ROBISON, L. M., FRENCH, T. K., FORD, M. H., SCHUMAN, K. L. and SORENSON, A.W. 1990, Tobacco, alcohol, coffee, and caffeine as risk factors for colon cancer in a low risk population. *Epidemiology*, **1**, 247–253.

SPRUCK , C. H., RIDEOUT, W. M., OLUMI, A. F., OHNESEIT, P. F., YANG, A. S., TSAI, Y. C., NICHOLS, P. W., HORN, T., HERMANN, G. G., STEVEN, K, ROSS, R. K., YU, M. C. and JONES, P. A. 1993, Distinct pattern of p53 mutations in bladder cancer: relationship to tobacco usage. *Cancer Research*, **53**, 1162–1166.

SUZUKI, H., TAKAHASHI, T., KUROISHI, T., SUYAMA, M., ARIYOSHI, Y., TAKAHASHI, T. and UEDA, R. 1992, p53 Mutations in non-small cell lung cancer in Japan: association between mutations and smoking. *Cancer Research*, **52**, 734–736.

TAJIMA, K. and TOMINAGA, S. 1985, Dietary habits and gastrointestinal cancers: a comparative case-control study of stomach and large intestinal cancers in Nagoya, Japan. *Japanese Journal of Cancer Research*, **76**, 705–716.

THOMAS, G. V., SZIGETI, K., MURPHY, M., DRAETTA, G., PAGANO, M. and LODA, M. 1998, Down-regulation of p27 is associated with development of colorectal adenocarcinoma metastases. *American Journal of Pathology*, **153**, 681–687.

TUYNS, A. J., PEQUIGNOT, G., GIGNOUX, M. and VALLA, A. 1982, Cancers of the digestive tract, alcohol and tobacco. *International Journal of Cancer*, **30**, 9–11.

VOBECKY, J., CARO, J. and DEVROEDE, G. 1983, A case-control study of risk factors for large bowel carcinoma. *Cancer*, **51** 1958–1963.

WILLIAMS, R. R. and HORM, J. W. 1977, Association of cancer sites with tobacco and alcohol consumption and socioeconomic status of patients: interview study from the Third National Cancer Survey. *Journal of the National Cancer Institute*, **58**, 525–547.

WYNDER, E. L., KAJITANI, T., ISHIKAWA, S., DODO, H. and TAKANO, A. 1969, Environmental factors of cancer of the colon and rectum. II: Japanese epidemiological data. *Cancer*, **23**,1210–1220.

YASUI, W., KUDO, Y., SEMBA, S., YOKOZAKI, H. and TAHARA, E. 1997, Reduced expression of cyclin-dependent kinase inhibitor p27^{Kip1} is associated with advanced stage and invasiveness of gastric carcinomas. *Japanese Journal of Cancer Research*, **88**, 625–629.

ZAHM, S. H., COCCO, P. and BLAIR, A. 1991, Tobacco smoking as risk factor for colon polyps. *American Journal of Public Health*, **81**, 846–849.

ZHANG, Z. F., SARKIS, A. S., CARDON-CARDO, C., DALBAGNI, G., MELAMED, J., APRIKIAN, A., POLLACK, D., SHEINFELD, J., HERR, H. W., FAIR, W. R., REUTER, V. E. and BEGG, C. 1994, Tobacco smoking, occupation and p53 nuclear overexpression in early stage bladder cancer. *Cancer Epidemiology, Biomarkers & Prevention*, **3** 19–24.