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Carcinogen DNA adducts and the risk of colon cancer: case–control study

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Abstract

Colorectal cancer represents 8.5% of all tumours at the King Faisal Specialist Hospital & Research Centre. Environmental and dietary carcinogens such as polycyclic aromatic hydrocarbons (PAHs) and heterocyclic amines (HCAs) have long been suspected to play a prominent role in colon cancer aetiology. We designed a case–control study to test the hypothesis of whether or not the presence of DNA adducts can play a role in the aetiology of colon cancer. DNA adducts were measured in 24 cancerous and 20 non-cancerous tissue samples of newly diagnosed colon cancer patients by ^{32}P-post-labelling technique. Normal tissue from 19 hospital patients served as controls. The mean levels of adducts per 10^{10} nucleotides in cancerous and non-cancerous tissue were 151.75 ± 217.27 and 114.81 ± 186.10, respectively; however, only adducts in cancerous tissue were significantly higher than controls (32.78 ± 57.51 per 10^{10} nucleotides) with p-values of 0.017. No BPDE-DNA adducts were found. No relationship was found between urinary cotinine as a marker of tobacco smoke and 1-hydroxypyrene as an indicator of an individual's internal dose of PAHs and DNA adducts. In a logistic regression model, only adducts in cancerous tissue were associated with the subsequent risk of colon cancer, with an odds ratio of 3.587 (95% confidence interval 0.833–15.448) after adjustment for age and the duration of living in the current region, but of a borderline significance ($p = 0.086$). Although it is difficult to arrive at a definite conclusion from a small dataset, our preliminary results suggest the potential role of DNA adducts in the colon carcinogenesis process. Additional studies with larger sample sizes are needed to confirm our preliminary finding. It is also important to identify the structural characterization of these unknown DNA adducts in order to have a better understanding of whether or not environmental carcinogens play a role in the aetiology of colon cancer.

Keywords: *DNA adducts, ^{32}P-postlabeling technique, colon cancer, Saudi Arabia, polycyclic aromatic hydrocarbons*

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Introduction

Colon cancer is one of the most frequent causes of cancer death in Western countries. A large body of evidence indicates that several dietary and lifestyle factors are likely to have a major influence on the risk of colon cancer. Its risk appears to be related to several dietary and lifestyle factors such as increased consumption of red meat, processed meat, refined carbohydrates and fibre (Giovannucci 2002). Meats cooked at high temperatures contain heterocyclic amines (HCAs) and other mutagens such as polycyclic aromatic hydrocarbons (PAHs) that play a role in increasing the risk of colon cancer in both animals and human (Sinha et al. 1999, Nakagama et al. 2005, Wu et al. 2006). Tobacco smoking is another risk factor for colon cancer (Huang et al. 2006). It contains thousands of chemical compounds, many of which are known carcinogens, such as PAHs and HCAs (Ding et al. 2006, Kalaitzoglou & Samara 2006).

As explained in the review article by Phillips (2005) any chemical carcinogen is either DNA reactive or undergoes metabolic activation to species that are DNA reactive, and DNA damage is an early event in the carcinogenic process. PAH and HAC compounds require activation to electrophilic metabolites to exert their mutagenic or carcinogenic effects via bay region dihydrodiol epoxide (DE) by cytochrome P450 enzymes (CYPs), via radical cation by one-electron oxidation, and the ortho-quinone pathway by dihydrodiol dehydrogenase (DD) (Xue & Warshawsky 2005). Among the carcinogenic PAHs, benzo[a]pyrene (BP) is one of the most potent carcinogens, acting as initiator and promoter of skin tumours in mice (Rubin 2001). However, it is more active as a tumour initiator (ATSDR 1995). Rubin (2001) revealed that its carcinogenicity increased when applied once as an initiator followed repeatedly by a promoter.

When benzo[a]pyrene is oxidized and hydroxylated, involving cytochrome P450 and epoxide hydrolase, it is converted to epoxides, phenols, diols, tetrols and quinone derivatives (IARC 1983). These polar, water-soluble compounds can be conjugated with glutathione, sulfate or glucuronic acid and are excreted in urine and bile for detoxification. An initial step in benzo[a]pyrene-induced tumorigenesis involves its metabolic conversion to (+)-r-7,t-8-dihydroxy-t-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*anti*-BPDE) which can react covalently with DNA to yield DNA adducts (WHO 1998). Such adducts, if they are not repaired or misrepaired, may initiate gene mutations and lead to adverse health effects in humans (Peltonen & Dipple 1995, Kriek et al. 1998). This could be a biomarker of biological effective dose of benzo[a]pyrene. Measuring carcinogen-DNA adducts is thought to be a useful biomarker in molecular epidemiological studies that attempt to determine cancer risk (Phillips 2005, Rundle 2006). A number of studies have reported a link between DNA adducts and colon or colorectal cancer (Hamada et al. 1994, Umemoto et al. 1994, Pfohl-Leszkowicz et al. 1995, Alexandrov et al. 1996, Malfatti et al. 2006). These adducts were originated either from exposure to HCAs or PAHs. It was suggested that detection of DNA adducts could constitute a useful approach to the early detection of colorectal cancer, surveillance of high-risk populations and prediction of the clinical effectiveness of chemopreventive drugs (Garcea et al. 2003).

Colorectal cancer seems to occur less in Saudi Arabia than in the West; it represents only 8.5% of all cancers diagnosed in the Kingdom of Saudi Arabia in 2002, whereas it constitutes 10–15% of newly diagnosed cancer cases in the USA and Europe (National Cancer Registry for the Kingdom of Saudi Arabia 2002). However, a recent

study by Al-Radi et al. (2000) reported that colorectal cancer is the most common gastrointestinal malignancy seen in the Western Region of Saudi Arabia. The authors related this to changes in the lifestyle and dietary habits of the Saudi population. According to Khan & Al-Kanhal (1998), an increasing trend in the per capita availability of total food (90%), oils and fats (200%), animal fat (171%), animal protein (207%), meat (313%), milk (120%), eggs (648%) and sugar (68%) has been observed in Saudi Arabia over the past two decades. Furthermore, in recent years, different types of smoking habits (cigarette and sheesha) in Saudi Arabia have become widely spread especially among the young generation (Al-Turki 2006). Jarallah et al. (1999) estimated an overall prevalence of 21% among males and 0.9% among females. The waterpipe is a device used to inhale tobacco where smoke passes through water. It is known under different names such as sheesha, maassel, jurâk, narghile, hookah, hubble bubble (Maziak et al. 2004, Asfar et al. 2005). They are now commonplace in Arab societies, and many restaurants and cafés in Saudi Arabia serve them. Waterpipe users may also be exposed to smoke constituents, although little research addresses this issue and little is known about its health effect. The amount of carbon monoxide in waterpipe smoke is similar to that of cigarettes (Sajid 1993). With regard to other constituents, waterpipe smoke produced by a machine contains significant amounts of dangerous constituents, including nicotine and heavy metals such as arsenic, cobalt, chromium and lead (Shihadeh 2003). Under normal waterpipe use conditions, the smoke produced from a single waterpipe use contains approximately the same amount of nicotine-free, dry particulate matter (tar) as 20 cigarettes (Shihadeh 2003), although it is generally believed that waterpipe smoking is not as dangerous as cigarette smoking because there is no tobacco in the mixture. Like cigarette smokers, waterpipe smokers are exposed to harmful substances, such as carbon monoxide and nicotine, which was found to be quite high (Al Mutairi et al. 2006, Bacha et al. 2007). Human colon cancer is a multifactorial, multistep disease wherein genetic and dietary factors represent important regulators of initiation, promotion and progression. While the aetiology of sporadic colon cancer remains largely unidentified, familial adenomatous polyposis (FAP) and hereditary non-polyposis colon cancer (HNPCC) represent predisposing genetic syndromes for early-onset familial/hereditary colon cancer (Telang et al. 2006). In the USA, up to 30% of reported colon cancer cases exhibit familial clustering, which means that tens of thousands of individuals have a disease with a potentially definable genetic component. Approximately 3–5% of colon cancers are associated with high-risk, inherited colon cancer syndromes (Kaz & Brentnall 2006). During recent years, the use of advanced molecular diagnostic tools has helped to uncover the genetics underlying an inherited predisposition to cancer (Calvert & Frucht 2002, Souglakos 2007). In addition to the multiple cancer family syndromes, Souglakos (2007) reported that several are known to be associated with the development of colorectal cancer.

All these aetiological factors might be involved in the development of colon cancer. The current study was carried out to investigate whether DNA adducts can predict the risk of colon cancer among Saudis. We measured bulky and lipophilic DNA adducts including aromatic adducts such as BPDE-DNA by ^{32}P-postlabelling technique in cancerous and non-cancerous colon tissue from the same patient and compared them with those taken from controls.

Materials and methods

Patient selection

Cancerous ($n = 24$) and adjacent non-cancerous ($n = 20$) colon tissue samples were obtained surgically from newly diagnosed Saudi patients with colon cancer who underwent either colectomy or colonoscopy, and who were between the ages of 16 and 83 years and had no past history of radiotherapy and/or chemotherapy. The criteria to determine a healthy looking section of the colon were based on the following: (1) it should be more than 5 cm away from the margins of the tumour; (2) histopathology should prove that it was normal mucosa without evidence of cancer or dysplasia; and (3) the mucosal samples were retrieved by the surgeon based on the criteria under item (1).

The clinical data of our cancer patients at presentation are shown in Table I. All the patients were diagnosed based on histology. The majority were rectal cancer patients. The 5-year survival for our patient population has been published and is as follows: the crude overall 5-year survival rate was 39%; and the 5-year survival rate for patients with Dukes' stage C cancers following 'curative' surgery was 25% (Rectal Cancer in the Kingdom of Saudi Arabia: the King Faisal Specialist Hospital Experience 2000).

The histological stage was based on how deeply the tumour invaded the bowel wall, and included the positivity or negativity of the lymph node. The clinical stage was a combination of histological stage plus the result of X-rays to rule out the distant metastasis and is reported by TNM classification. In this study the stages of colon cancer at presentation were 44.1% with stage 1 and 2 (T1-2N0M0); 22.1% with stage

Table I. Clinical data of the patients with cancer.

ID	Diagnosis	Age (years)	Gender	Tumour differentiation
1	Adenocarcinoma grade (2)	55	F	Well to moderately differentiated
5	Adenocarcinoma grade (3)	73	F	Invasive moderately differentiated
6	Adenocarcinoma grade (2)	44	M	Well to moderately differentiated
8	Adenocarcinoma grade (2)	75	M	Well to moderately differentiated
13	Adenocarcinoma grade (2)	30	M	Well to moderately differentiated
17	Adenocarcinoma grade (2)	80	F	Well to moderately differentiated
19	Adenocarcinoma grade (2)	48	F	Well to moderately differentiated
22	Adenocarcinoma grade (1)	71	F	Mucinous
23	Adenocarcinoma grade (2)	45	M	Well to moderately differentiated
25	Adenocarcinoma grade (3)	56	M	Invasive moderately differentiated
26	Adenocarcinoma grade (3)	76	F	Invasive moderately differentiated
27	Adenocarcinoma grade (2)	83	M	Well to moderately differentiated
29	Adenocarcinoma grade (2)	69	F	Well to moderately differentiated
30	Adenocarcinoma grade (2)	47	M	Well to moderately differentiated
32	Adenocarcinoma grade (1)	48	M	Mild dysplasia
33	Villous adenoma	57	F	Severe dysplasia
34	Adenocarcinoma grade (2)	55	F	Well to moderately differentiated
35	Adenocarcinoma grade (1)	60	F	Mild dysplasia
36	Adenocarcinoma grade (2)	63	M	Well to moderately differentiated
37	Adenocarcinoma grade (2)	58	M	Well to moderately differentiated
38	Adenocarcinoma grade (3)	51	F	Invasive well to moderately differentiated
39	Adenocarcinoma grade (2)	54	M	Well to moderately differentiated
40	Adenocarcinoma grade (2)	53	M	Well to moderately differentiated
43	Adenocarcinoma grade (1)	46	F	Sever dysplasia

3 (T1-4N1-2M0); 22.1% with stage 4 (T1-4N1-2M1) and 11.4% unknown. Due to the difficulty of finding an ideal control population, we chose patients who underwent colectomy or colonoscopy for a benign pathology other than adenomatous polyps or cancer as controls. In this population, the resection of colon tissues ($n = 18$) was performed from a healthy looking part of the colon. With non-cancerous and normal colon tissue samples, DNA could not be extracted for DNA adduct analysis; this was due partly to the small amount of tissue available and also because some tissues were very fatty, which hinders the extraction process. Out of the controls and non-cancerous colon tissue samples, we were not able to isolate DNA from six and four patients, respectively. DNA adducts were determined in 12 controls and 20 non-cancerous tissue samples only. Pregnant female patients were excluded. All subjects signed an informed consent form. Information regarding age, body mass index (BMI; weight in kg divided by height in m^2), gender, marital status, residential history, socioeconomic status, smoking, dietary habits, medical history, use of medications and other characteristics of the patients were recorded in a questionnaire that was completed by a trained interviewer. Cancerous and non-cancerous colon tissue samples were examined and separated by the histopathologist. Tissue samples were kept at -80°C before DNA extraction. A urine sample (100 ml) was collected from each patient in a plastic container without any preservatives immediately after the surgery and stored at -20°C until analyzed for 1-hydroxypyrene and cotinine.

This research project was evaluated and approved by both the Research Basic Committee and the Research Ethic Committee of King Faisal Specialist Hospital and Research Centre.

Analysis of DNA adduct by ^{32}P-postlabelling

DNA isolation. DNA was isolated by a phenol extraction procedure as described elsewhere (Gupta 1996). Briefly, colon tissues were homogenized in 50 mM Tris–HCl (pH 8.0) containing 10 mM EDTA. Isolated crude nuclei were digested with ribonucleases A (150 μg ml^{-1}) and T1 (1 unit μl^{-1}) (Sigma Chemical Co., St Louis, MO, USA), followed by digestion with proteinase K (150 μg ml^{-1}) (Boehringer Mannheim Corp., Indianapolis, IN, USA). The samples were extracted sequentially with phenol, phenol:Sevag (chloroform:*iso*amylalcohol, 24:1) and Sevag and DNA was precipitated with 1 volume of ethanol and 0.5 M sodium chloride. The DNA concentration was estimated spectrophotometrically using 1 A_{260} unit as equal to 50 μg DNA.

DNA adduct analysis. DNA (50 μg) was digested with a mixture of micrococcal nuclease (Sigma Chemical Co.) and spleen phosphodiesterase (Boehringer Mannheim) (enzyme:DNA = 1:5, w/w, 5 h at 37°C). DNA adducts were enriched by treatment with nuclease P1 (Calbiochem-Novabiochem Corp., San Diego, CA, USA) (enzyme:DNA = 1:2.5, w/w, 45 min at 37°C) (Gupta 1996). Enriched adducts (40 μg) were labelled by 0.4 μl T4 polynucleotide kinase (10 U μl^{-1}) in the presence of molar excess of commercial [γ-^{32}P]ATP (20 μCi; 7000 Ci $mmol^{-1}$ specific activity) Pl (ICN Pharmaceutical, Inc., Costa Mesa, CA, USA) (Gupta 1996), and resolved by multidirectional polyethyleneimine (PEI)-cellulose-TLC in the solvent system: D1 = 1 M sodium phosphate, pH 6.0; D3 = 4 M lithium formate/8.5 M urea, pH 3.5; D4 = 0.8 M lithium formate/8.5 M urea/0.5 M Tris–HCl, pH 8.0; and D5 = 1 M sodium phosphate, pH 6.0. An aliquot of diluted DNA digest (2 ng) was also labelled in

parallel with adducts and normal nucleotides converted to 5′-monophosphate were resolved using 0.5 M acetic acid (Gupta & Arif 2001). For quantification purposes, only standard of BPDE-modified DNA with known concentration (0.62 μg ml^{-1}) was run in parallel in all experiments. Adduct DRZs were visualized and quantified using a Typhoon 8600 variable mode imager and followed by quantification by ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA) (Arif et al. 2004). The relative adduct labelling (RAL) was calculated as follows: RAL = cpm in adducts/cpm in total nucleotides × 1/dilution factor. Adducts were expressed as adducts per 10^{10} nucleotides.

1-Hydroxypyrene assay

Hydrochloric acid and methanol were obtained from (Fisher Scientific, Pittsburgh, PA, USA). Sodium acetate and glucuronidase with aryl sulfatase were purchased from Sigma-Aldrich Co. 1-Hydroxypyrene used as a standard in the analytical system was purchased from TRC (Toronto Research Chemicals Inc., North York, Ontario, Canada).

A 10 ml urine sample was adjusted to pH 5.0 by 2 M hydrochloric acid. Then, 1 ml glucoronidase–arylsulphatase was added to the sample, vortexed sonicated for 20 min and incubated for 3 h at 40°C in shaking water bath. The hydrolyzed urine samples were loaded into 0.5 g C18 reversed-phase cartridge after it had been preconditioned with 5 ml of methanol and 5 ml of water. The cartridge was sequentially washed with 10 ml of water. The flow rate of the treated urine passing through the cartridge was ~3 ml min^{-1}. Final elution of 1-hydroxypyrene was performed with 8 ml of methanol. The solution was evaporated to dryness under nitrogen gas, reconstituted with 1 ml of methanol and sonicated for 20 min. It was then centrifuged at 10 000 r.p.m. for 5 min and then stored at −20°C until analysis. All samples were analyzed using the Alliance Waters HPLC 2695 system and a multi λ fluorescence detector 2475 was used to determine 1-hydroxypyrene. A Dell Optiplex GX1 computer and Millennium32 software was used to operate this system. An aliquot of 5 μl was injected onto a Waters Symmetry™ C_{18} (150 × 4.6 mm) column packed with Waters Symmetry™ C_{18} (5-μm particle size) and eluted with a water/methanol gradient at 0.8 ml min^{-1} as follows (percentage water, time): 54%, 0–5 min and 6%, 5–40 min. A guard column, Waters Symmetry™ C_{18} (4.6 mm × 2 cm, 5-μm particle size) with the same packing materials was placed in front of the analytical column for protection. The column temperature was at 40°C. The excitation wavelength (λ_{ex}) of the fluorescence detector was set to 242 nm and the emission wavelength (λ_{em}) to 388 nm. Peak heights were used for quantification. The retention time for 1-OHP was 19.5 min. The detection limit of the method calculated as 3 × SD was 0.201 μg l^{-1}. The urinary 1-hydroxypyrene concentrations were normalized by their corresponding creatinine concentrations and were expressed as μg g^{-1} creatinine. The extraction efficiency 1-hydroxypyrene from urine using this method was ~98%. Urinary creatinine was measured at the Department of Pathology, King Faisal Specialist Hospital using a Hitachi 717 analyzer (Boerhinger-Mannheim, Germany).

Cotinine assay

Urine cotinine was quantified by an enzyme-linked immunosorbent assay (ELISA) method using a commercially available cotinine direct ELISA kit (Bio-Quant Inc., San

Diego, CA, USA). This assay is based upon the competitive binding to antibody of enzyme-labelled antigen and unlabelled antigen, in proportion to their concentration in the reaction mixture.

A 10-µl volume each of urine, standard and control were added to separate 96-well microplates. A 100-µl volume of cotinine conjugate (a cotinine derivative labelled with horseradish peroxidase in a buffered, protein solution with stabilizers, pH 7.6 containing non-azide preservatives) was added to each well and left to stand at room temperature for 60 min. A 350-µl volume of water was used to wash the plates six times. A 100-µl volume of TMB chromogenic substrate reagent (3,3′,5,5′ tetramethylbenzidine and urine peroxidase in buffer) was added to each well and left to stand for 30 min at room temperature in the dark. A 100-µl volume of stop reagent (1 N hydrochloric acid) was then added to each well. After 1 h, an ELISA reader with a wavelength of 450 nm was used to measure absorbency. Each series of measurements was calibrated using five sets of cotinine calibrators (5, 10, 25, 50 and 100 µg l^{-1}). Two sets of negative and positive standards representing low and high levels of cotinine were run with each assay. Cotinine negative control contains drug-free synthetic urine matrix containing azide-free preservatives while the positive control contains 500 µg l^{-1} of cotinine in a synthetic urine matrix containing azide-free preservatives. The urinary cotinine concentrations were expressed as µg g^{-1} creatinine.

Statistical analysis

Adduct levels expressed as 10^{10} nucleotides were log-transformed (base 10) because of the extreme non-normality of its distribution. Because logarithms are not defined for values of zero or less, we added a value of 1 to the adduct data in order to account for values of zero (0). Potential differences in demographic variables and levels of DNA adducts between cases and controls were analyzed by the paired Student's test for continuous data and χ^2 test for categorical data. For the χ^2 test, Fisher's exact test was used if any one of the expected frequencies was <5. The paired t test was applied for comparing non-cancerous and cancerous tissue within patients. Logistic regression was applied to calculate the odds ratios (OR) and 95% confidence intervals (CI) for the association between levels of adducts and colon cancer risk after adjusting for confounding variables. A number of demographic, socioeconomic, environmental and health-related variables were used as potential confounding variables. Due to the limited number of smokers, we combined different smoking habits (cigarettes, sheesha and muaasal) into one variable. The region of living variable was created to reflect the location of the participants. Saudi Arabia is divided into five regions: Central, Northern, Southern, Western and Eastern. Due to the limited sample size, we had to group our participants into two groups: Central region and other regions. All p values were two-tailed (at a significance level of $p<0.1$). All statistical analyses were performed with SPSS version 10.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results and discussion

Although BPDE-DNA adducts were not detected in the normal, cancerous and non-cancerous tissue of patients, our data demonstrated clearly the presence of other unknown DNA adducts in both tissues. The absence of BPDE-DNA adducts in our

patients might result from either low levels of benzo[*a*]pyrene exposure or be influenced by polymorphisms in carcinogen metabolizing genes such as *GSTM1* (Boysen & Hecht 2003). Moore et al. (2005) found associations between colorectal adenomas and *GSTM1* wild-type and *GSTT1* null allele among smokers, while Huang et al. (2006) reported that *GSTT1* and *GSTM1* polymorphisms weakly related to colorectal cancer risk and there may be racial differences in gene–smoking interactions. A histogram of DNA adducts in cancerous, non-cancerous and normal tissue is shown in Figure 1.

The wide range in the DNA adduct levels most likely reflects interindividual variability in exposure to carcinogen and metabolic activation/inactivation. Out of the 24 cancer patients, DNA adducts were detected in 20 cancerous tissue samples (83.3%), whereas DNA adducts were found in 12 non-cancerous tissue samples (60.0%). The higher adduct levels in the cancerous and non-cancerous tissue compared with controls provides evidence for the involvement of carcinogen exposure in cancer risk. The total level of DNA adducts in the cancerous tissue of 24 cancer patients was 151.75 ± 217.27 per 10^{10} nucleotides. Although, it was higher than the total adducts in the non-cancerous tissue of 20 cancer patients (114.81 ± 186.10 per 10^{10} nucleotides), statistically it was not significant ($p = 0.343$). This increase might be related to the stage of cancer. Approximately half of the patients presented late, stage 3–4, as there is no national programme of screening for colorectal cancer. The cost-effectiveness of such a programme is doubtful in the face of an annual incidence of 502 cases of colorectal caner per year in a population of approximately 26 million, according to the 2002 census. The overall age-standardized rate was 5.6/100 000 (King Faisal Specialist Hospital & Research Centre Cancer Registry 2002). On the other hand, a positive correlation coefficient was found between total adducts in cancerous and non-cancerous tissues ($r = 0.659$, $p = 0.002$) suggesting that both

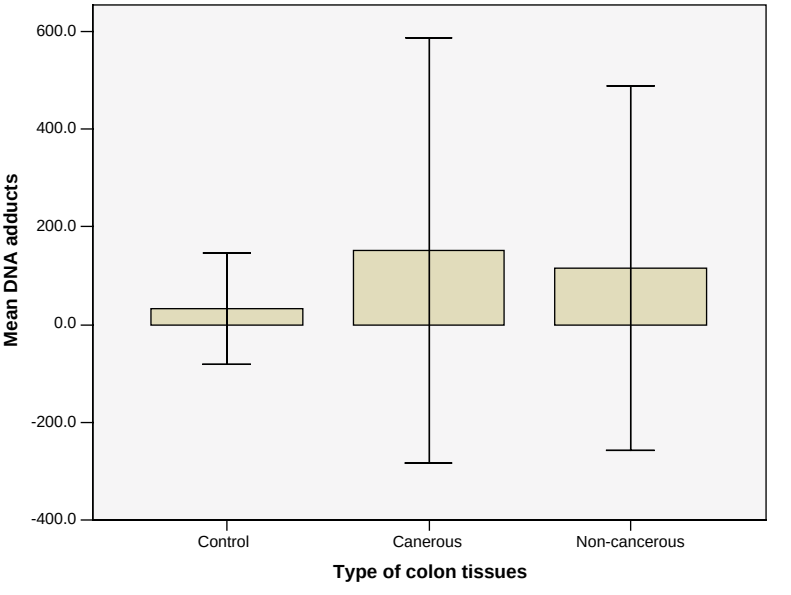

Figure 1. Comparison of DNA adduct levels in tissue from controls and patients with colon cancer. Cancerous and non-cancerous tissue samples were taken from the same patient. Error bars show mean ± SD.

adducts are formed from the same source of exposure. When we compared the total levels of adducts in the cancerous tissue of 24 cancer patients with the total in the controls (32.78 ± 57.51 per 10^{10} nucleotides), the difference was statistically significant ($p = 0.017$), whereas, the total adduct levels in the controls were not significantly different from those in the non-cancerous tissue samples ($p = 0.156$). These differences may be due to various factors such as excess exposure or metabolic activation processes as well as variations in the DNA repair mechanism (Povey et al. 2002). As shown in Table II, there were differences in the levels of DNA adducts between different grades of the cancerous tissues. The highest level of adducts was seen in patients with grade 1 and 2, whereas those with grade 3 had the lowest level of adducts. Interestingly, a similar pattern was observed in adjacent non-cancerous tissue. DNA-adduct formation is regarded as an initial step in the multistage process of carcinogenesis (Garcea et al. 2003). This could explain the higher level of DNA adducts we observed in non-cancerous tissue samples that were adjacent to the cancerous tissue. However, this finding is not conclusive because of the small sample size, and it should be given more attention in future studies.

Colon cancer has been attributed to a number of environmental carcinogens, especially those in the diet such as HCAs (Wu et al. 2006). The first demonstration of DNA adducts in human colon was reported by Umemoto et al. (1994) suggesting exposure to environmental carcinogens. In 1991, Ito et al. reported that 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyrldine (PhIP) is the most abundant of the mutagenic HCAs in cooked meat and fish. Dingley et al. (1999) found PhIP adducts in colon tissue of cancer patients following dietary exposure to the carcinogen PhIP. A recent study by Malfatti et al. (2006) measured one of the PhIP metabolites (N-hydroxy-PhIP-N(2)-glucuronide) in urine and its involvement in DNA-adduct formation after low dietary exposure to PhIP. Our study has limitations with regard to adduct identification due to unavailability of reference adducts. Researchers have identified other risk factors which are associated with colon cancer such as high meat intake, vegetable consumption, tobacco smoke and alcohol (Potter 1996, Emmons et al. 2005, Ji et al. 2006). In this study, it was hard to relate the association between DNA adducts and other risk factors such as smoking, meat and/or fish consumption because of the small sample size. Added to this, there was no consistent history of exposure to carcinogens in the patients studied. A family history of colorectal cancer has also been associated with an increased risk of the disease, especially among younger people (Fuchs et al. 1994, Soliman et al. 1998, Mitry et al. 2001, Pinol et al. 2004, Mahdavinia et al. 2005). Among the studied patients, one cancer patient had a family history of colon cancer and the others had relatives with different types of cancer. Our sample population is too small to perform any significant analysis.

Table II. The total DNA adducts (per 10^{10} nucleotides) in cancerous and non-cancerous tissue of patients as classified by clinical tumour grade.

	Total adduct levels per 10^{10} nucleotides	
Tumour grade	Cancerous tissue	Non-cancerous tissue
1	153.88 ± 259.72 ($n = 5$)	167.45 ± 203.69 ($n = 5$)
2	171.31 ± 230.26 ($n = 15$)	116.72 ± 202.18 ($n = 12$)
3	75.78 ± 122.20 ($n = 4$)	19.47 ± 25.18 ($n = 3$)

Our study revealed that neither urinary cotinine nor hydroxypyrene levels were correlated with the levels of DNA adducts in cancerous and/or non-cancerous tissue ($p > 0.05$). The lack of association may be related to the different kinetics of these variables. Both urinary cotinine and 1-hydroxypyrene reflects short-term exposure (Strickland & Kang 1999, Hukkanen et al. 2005), whereas DNA adducts (especially bulky DNA adducts) may serve as biomarkers of long-term cancer risk (Kyrtopoulos 2006). Despite the fact that urinary cotinine reflects recent tobacco exposure, the number of smokers was low in this study. There were only three controls and three cancer patients who had cotinine values ≥ 150 μg g^{-1} creatinine. All the cancer patients were current cigarette smokers. Among the controls, there were two current cigarette smokers and one non-smoker. The level of adducts in the cancerous (476.43 per 10^{10} nucleotides) and non-cancerous (373.37 per 10^{10} nucleotides) tissue of former smokers tended to be higher than that in the current smokers (21.60 per 10^{10} nucleotides for cancerous tissue and 11.63 per 10^{10} nucleotides for non-cancerous tissue). Adducts in former smokers are likely to reflect multiple sources (environmental tobacco smoke, air pollution, food carcinogen, etc.) or exposure to other carcinogens not related to tobacco smoking which we were unable to confirm because of the small number of participants. Overall, urinary 1-hydroxypyrene concentrations were relatively low but detectable. In 70.6% of the controls, the average was 0.04 μg g^{-1} creatinine in the range of 0.011–0.139 μg g^{-1} creatinine, and in 72.7% of the cancer patients, the average was 0.034 μg g^{-1} creatinine in the range of 0.010–0.07 μg g^{-1} creatinine. At low environmental levels, non-occupational sources, such as cigarette smoke and diet, may be major contributors to 1-hydroxypyrene excretion (Yang et al. 2003). In this study, we found no correlation between urinary 1-hydroxypyrene and cotinine in cancer patients ($p > 0.05$), but a borderline positive correlation was found in the controls ($r = 0.424$, $p = 0.09$). The mean urinary concentrations of 1-hydroxypyrene adjusted for creatinine among non-smokers and smokers were the same (0.027 μg g^{-1} creatinine) but higher than former smokers (0.021 μg g^{-1} creatinine). These levels were much lower than the reported background levels of urinary 1-hydroxypyrene for non-smokers in the general population of 0.3 μg g^{-1} creatinine (German Federal Environmental Agency 2005). It seems the exposure level in the studied patients was lower than that of an occupationally exposed population or of a population that resides in a region with high environmental air pollution. Because of the very low levels of urinary 1-hydroxypyrene detected and the small sample size, it is difficult to assess with certainty the possible increased cancer and health risks associated with these low-level exposures. In addition to smoking, many studies have shown that diet is also another significant contributor to 1-hydroxypyrene (van Maanen et al. 1994, Ruchirawat et al. 2005, Cocco et al. 2007). We did not assess dietary contributions to 1-hydroxypyrene or have sufficient environmental monitoring data to interpret perform a multimedia exposure analysis.

In order to consider possible risks factors that might be associated with the presence of these adducts we compared cases and controls with regard to continuous and categorical confounding variables. The Student *t*-test for continuous variables revealed that only age and duration of living in the current region in years were significantly related to cancer risk with *p*-values of 0.0 and 0.005, respectively, as shown in Table III. The mean age of the controls was significantly younger (30.50 ± 12.78 years) than those of the patients (58.21 ± 13.17 years). This unbalanced design

raises the question of whether the higher level of adducts detected in the patients compared with the controls was a consequence of the differences in age distributions between the two groups. The incidence of colon cancer increases at age 50, approximately (Borum 2001). Because colon cancer is more common in older people and the controls were much younger than the cancer patients in this study, the possibility that the observations described here were an artefact of subject selection was examined. For categorical variables, using the χ^2 test, we found that variables such as marital status and attending school were significantly associated with the risk of cancer ($p < 0.05$) as shown in Table IV. It is also possible that the high risk of colon cancer in relation to marital status and duration of living in the current region might be a spurious finding due to age factor. The study by Lee et al. (2005) provided evidence that there is a progressive age-dependent accumulation of oxidative DNA damage and of BPDE-DNA adducts in human stomach tissues.

In the multiple logistic regression model, we included only confounding variables that were significantly associated with cancer risk such as age and the duration of living in the current region ($p < 0.05$). Due to limited sample size, we did not include marital status and attending school. Adducts in cancerous tissues were associated with the subsequent risk of colon cancer, with an OR of 3.587 and 95% CI 0.833–15.448 after adjustment for age and duration of living in the current region, but of a borderline significance ($p = 0.086$). On the other hand, a non-statistically significant association was seen between adducts in the non-cancerous and cancer risk with a p-value of 0.457. DNA adducts would appear to have a potential as biomarkers of cancer risk.

During recent years, a considerable number of studies have supported the relationship between DNA adducts and cancer risk at various sites such as liver, lung, prostate, breast and bladder (Santella et al. 2005, Bak et al. 2006, Rybicki et al. 2006, Saad et al. 2006, Zhang et al. 2006, Zhu et al. 2006). Although, Kyrtopoulos (2006) in his recent review referred to the potential of DNA adducts as biomarkers of cancer risk, the progress achieved in terms of the identification of environmental or genetic factors

Table III. Mean ± SD of studied variables in cases and controls.

Variables	Control Mean ± SD, *n*, range	Cases Mean ± SD, *n*, range	*p*-Value[c]
Urinary cotinine ($\mu g\ g^{-1}$ creatinine)	376.41 ± 970.47 $n = 17$ (0.875–3075.0)	193.03 ± 554.62 $n = 22$ (1.15–2265.5)	0.461
Urinary 1-hydroxypyrene ($\mu g\ g^{-1}$ creatinine)	0.028 ± 0.034 $n = 17$ (0–0.139)	0.025 ± 0.022 $n = 22$ (0–0.070)	0.691
Total DNA adducts (per 10^{10} nucleotides)	32.78 ± 57.51 $n = 12$ (0–166.10)	151.75 ± 217.27[a] $n = 24$ (0–644.50)	0.017
		114.81 ± 186.10[b] $n = 20$ (0–691.80)	0.156
Age (years)	30.50 ± 12.78 $n = 18$ (16–61)	58.21 ± 13.17 $n = 24$ (30–83)	0
Body mass index ($kg\ m^{-2}$)	26.61 ± 6.47 $n = 13$ (12.60–36.0)	27.65 ± 7.46 $n = 24$ (17.29–45.17)	0.225
Party (number of live children)	5.44 ± 3.58 $n = 9$ (1–11)	7.61 ± 4.81 $n = 24$ (1–25)	0.232
Length of time living in the current region (years)	15.61 ± 11.18 $n = 18$ (1–40)	35.21 ± 25.87 $n = 24$ (2–83)	0.005

[a]Total adducts in cancerous tissue of cancer patients; [b]total adducts in non-cancerous tissue of cancer patients; [c]Student's *t*-test.

Table IV. Distribution of selected characteristics of study subjects.

Variables	Control *n* (%)	Cases *n* (%)	*p*-Value[a]
Gender			0.721
Male	10 (45.5)	12 (54.5)	
Female	8 (40.0)	12 (60.0)	
Marital status			0.033[b]
Married	10 (32.3)	21 (67.7)	
Not married	8 (72.7)	3 (27.3)	
Current living region			0.08[b]
Central region	16 (51.6)	15 (48.4)	
Other regions	2 (18.2)	9 (81.8)	
Living close to busy or/and industrial area			1.0[b]
Yes	3 (37.5)	5 (62.5)	
No	15 (44.1)	19 (55.9)	
Other illnesses			0.09
Yes	5 (26.3)	14 (73.7)	
No	13 (56.5)	10 (43.5)	
Family history of cancer			0.706[b]
Yes	4 (50.0)	4 (50.0)	
No	14 (41.2)	20 (58.8)	
Use of herbal remedy			1.0[b]
Yes	2 (50.0)	2 (50.0)	
No	16 (42.1)	22 (57.9)	
Attending school			0.011[b]
Yes	15 (60.0)	10 (40.0)	
Never	3 (17.6)	14 (82.4)	
Working status			0.327
Yes	7 (35.0)	13 (65.0)	
Never	11 (50.0)	11 (50.0)	
Smoking status			0.656[b]
Current	2 (33.3)	4 (66.7)	
Former	1 (25.0)	3 (75.0)	
Never	15 (46.9)	17 (53.1)	
Fish consumption			0.685[b]
Yes	16 (44.4)	20 (55.6)	
No	2 (33.3)	4 (66.7)	
Frequency of eating fish			0.073[b]
Frequent	7 (70.0)	3 (30.0)	
Seldom	9 (34.6)	17 (65.4)	
Method of cooking			0.677
Grilled	11 (42.3)	15 (57.7)	
Others	5 (50.0)	5 (50.0)	
Frequency of eating meat			1.0[b]
Frequent	14 (46.7)	16 (53.3)	
Seldom	4 (40.0)	6 (60.0)	
Method of heating			0.18**
Electrical	15 (50.0)	15 (50.0)	
Others	3 (25.0)	9 (75.0)	
Use of incense			0.247[b]
Yes	18 (46.2)	21 (53.8)	
No	0	3 (100.0)	
Frequency of incense use			0.510
Frequent	12 (42.9)	16 (57.1)	
Seldom	6 (54.5)	5 (45.5)	

[a]χ^2 test for the distribution between cases and controls; [b]Fisher's exact test.

which substantially affect cancer risk among the general population is still relatively limited. He suggests that more efforts are needed to promote thorough validation of chemical-specific biomarkers of exposure, the development of reliable and high-throughput phenotypic assays of biomarkers of susceptibility and the formulation of a systems biology approach to the analysis of the modulation of biomarkers by environmental and genetic factors. The latter utilizes mathematical models to describe the structure and behaviour of the biological system (Assmus et al. 2006, Hornberg et al. 2006). Major systems biology efforts have been applied to cancer in order to understand disease mechanisms and the ability to devise effective therapeutics (Celis et al. 2005, Khalil & Hill 2005, Chen et al. 2006, Wang et al. 2007).

Another recent review by Rundle (2006) confirmed the concerns expressed by Kyrtopoulos and discussed the drawback of methodological issues such as the impact of using target versus surrogate tissues on the selection of control, the effect of diseases on adduct, the time period reflected by adduct levels, the use of inappropriate statistical analyses and small sample sizes. He suggested a greater focus should be placed on designs that allow measurements of adduct levels in tissues collected years prior to cancer diagnosis and proposed that there is little need for further hospital-based case–control studies in which adducts are measured at the time of or after diagnosis.

Although it is difficult to make a definite conclusion from such a small dataset, significantly higher levels of DNA adducts were found in cancer patients compared with the controls suggesting their potential role in the carcinogenesis process. An additional study with a larger sample size is needed to confirm our preliminary findings. It is also important to identify the structural characterization of these unknown DNA adducts in order to have a better understanding of whether or not environmental carcinogens play a role in the aetiology of colon cancer.

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