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White blood cell DNA adducts and fruit and vegetable consumption in bladder cancer

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The 'Mediterranean diet', a diet rich in cereals, fruit and vegetables, has been associated with lowering the risk of a variety of cancers of the digestive tract and the bladder. In a previous study, we showed that the high phenolic content these dietary components produce in the urine could be associated with higher antimutagenic properties of the urine and lower arylamine–DNA adducts in exfoliated bladder cells. We have conducted a case–control study on 162 bladder cancer patients and 104 hospital controls. Total aromatic DNA adducts were measured in white blood cells (WBC) of all subjects by ^{32}P-post-labelling. Genetically based metabolic polymorphisms were analysed by PCR–RFLP (NAT2, GSTM1, GSTT1, GSTP1, COMT and NQO1). All subjects were interviewed about their tobacco use, dietary habits and other risk factors. The odds ratio (OR) for the risk of bladder cancer according to the presence/absence of WBC DNA adducts (detection limit 0.1 RAL$\times 10^8$) was 3.7 [95% confidence interval (CI) 2.2–6.3] and a dose–response relationship with levels of adducts was apparent. The association between case/control status and the presence of WBC DNA adducts was significantly stronger in the subjects who consumed fewer portions of fruit or vegetables per day (OR 7.80, 95% CI 3.0–20.30 for 0–1 portions of vegetables) than in the heavy consumers (OR 4.98 for consumers of 2 portions daily, OR 1.97 for consumers of $\geqslant$3 portions; similar but lower estimates were found for the intake of fruit). No association was noticed between tobacco smoking and WBC DNA adducts. Only NAT-2, among the several genotypes considered, was associated in a statistically significant way with the risk of bladder cancer (OR 1.72, 95% CI 1.03–2.87) and with the levels of WBC DNA adducts. Our report suggests that fruit and vegetables could protect against bladder cancer by inhibiting the formation of DNA adducts.

Introduction

The 'Mediterranean diet', a diet rich in cereals, fruit and vegetables, has been associated with lowering the risk of cancers of the colon, breast, bladder and prostate (1,2). Flavonoids are a particularly relevant group of antioxidants found in fruits, vegetables and beverages. Their dietary intake in humans ranges between 23 (3) and 1000 mg/day/person (4). Urinary excretion of these substances has been reported (5); some have been shown to inhibit the mutagenicity of various aromatic and heterocyclic amines (6). Heterocyclic amines, including 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP), a potent experimental mutagen and carcinogen, have been implicated as an important class of human dietary carcinogens (7).

The consumption of phenolics or flavonoids has been shown to decrease the level of DNA adducts in experimental studies in humans and animals: moderate wine consumption (a source of phenolics) inhibited peroxide-induced micronucleated cells (8) and the consumption of flavonoids inhibited DNA damage related to lipid peroxidation (9). A high concentration of flavonoids and other phenolics can be found in onions, lettuce, red wine and other components of the Mediterranean diet.

We have shown previously that urine extracts contain substances that strongly inhibit urinary mutagenicity related to smoking (10). Building on these findings, we conducted two pilot investigations in volunteers to determine whether a high phenolic content of urine was associated with higher antimutagenic properties of urine and lower arylamine–DNA adducts in exfoliated bladder cells (11,12). We hypothesized that antimutagenic substances in the urine of smokers: (i) are of dietary origin and so could be associated with Mediterranean dietary habits; (ii) are likely to be phenolics; (iii) can inhibit the formation of carcinogen–DNA adducts in exfoliated bladder cells. DNA adducts reflect an enhanced risk of developing a mutation-related disease more realistically than external exposure doses; some adduct profiles reflecting oxidative stress are influenced by antioxidative dietary profiles (13).

The risk of bladder cancer can be modulated by genetically based metabolic polymorphisms. Cytosolic glutathione *S*-transferases (GSTs) are a large family of isozymes involved in detoxification of many electrophilic substrates by their conjugation with reduced glutathione. They can be divided by electrophoresis into four classes: α, θ, μ and π, (14). The polymorphic GSTM1 plays a role in the metabolism of organic epoxides and peroxides and, in particular, conjugates known carcinogens such as epoxides of polycyclic aromatic hydrocarbons (PAHs). This suggests that people without the gene are at greater risk of developing cancers associated with exposure to PAHs. Other GSTs potentially relevant to the risk of bladder cancer are T1 and P1. *N*-Acetyltransferase (NAT) has been shown to polymorphically acetylate arylamines, well-known bladder carcinogens, to arylamides. This metabolic polymorphism depends on a mutation in the *NAT2* gene, transmitted in an autosomal dominant way. Slow acetylators are homozygous recessive individuals, whereas fast acetylators are either wild-type homozygous or heterozygous. It is the slow acetylators who are at higher risk of developing bladder cancer (15).

Abbreviations: ABP-dG, *N*-(deoxyguanosine-8-yl)-4-aminobiphenyl; 95% CI, confidence interval; COMT, catechol *O*-methyltransferase; GST, glutathione *S*-transferase; NAT, *N*-acetyltransferase; NQO1, NAD(P)H:quinone oxidoreductase; OR, odds ratio; PAHs, polycyclic aromatic hydrocarbons; PhIP, 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine; RAL, relative adduct labelling; WBC, white blood cells.

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Other polymorphisms potentially relevant to the risk of environmentally induced cancer are catechol-*O*-methyltransferase (COMT) and NAD(P)H:quinone oxidoreductase (NQO1) (14).

We report here a case–control study on bladder cancer in which we compare DNA adducts measured in white blood cells with fruit and vegetable intake and with several metabolic polymorphisms potentially relevant to bladder cancer.

Materials and methods

Subjects

We conducted a hospital-based case–control investigation at the urology departments of two Turin hospitals (Gradenigo and S.Giovanni Battista), where about half the cases of newly diagnosed bladder cancers in the Turin metropolitan area population are treated. The men making up the case group were aged 45–74, resident in the Turin metropolitan area and treated from 1994 to 1996 in the hospital urology departments for histologically confirmed bladder cancer. These were all incident (newly diagnosed) cases of the disease. The cases were identified by daily contacts between a trained interviewer and the urology departments. Histological confirmation was obtained from the pathology departments.

The controls were recruited daily in random fashion: (i) from patients treated at the same urology departments for benign diseases, mainly prostatic hyperplasia and cystitis (all newly diagnosed); (ii) from patients treated at the medical and surgical departments for hernias, vasculopathies, diabetes, heart failure, asthma or other benign diseases. Patients with cancer, liver or renal diseases and smoking-related conditions were excluded from the study. Like the cases, the controls were men aged 40–74 and living in the Turin metropolitan area.

Before therapy began, a trained interviewer used a standard questionnaire to interview the cases and the controls on their history of tobacco smoking (including brands and tobacco type) and a 24 h recall interview to collect information about their dietary habits, drug use and occupational history. Obviously, the use of a 24 h dietary recall interview to assess habitual dietary consumption was somewhat limited, but because white blood cell (WBC) DNA adducts reflect recent biological phenomena (chiefly lasting weeks), it proved helpful for a comparison with adducts. It was found that 60 cases and 18 controls had, in fact, changed their usual dietary habits as a consequence of the current disease. Most of these individuals followed a pre-operative dietary regime, poor in vegetables and fruit. Because of such changes, their dietary histories were not collected in any detail and they were considered separately in the analysis.

Biological samples: collection and analysis

Before therapy and after informed consent, blood was collected from the cases and the controls (40 ml) and immediately centrifuged. Buffy coats were separated from coded blood samples (8 ml) by centrifugation at 800 *g* for 45 min, followed by lysis of the red cells by suspension in 3 vol 0.17 M ammonium chloride at 4°C for 10 min and centrifugation at 800 *g* for 10 min. The pellet containing WBC was washed with ammonium chloride and stored at –80°C. WBC DNA was isolated and purified from the stored cell pellets by enzymatic digestion of RNA and proteins followed by phenol/chloroform extractions. The WBC DNA samples were digested to 3′-mononucleotides with 0.46 U of micrococcal nuclease (0.312 U/µl; Sigma Chemical Co., St Louis, MO) and 0.352 U of spleen phosphodiesterase (0.058 U/µl; Sigma Chemical Co.) in 17 mM sodium succinate, 8 mM calcium chloride, pH 6.0 at 37°C, for 4.5 h. DNA hydrolysates were evaporated to dryness and redissolved appropriately. Enrichment of carcinogen–DNA adducts in DNA digests was done by selective destruction of normal nucleotides by the nuclease P1 procedure. Normal nucleotides (10 µg) were dephosphorylated at the 3′-position by treatment with 5 µg of nuclease P1 (Sigma Chemical Co.) for 40 min at 37°C. Parts of the DNA samples of the bladder cancer cases and the referents were also analysed blind using the butanol extraction technique, which adds tetrabutylammonium chloride to the aqueous solutions of the DNA digests and then extracts the adducts with butanol. The hydrolysates enriched in adducted nucleotides were labelled by incubation with 55 µCi of carrier-free [γ-^{32}P]ATP (3000 Ci/mmol, DuPont, Wilmington, DE) and 5 U of T4 polynucleotide kinase (Pharmacia, Uppsala, Sweden) at 37°C for 40 min in 25 µl of bicine buffer mixtures (pH 9). Resolution of ^{32}P-labelled DNA digests treated with nuclease P1 or extracted with butanol were carried out on PEI–cellulose TLC plates (Merck, Darmstadt, Germany) using the contact transfer technique. The solvent systems selected were: D1, 1 M sodium phosphate, pH 6.0; D3, 4 M lithium formate, 7.5 M urea, pH 3.5; D4, 0.7 M LiCl, 0.45 M Tris–HCl, 7.7 M urea, pH 8.0; D5, 1.7 M sodium phosphate, pH 5.0. The adduct spots were detected by autoradiography for 72 h at –80°C using Kodak XAR-5 film and intensifying screens. The levels of total aromatic DNA adducts were determined by excising areas of chromatograms and measuring the levels of radioactivity present by Cerenkov counting. Quantitation of normal nucleotides was carried out as already reported (25). For the samples with no detectable DNA adducts after autoradiography, the nominal DNA adduct levels were calculated from the radioactivity in an average sized diagonal area of the TLC plates in the region of adduct migration; the detection limit value was 0.1 adducts/10^8 normal nucleotides. The reproducibility of the nuclease P1 technique was verified by analyzing 56% of all the WBC DNA samples in a second independent ^{32}P-post-labelling experiment; the results of the two analyses were in good agreement (r = 0.71). All analyses concerning WBC DNA adducts were carried out at IST, Genova.

Three known slow acetylator alleles (NAT2*5A/5B, 6A/6B and 7A/7B) were identified as described (16), with slight modifications. PCR was carried out in a total volume of 50 µl, using primers N5 and N4. Five microlitres of whole blood purified DNA solution were added to the PCR mixture containing 50 pmol of each primer, 200 µmol of dNTPs, 1.5 U *Taq* polymerase (AmpliTaq; Perkin Elmer), 10 mM Tris–HCl, pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$. PCR conditions were exactly as described (17). Four microlitre aliquots of PCR products were incubated with the restriction enzymes *Kpn*I, *Taq*I and *Bam*HI (Gibco BRL). The restriction digests were electrophoresed and categorized as described (17). Rapid acetylator genotypes are wild-type allele homo/heterozygotes; slow acetylator genotypes are those with two slow acetylator alleles. GSTM1 null, GSTM1A, GSTM1B and GSTM1A/B polymorphisms at the GSTM1 locus were identified using a slightly modified version of the method described by Fryer *et al.* (18). By similar methods, GSTT1 (19), GSTP1 (20), NQO1 (21,22) and COMT (23) polymorphisms were also analysed.

Statistical analyses

We computed the means and the medians of WBC DNA adducts. However, since the distribution of the adducts was skewed, most analyses were based simply on the presence or absence of detectable adducts (detection limit 0.1 RAL×10^8). When quantitative analyses are shown they are limited to the subjects with detectable levels. Non-parametric analysis of variance, age-adjusted odds ratios (OR), the corresponding 95% confidence intervals (95% CI) and multivariate analyses were performed with the SAS package for the personal computer. Logistic regression was performed using both continuous and dummy variables.

The distribution of WBC DNA adducts according to the types of controls has been considered in a separate, methodological paper (24).

Results

We recruited 162 bladder cancer cases and 104 controls who provided biological samples. All were interviewed except for two cases and two controls who refused and three cases who were too ill to respond. We also interviewed 42 cases who did not provide biological samples. Although they did not differ from the others in terms of their clinical or pathological characteristics or smoking habits (25), they were excluded from the present analysis nonetheless. In studies on tobacco use, smoking has been clearly associated with the risk of bladder cancer (25), yet no relationship between smoking and WBC DNA adduct levels ($P > 0.05$) has been established in our study.

The level of DNA adducts was strongly associated with case/control status (Table I). The age-adjusted OR associated with an adduct level above the limit of detection was 3.7 (95% CI 2.2–6.3) and a dose–response relationship with levels of adducts was apparent (Table I). To study the dose–response relationship, categories were defined by subdividing the values above the detection limit into tertiles. All OR in Table 1 are highly statistically significant, with confidence intervals that largely exceed one. WBC DNA adducts were associated with the case/control status independently of tobacco use and fruit or vegetable consumption (Table II, regression models).

The level of WBC DNA adducts fell as vegetable consumption rose (Table III), but the decrease was statistically significant for the controls only. The trend with fruit intake was ambiguous. The association between case/control status and the level of

WBC DNA adducts was stronger in the subjects who consumed fewer portions of fruit or vegetables per day (Table III) (OR 7.80 for 0–1 portions of vegetables, 95% CI 3.00–20.30) than in heavy consumers (OR, 4.98, 95% CI 1.56–15.92, for consumers of 2 portions daily; OR 1.97, 95% CI 0.48–8.02, for consumers of ⩾3 portions).

The age-adjusted OR for NAT2 was 1.72 (95% CI 1.03–2.87). NAT2 was also predictive of the DNA adduct levels; the difference between slow and rapid acetylators was statistically significant among the cases, but not among the controls (Table IV). WBC DNA adducts were slightly higher among subjects with the mutated genotype for COMT; although the differences were not statistically significant, there may be a dose–response relationship with the number of mutations (Table IV).

Table I. Case–control study on bladder cancer (162 cases, 104 controls): distribution by WBC DNA adducts (^{32}P-post-labelling)[a]

Quartiles of DNA adducts	Cases	Controls	OR	95% CI
0.1 (detection limit)	32	50	1.0	
>0.1	130	54	3.7	2.2–6.3
Below median (0.23)	64	72	1.0	
Above median	98	32	3.6	2.1–6.1
0.1 (detection limit)	32	50	1.0	
Tertiles above 0.1				
0.11–0.23	33	24	2.1	1.1–4.2
0.23–0.51	44	19	3.5	1.7–7.1
>0.51	54	11	7.6	3.6–16.1

OR, odds ratio; CI, confidence interval. ORs are adjusted by age. Adducts are expressed as RAL$\times 10^8$.
[a]Data are missing for seven cases and one control.

Discussion

A number of studies have previously reported that DNA adducts in the peripheral blood may be associated with the risk of cancer (26–28). The increased levels of DNA adducts in the peripheral blood of bladder cancer patients may be a consequence of the cancer process itself or, alternatively, may reflect the susceptibility of a group of individuals to form higher levels of DNA adducts for a similar level of environmental exposure.

Table II. Case–control study on bladder cancer (162 cases, 104 controls): logistic regression models

	Estimate	SE	*P* value	OR	95% CI
Model I					
Age	0.036	0.015	0.015	1.037	1.007–1.068
Number of cigarettes/day	0.054	0.011	0.0001	1.056[a]	1.033–1.080
Model II					
Age	0.048	0.019	0.015	1.034	1.014–1.055
Number of cigarettes/day	0.06	0.015	0.0001	1.068[a]	1.038–1.10
Fruit intake	–1.72	0.39	0.0001	0.18	0.08–0.39
Vegetable intake	–0.80	0.39	0.039	0.45	0.21–1.0
Model III					
Age	0.035	0.01	0.0016	1.035	1.013–1.058
Number of cigarettes/day	0.07	0.015	0.0001	1.07[a]	1.04–1.10
Fruit intake	–1.55	0.42	0.0002	0.21	0.09–0.48
Vegetable intake	–0.91	0.41	0.03	0.40	0.18–0.91
WBC DNA adducts: yes/no	1.66	0.43	0.0002	5.25	2.21–12.43

SE, standard error; CI, confidence interval; na, not applicable. Age and smoking are continuous variables. Fruit and vegetables are >3 portions per day versus ⩽3.
[a]Increase per single cigarette smoked.

Table III. Case–control study on bladder cancer (162 cases, 104 controls): distribution of DNA adducts in WBC according to fruit/vegetable consumption[a]

	WBC DNA adducts						OR (CI)
	Controls			Cases			
	⩽0.1	>0.1	Mean	⩽0.1	>0.1	Mean	
Intake of vegetables							
0–1	15	10	0.69	9	53	0.62	7.80 (3.00–20.30)
2	18	18	0.38	4	22	0.57	4.98 (1.56–15.92)
3+	12	13	0.28	5	10	0.49	1.97 (0.48–8.02)
P value			0.027			0.90	
Intake of fruit							
0–1	14	12	0.42	10	65	0.59	7.60 (2.91–19.78)
2	16	16	0.46	5	18	0.61	3.10 (0.96–10.07)
3+	15	13	0.39	3	2	0.30	0.76 (0.13–4.51)
P value			0.66			0.87	
Changed habits[a]	5	13	0.29	14	46	0.60	1.38 (0.40–4.79)

P values are based on non-parametric analysis of variance. ORs refer to levels of adducts >0.1 versus ⩽0.1. Means of DNA adducts exclude levels ⩽0.1.
[a]Subjects who reported major dietary changes due to symptoms.

Table IV. Levels of WBC DNA adducts according to selected metabolic polymorphisms in 162 cases and 103 controls (one missing value; RAL×10^8)

	NAT2		GSTT1		COMT			GSTP1[a]		GSTM1		NQO1	
	Slow	Rapid	Null	Wild-type	mm	mw	ww	Slow	Rapid	Null	Wild-type	mm/mw	ww
Cases													
Mean	0.67	0.39	0.57	0.60	0.68	0.62	0.49	0.58	0.63	0.63	0.55	0.62	0.58
Median	0.5	0.34	0.5	0.43	0.6	0.5	0.3	0.48	0.46	0.5	0.42	0.49	0.47
SE	0.06	0.06	0.09	0.05	0.10	0.06	0.08	0.05	0.08	0.07	0.05	0.07	0.06
n	84	39	14	108	25	63	35	73	50	61	69	54	69
P value	0.014		0.83		0.18			0.58		0.33		0.63	
Controls													
Mean	0.47	0.30	0.22	0.41	0.43	0.41	0.31	0.36	0.41	0.36	0.43	0.32	0.46
Median	0.31	0.27	0.20	0.30	0.25	0.30	0.26	0.20	0.32	0.23	0.33	0.27	0.31
SE	0.08	0.03	0.025	0.05	0.10	0.08	0.06	0.10	0.05	0.06	0.08	0.04	0.09
n	30	24	6	48	14	28	12	22	32	29	25	26	28
P value	0.09		0.22		0.42			0.63		0.46		0.18	

RAL values equal to or lower than the detection limit (0.1) have been excluded. *P* values are based on non-parametric analysis of variance.
[a]aa homozygous versus others.

The presence of aromatic DNA adducts was analysed using both the nuclease P1 and the butanol extraction procedures. The nuclease P1 method is mainly effective for bulky hydrophobic aromatic DNA adducts, such as those induced by PAH and some arylamines bound to the exocyclic position of guanine or adenine, while the butanol extraction technique is capable of detecting mostly arylamines bound to the C-8 position of guanine and some low molecular weight alkylating agents (29).

Our data suggest that fruit and vegetables may protect against bladder cancer by interfering with the formation of DNA adducts in WBC. The protection may be due to induction or inhibition of enzymes involved in carcinogen metabolism or in DNA repair (8,9,14,30). DNA adducts enhance the risk of developing a mutation-related disease; some adduct profiles have been found to be influenced by antioxidative dietary profiles (13).

The present findings are consistent with our previous studies. Our first investigation into the nature of DNA adducts in the exfoliated bladder cells of smokers showed that they were related to cigarette smoking. One of them was the putative *N*-(deoxyguanosine-8-yl)-4-aminobiphenyl (ABP-dG) adduct (11), which was found to be linearly related to the number of cigarettes smoked per day. In a second study, both ABP-dG and total DNA adducts were found to be inversely related to bacterial antimutagenicity, expressed as the decrease in the number of revertants of *Salmonella typhimurium* TA98 per ml urine equivalent (PhIP-induced mutations). The logarithm of DNA adducts versus antimutagenicity were linearly and inversely related in a statistically significant way ($R = -0.81$, $P < 0.01$) (12). To identify the chemical nature of the antimutagens, we also measured the amount of phenolics in urine extracts from a group of volunteers ($n = 19$) by spectrophotometric assay. The concentrations were then compared with the urinary antimutagenic activity, again, using PhIP as mutagen in *S.typhimurium* TA98. All the urine extracts contained measurable quantities of phenolics, ranging from 3.7 to 12.5 µg/10 µl extract solution. A statistically significant linear relationship was found between the antimutagenicity of the urine extracts and their content of phenolic substances ($R = 0.58$, $P < 0.02$) (12).

The present case–control study extends our previous investigations by showing that there are fewer WBC DNA adducts or carcinogen–DNA adducts in bladder biopsies among heavy consumers of fruit or vegetables. Inhibition of DNA adduct formation could be one of the mechanisms by which the 'Mediterranean diet' protects against cancer. The lack of association between WBC DNA adducts and smoking habit, though puzzling, is not new (25).

The present investigation was flawed in two ways. One was collecting information by a 24 h recall dietary history, which cannot claim to represent long-term dietary habits. Recent dietary history is, however, likely to have a more important impact on DNA adduct levels, which reflect recent events (recent weeks). The other flaw was the use of a simplified questionnaire to investigate dietary history, without estimating caloric intake or specific nutrients. Even so, the average number of portions consumed by cases and controls was reasonably high enough to argue for considering portions of fruit and vegetables sound indicators.

Although the relationship between cigarette smoking and bladder cancer has been shown to be highly statistically significant in our study (25), no relationship has been found between WBC DNA adducts and cigarette smoking using both the nuclease P1 and butanol extraction procedures. Conflicting results on the correlation between WBC DNA adducts and tobacco smoking have been reported by previous investigations (31–33). The lack of a correlation with cigarette smoking may reflect marked interindividual variability in carcinogen metabolism and in DNA binding, which may result in different levels of DNA adducts for a similar degree of exposure (34). Alternatively, the effects of cigarette smoking on WBC DNA adducts might be masked by those of other environmental carcinogens, such as urban pollutants and dietary constituents.

The presence of a variety of inflammatory disorders, such as cystitis, in the referent group might have influenced the DNA adduct levels by producing endogenous electrophilic compounds (24) or, alternatively, by changing the proportion of leukocytes in peripheral blood, i.e. lymphocytes and neutrophils. The presence of inflammatory disorders in controls was analysed as a potential confounding factor in a previous methodological paper (24).

Concerning metabolic polymorphisms, the only significant association we detected was with the NAT2 slow genotype, which was in agreement with several previous findings (14). Adduct levels were higher in slow acetylators and in subjects with mutations in COMT. The association with the NAT2

polymorphism is suggestive of a role played by arylamines in tobacco-induced bladder cancer.

In conclusion, our findings suggest that the Mediterranean diet may protect against bladder cancer through a mechanism that interferes with carcinogen–DNA adduction by inducing/inhibiting metabolizing enzymes or inducing DNA repair. Phenolics could be involved in such a mechanism.

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