Bladder Tumor Contains Higher N7-Methylguanine Levels in DNA than Adjacent Normal Bladder Epithelium

Abir A. Saad,[1,3] Peter J. O'Connor,[1] Mostafa H. Mostafa,[3] Nabila E. Metwalli,[4] Donald P. Cooper,[1] Geoffrey P. Margison,[1] and Andrew C. Povey[1,2]

[1]Cancer Research UK Carcinogenesis Group, Paterson Institute for Cancer Research, Christie Hospital NHS Trust; [2]Centre for Occupational and Environmental Health, University of Manchester, Manchester, United Kingdom; [3]Institute of Graduate Studies and Research and [4]Department of Pathology, University of Alexandria, Alexandria, Egypt

Abstract

***Schistosoma haematobium*–infected patients are more likely to develop bladder cancer and be more exposed to carcinogenic *N*-nitroso compounds than uninfected patients. As N7-methylguanine is a marker of exposure to methylating agents of this type, we have measured N7-methyldeoxyguanosine 3′-monophosphate (N7-MedGp) by ^{32}P postlabeling. DNA was isolated from 42 paired normal and tumor tissue of Egyptians with bladder cancer. N7-MedGp was detected in DNA from 93% of the tumors and 74% of the normal bladder tissue samples. Adduct levels were highly variable and ranged from 0.04 to 6.4 and from 0.02 to 0.72 μmol/mol deoxyguanosine 3′-monophosphate (dGp) in tumor and normal DNA, respectively. N7-MedGp levels in normal and tumor DNA were highly correlated with one another ($P = 0.007$). The mean difference (95% confidence interval) in adduct levels between tumor and normal DNA was 0.21 (0.13-0.32) μmol/mol dGp and this was statistically significant ($P < 0.001$). The adduct ratio (tumor DNA/normal DNA) varied between 0.2 and 136 (median, 4.6). N7-MedGp levels were not associated with gender, age, or the presence of schistosomiasis. However, lower N7-MedGp levels were found in normal DNA from individuals lacking the *GSTM1* gene ($P = 0.03$) but not the *GSTT1* gene or in subjects with the *Ile105Val GSTP1* polymorphism. These results show that exposure to methylating agents is widespread and suggest that such exposure may play a role both in tumor initiation and progression.** (Cancer Epidemiol Biomarkers Prev 2006; 15(4):740–3)

Introduction

Cancer of the urinary bladder is one of the most common human malignancies and the most frequent malignant neoplasm of the urinary tract (1). In industrialized countries, bladder cancer is strongly linked to occupational and environmental exposures to chemical carcinogens and, in Europe, one half of male and one third of female cases may be attributed to smoking (2). Aromatic amines represent a common factor among cigarette smoking, many occupational exposures and urinary bladder cancer: compounds such as 4-aminobiphenyl and 2-naphthylamine were among the first to be identified as human bladder carcinogens (3). In contrast to western countries, the high incidence of bladder cancer seen in East Africa and the Middle East and other subtropical countries is associated with chronic urinary infection with *Schistosoma haematobium* (4-6). Carcinoma of the urinary bladder is the most common malignancy among Egyptian males, accounting for 40% of total cancer incidence, whereas in females it ranks second to breast cancer (7).

The etiology of bladder cancer arising in association with schistosome infection is thought to be multifactorial (8). Most attention, however, has focused on the possible role of urinary chemical carcinogens, particularly the *N*-nitroso compounds, in this process (9). Carcinogenic *N*-nitroso compounds or their metabolites are alkylating agents that can react with cellular DNA to form a wide range of adducts (10). Although apparently innocuous, N7-alkylguanine lesions are used as monitors of exposure to *N*-nitroso compounds, in part because of their relatively high levels in DNA and also because their repair rate is very much slower than that of other promutagenic and carcinogenic lesions such as O^6-alkylguanine (10). Nitrosamines have been detected in urine from *S. haematobium*–infected patients including those with bladder cancer at levels significantly higher than in uninfected individuals (11, 12). As this might potentially lead to increased DNA alkylation in bladder DNA and hence bladder cancer, we have examined whether DNA damage (N7-methylguanine) can be detected in DNA from normal bladder tissue and from bladder tumors.

Materials and Methods

Bladder Tissue Samples. Human urinary bladder tissue specimens were obtained during radical cystectomy of Egyptian bladder cancer patients attending the Department of Urology, Faculty of Medicine, Alexandria University. Paired samples comprising a sample of bladder tumor and bladder mucosa without macroscopic signs of tumor invasion (referred to as uninvolved tissue) were collected from the urinary face of the bladder mucosa and were frozen immediately on dry ice and stored at −70°C.

Samples were collected from 42 patients (35 men, 7 women) with a mean age of 56.0 ± 8.0 years. Twenty-six patients had transitional cell carcinoma, eight had squamous cell carcinoma, six had transitional cell carcinomas with foci of squamous differentiation, and two had adenocarcinoma. Among these patients, 86% had a history of schistosomiasis (i.e., either a clinical history of schistosomiasis or schistosome ova were detected in histologic specimens).

Analysis of N7-Methyldeoxyguanosine 3′-Monophosphate by ^{32}P Postlabeling. N7-Methyldeoxyguanosine 3′-monophosphate (N7-MedGp) was quantified by ^{32}P postlabeling as

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Note: D. Cooper is currently at Waters Corporation, Micromass UK Ltd., Manchester, United Kingdom. Contacts between Alexandria and Manchester were facilitated by the British Council.

Requests for reprints: Andrew C. Povey, Centre for Occupational and Environmental Health, University of Manchester, Humanities Building, Manchester M13 9PL, United Kingdom. Phone: 161-275-5232; Fax: 011-44-161-275-5595. E-mail: apovey@manchester.ac.uk

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previously described (13). Bladder tissue DNA was extracted and purified using Qiagen genomic tip columns (containing an anion exchange resin) according to the protocol specified by the supplier. The purified DNA was quantitated by microtitre plate fluorimetry using Hoechst dye. Then, DNA (50 μg) was digested to 3′-nucleotides using calf spleen phosphodiesterase and micrococcal nuclease. This digest was then subjected to a two-stage high-performance liquid chromatography purification process with an anion exchange high-performance liquid chromatography initial step (Synchropak AX300), followed by reverse-phase high-performance liquid chromatography (Chromex Hypersil 5ODS column). N7-MedGp-containing fractions were postlabeled for 1 hour at 37°C with an internal standard [deoxyguanosine 3′-monophosphate (dGp)] and 20 μCi [γ-^{32}P]ATP in a labeling buffer (pH 8.6) using 2 units of T_4 polynucleotide kinase. After nuclease P1 treatment, the resulting 5′ ^{32}P-labeled mononucleotides were separated by two-dimensional TLC on polyethyleneimine-cellulose (20 × 20 cm) TLC plates using D1 (1 mol/L ammonium acetate pH 6.5/isopropanol, 90:10) and then D2 (saturated sodium citrate/saturated ammonium sulfate/isopropanol, 50:5:1).

Radioactivity from the N7-me-[^{32}P]pdG and [^{32}P]pdG-labeled nucleotides was detected using storage-phosphor screens that were scanned using a Molecular Dynamics Storm 860 phosphorimager at a resolution of 176 μm. The N7-medGp [^{32}P] postlabeling reaction was shown to be linear over 3 orders of magnitude. The labeling efficiency, determined by percentage of radioactivity incorporated in dGp [(radioactivity of dGp spot / total radioactivity of TLC plate) × 100], ranged from 14.5% to 37.7% (mean, 24.8 ± 7.4%). The detection limit was 0.019 μmol N7-medGp/mol dGp.

For each batch of samples, positive (N7-medGp standard; e.g., 10 or 100 fmol + 1 pmol dGp) and negative (no sample or standard) controls were included. N7-medGp standard was synthesized by reacting dGp with iodomethane and purified by reverse-phase Hypersil ODS column chromatography.

Analysis of *GST* Genotypes. A multiplex PCR method was used to detect the presence (or absence) of the *GSTT1* and *GSTM1* genes in genomic DNA samples using the exon 3/intron 4 boundary of the *CYP2D6* gene as an internal control (14). The *GSTP1* (*Ile105Val*) genotype was determined by *Alw*26I digestion of a PCR-amplified fragment (14), which distinguishes between the restriction site on the *Val* allele (ACG TCT) and the resistant *Ile* allele (ACA TCT).

Statistical Analysis. Transformation of N7-MedG levels into log N7-MeG resulted in a more normal distribution and analysis was then done using parametric procedures. Results are presented as the geometric mean (95% confidence interval).

Results

Of the 42 paired DNA samples analyzed, 93% from tumor tissue and 74% from normal bladder tissue had detectable N7-MedGp levels. Figure 1 shows representative phosphorimages of enzyme-digested, high-performance liquid chromatography–purified, and ^{32}P-postlabeled DNA from bladder samples. There were large interindividual differences in N7-MedGp levels, ranging from 0.04 to 6.4 μmol N7-medGp/mol dGp in tumor tissue DNA (i.e., 160-fold variation) and from 0.02 to 0.72 μmol N7-medGp/mol dGp for uninvolved normal tissue DNA (a 36-fold variation).

N7-MedGp in normal and tumor DNA were highly correlated with one another (r^2 = 0.413; P = 0.007). The difference in adduct levels (95% confidence interval) between tumor and normal DNA was 0.21 (0.13-0.32) μmol/mol dGp and this was statistically significant (P < 0.001; Table 1). The ratio of adduct levels (tumor DNA/normal DNA) was highly variable and varied from 0.2 to 136; in 36 subjects the adduct ratio was >1. The median adduct ratio was 4.6 and did not vary whether the tumor was a transitional cell carcinoma or a squamous cell carcinoma (Table 1). There was no detectable difference in N7-MedGp levels in DNA from men or women, nor were N7-MedGp levels correlated with age (data not shown). N7-MedGp levels (geometric mean; 95% confidence interval) in normal tissue from patients with evidence of schistosomiasis infection tended to be lower than those without infection (0.05; 0.03-0.08 versus 0.11; 0.05-0.26 μmol N7-medGp/mol dGp) but this was not statistically significant (P = 0.17). Adduct levels in tumor DNA were not different between patients with or without evidence of schistosomiasis (data not shown).

The possible influence of *GST* genotypes on the level of N7-medGp was also examined (Table 2). Adduct levels in normal DNA, but not tumor DNA, were significantly higher in *GSTM1*1* than *GSTM1*2* patients (P = 0.03). There was no significant difference in adduct levels in subjects with the wild-type *GSTT1* allele compared with the null individuals. When patients were grouped according to their *GSTP1* genotypes, there was no association in normal tissue: adduct levels were higher in tumor DNA from patients with the **BB* genotype, but not significantly so. After patients were categorized in

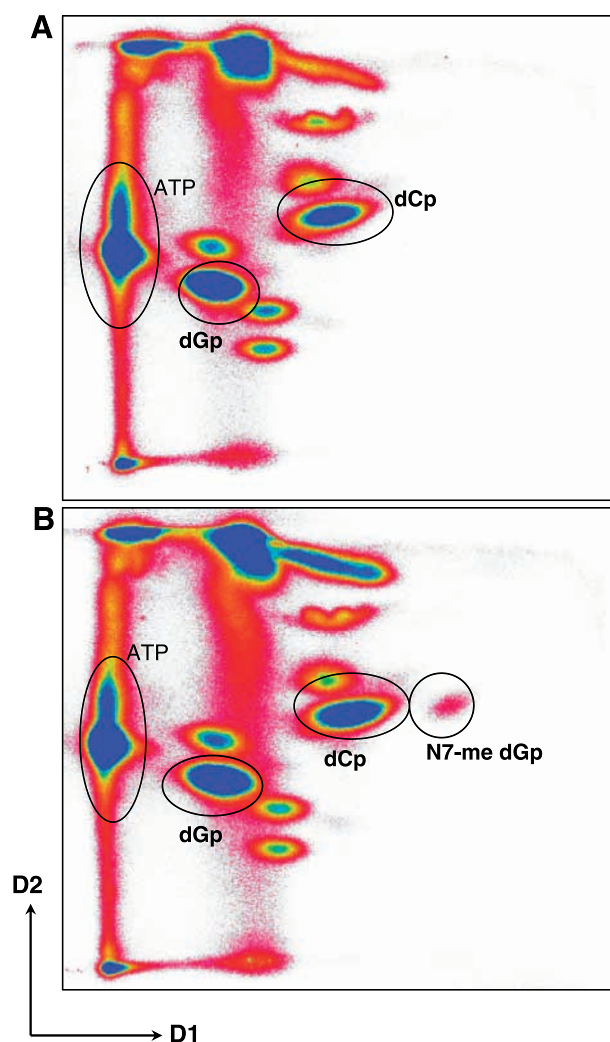

Figure 1. Scanned phosphorimages of two-dimensional TLC analyses of [^{32}P]-postlabeled N7-medGp adducts in DNA digests from human bladder tissue. **A.** DNA sample with no detectable radioactivity arising from N7-medGp in the original DNA digest. **B.** DNA sample with detectable N7-medGp in the original digest.

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Table 1. N7-MedGp levels in matched pairs of normal and tumor DNA

Sample	n	Geometric mean N7-MedGp levels (μmol/mol dGp)		Mean difference in adduct levels (95% confidence interval)	Tumor/normal adduct ratio. Median (range)
		Tumor	Normal		
All	42	0.26	0.05	0.21 (0.13-0.32)*	4.6 (0.2-136)
Transitional cell carcinoma	26	0.29	0.05	0.19 (0.11-0.33)*	4.7 (0.5-136)
Squamous cell carcinoma	8	0.25	0.05	0.19 (0.06-0.58)†	4.9 (1.0-51)

*$P < 0.001$.
†$P = 0.009$.

terms of the number of potentially high-risk genes (i.e., the presence of *GSTM1*1*, *GSTT1*1*, or *GSTP1*BB*; ref. 14), there was no difference in adduct levels in normal and tumor DNA between patients with one high-risk gene and those with two or more high-risk genes (data not shown).

Discussion

Bladder DNA has been shown to contain DNA damage that can be measured by ^{32}P-postlabeling techniques that detect ''bulky'' DNA adducts (e.g., ref. 15). Specific DNA adducts arising from exposure to aromatic amines such as benzidine, 4,4′-methylene-bis(2-chloroaniline), and 4-aminobiphenyl have also been detected in human bladder tissue or exfoliated bladder cells (16-19). This study shows for the first time that DNA from bladder tumors and uninvolved normal tissue from the same patient contains detectable levels of N7-MedGp, indicating that bladder tissue is exposed to a methylating agent, confirming a previous report of the presence of O^6-methylguanine in bladder DNA from cancer patients with schistosomiasis (20). DNA adduct levels in bladder DNA have been shown to vary considerably but tend to be of the order of tens or hundreds of adducts per 10^9 normal nucleotides, although higher levels have been reported (19), particularly if an individual has been accidentally exposed (17). N7-MedGp levels in this study are thus in the same range as other adduct levels previously reported.

Previous studies with bladder (21) and other tissues (22, 23) have indicated that higher levels of the DNA repair protein (i.e., O^6-alkylguanine DNA alkyltransferase) may result in lower levels of O^6-methylguanine in DNA, but in this case, levels of the DNA repair protein are unlikely to provide an explanation. The wide range in N7-MedGp levels most likely then reflects interindividual variability in exposure to methylating agents and metabolic activation/inactivation. The source of these agents remains to be determined. It is known that urine samples from subjects infected with *S. haematobium* contain *N*-nitroso compounds that can methylate DNA (11, 12), and in one experimental study, increasing multiplicity of infection led to increasing levels of O^6-alkylguanine in host mouse liver DNA (24). Adduct levels were similar in patients with or without infection but this may reflect the limited power of the study to examine the relationship between adducts and *S. haematobium* infection as most subjects were infected. Methylating agents may also only contribute a fraction of the DNA damage induced by alkylating agents in total.

Previously we reported that polymorphisms in the *GST* genes are associated with increased risk of bladder cancer among Egyptians, and in particular the presence of two or more a priori at risk genotypes was associated with increased bladder cancer risk (14). In this study, we found an association between the null *GSTM1* genotype and lower adduct levels in normal, but not tumor, DNA. It is possible that this association occurred by chance or was the result of some as yet unknown bias, particularly as individuals lacking *GSTM1* would be expected to have higher levels of DNA adducts (e.g., ref. 25) although such associations are not consistently found (e.g., ref. 26).

Although N7-MedGp is not currently thought to be a lesion that plays an important role in the carcinogenic effects of alkylating agents, its presence does confirm that exposure to such agents has taken place. N7-MedGp levels have been associated with increased risk of treatment failure in cervical cancer (27) and the presence of N7-MedGp in bladder DNA is then consistent with the hypothesis that exposure to methylating agents may play an etiologic role in the development of this cancer. In contrast to previous studies which have reported lower DNA adduct levels in tumor DNA compared with DNA from adjacent normal tissue (28, 29), in this study, N7-MedGp levels were much higher in tumor than normal DNA. The very clear differences shown here indicate that much more detailed studies are needed to understand the close correlation between levels of DNA alkylation in normal and tumor tissue despite the very large difference in the levels of DNA damage in the tissues from these two sources. This difference is unlikely to be an artifact of tissue processing as samples were processed at the same time and in the same manner. Potentially these different adduct levels may indicate

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Table 2. N7-MedGp levels in normal and tumor DNA by *GST* genotype

Gene	Genotype	n	Geometric mean (95% confidence interval) N7-MedGp levels (μmol/mol dGp)	
			Normal	Tumor
GSTM1	**1*	16	0.10 (0.05-0.19)*	0.30 (0.15-0.59)
	**2*	26	0.04 (0.02-0.06)*	0.24 (0.12-0.46)
GSTT1	**1*	24	0.05 (0.03-0.09)	0.21 (0.10-0.42)
	**2*	18	0.06 (0.03-0.12)	0.35 (0.19-0.62)
GSTP1	**AA*	26	0.05 (0.03-0.07)	0.23 (0.13-0.40)
	**AB*	9	0.08 (0.02-0.26)	0.23 (0.06-0.83)
	**BB*	7	0.07 (0.02-0.28)	0.50 (0.10-2.54)

*$P = 0.03$.

a switch in modes of alkylating agent metabolism and/or DNA repair pathways (e.g., refs. 30, 31) that may result in higher levels of DNA damage as part of the change towards a malignant phenotype. Bladder tissue does indeed contain enzymes, such as CYP2E1, which are capable of metabolizing nitrosamines to alkylating species (32-34). Members of the CYP2A subfamily have not been thus far reported in bladder tissue (34). CYP expression is altered by *S. haematobium* infection (33, 35) but whether there is increased CYP expression in tumor tissue remains to be clarified (32, 35). Cyclooxygenase-2, also capable of metabolizing nitrosamines, has been reported to be up-regulated in bladder tumors (36).

Continuing exposure to chemical carcinogens and the consequent formation of DNA adducts may contribute to the further development of bladder cancers. Further work is required to identify the sources of this exposure, the host-mediated and environmental determinants of adduct levels, as well as the biological significance of these adducts in normal and tumor DNA.

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