

p53 Mutations in Cyclophosphamide-associated Bladder Cancer

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Abstract

Cyclophosphamide is a known bladder carcinogen, with cumulative dose directly related to increased risk. There is no consensus, however, on which major cyclophosphamide metabolite (*i.e.*, acrolein or phosphoramidate mustard) drives bladder carcinogenesis. We examined 19 cyclophosphamide-related bladder tumors to test the hypothesis that they might contain somatic mutations in the *p53* tumor suppressor gene that could link a specific metabolite to the etiology of these cancers. Forty-three % (9 of 19) of the cases had a mutation in *p53*, with a predominance at G:C bp (7 of 9, 77%), a preference for non-CpG sites (6 of 7, 86%), and frequent G:C→A:T transitions (5 of 7, 71%). The *p53* mutation spectrum of these cyclophosphamide-associated bladder cancers differed significantly from patterns reported for sporadic ($P = 0.020$), smoking-related (0.043), and schistosomiasis-linked ($P = 0.002$) tumors but not arylamine-associated neoplasms ($P = 0.860$). Differences between the cyclophosphamide and arylamine-associated spectra included an unusual degree of clustering of exon 6 mutations (43% versus 17%, respectively) and an absence of multiple mutations in the former. Notably lacking in our series were G:C→T:A transversions, the principal mutation associated with

acrolein. Instead, the mutation spectrum matches the phosphoramidate mustard adduction sequences determined by a repetitive primer-extension assay ($P = 0.024$), indicating that this metabolite might be a key mutagen in cyclophosphamide-related bladder cancer.

Introduction

Cyclophosphamide is a known bladder carcinogen with a highly significant relationship between cumulative dose and bladder cancer risk (1). This cytotoxic agent is prescribed to 500,000 patients each year worldwide (2), and the resulting bladder cancers may have both early onset and an aggressive course (3). Although three major, DNA-binding metabolites (phosphoramidate mustard, normitrogen mustard, and acrolein; Fig. 1A) result from the metabolism of cyclophosphamide by mixed function oxidases, there is no consensus on which product drives bladder carcinogenesis. Many investigators consider the primary mutagen to be phosphoramidate mustard (4-6), which forms monofunctional and bifunctional guanine adducts (7); normitrogen mustard may also play a role because it has similar chemical properties (Ref. 7; Fig. 1B). Acrolein is mutagenic in animal models (4, 5, 7, 8) and human cells *in vitro* (9, 10), but it is not a proven carcinogen, possibly because of its extreme toxicity (11). Clinical data correlating acrolein-induced hemorrhagic cystitis with subsequent bladder cancer is conflicting (12-14). Given the uncertainty over the role of acrolein as a bladder carcinogen, we evaluated a series of bladder cancers which followed cyclophosphamide therapy (1) to test the hypothesis that patterns of somatic mutations might link acrolein, phosphoramidate mustard, or normitrogen mustard to the etiology of these cancers. We tested for mutations in the *p53* tumor suppressor gene because it plays a prominent role in the development of bladder cancer (reviewed in Ref. 15) and because characteristic mutational spectra have been reported for two other bladder carcinogens, tobacco (16) and schistosomiasis (17).

Materials and Methods

Study Population. All available tissue samples were obtained from a case-control study of secondary bladder cancer (1) conducted within a cohort of 6171 2-year survivors of non-Hodgkin's lymphoma (18). In this analytic investigation, which represents the largest study to date of cyclophosphamide-associated bladder cancer, the relative risk associated with cumulative doses of cyclophosphamide <20, 20-50, and >50 g were 2.4, 6.3, and 14.5, respectively (P -trend = 0.004; Ref. 1). With the approval of local boards governing research on human subjects, resection or biopsy tissues were requested for all patients in the case-control study, with specimens available for 19 primary bladder cancers from 18 patients. The second bladder cancers occurred an average of 12 years after lymphoma diagnosis. Data on cumulative cyclophosphamide dose, radiation dose to bladder, interval between lymphoma and bladder cancer, and other pertinent clinical information for the 18 patients are summarized in Table 1. All subjects were treated

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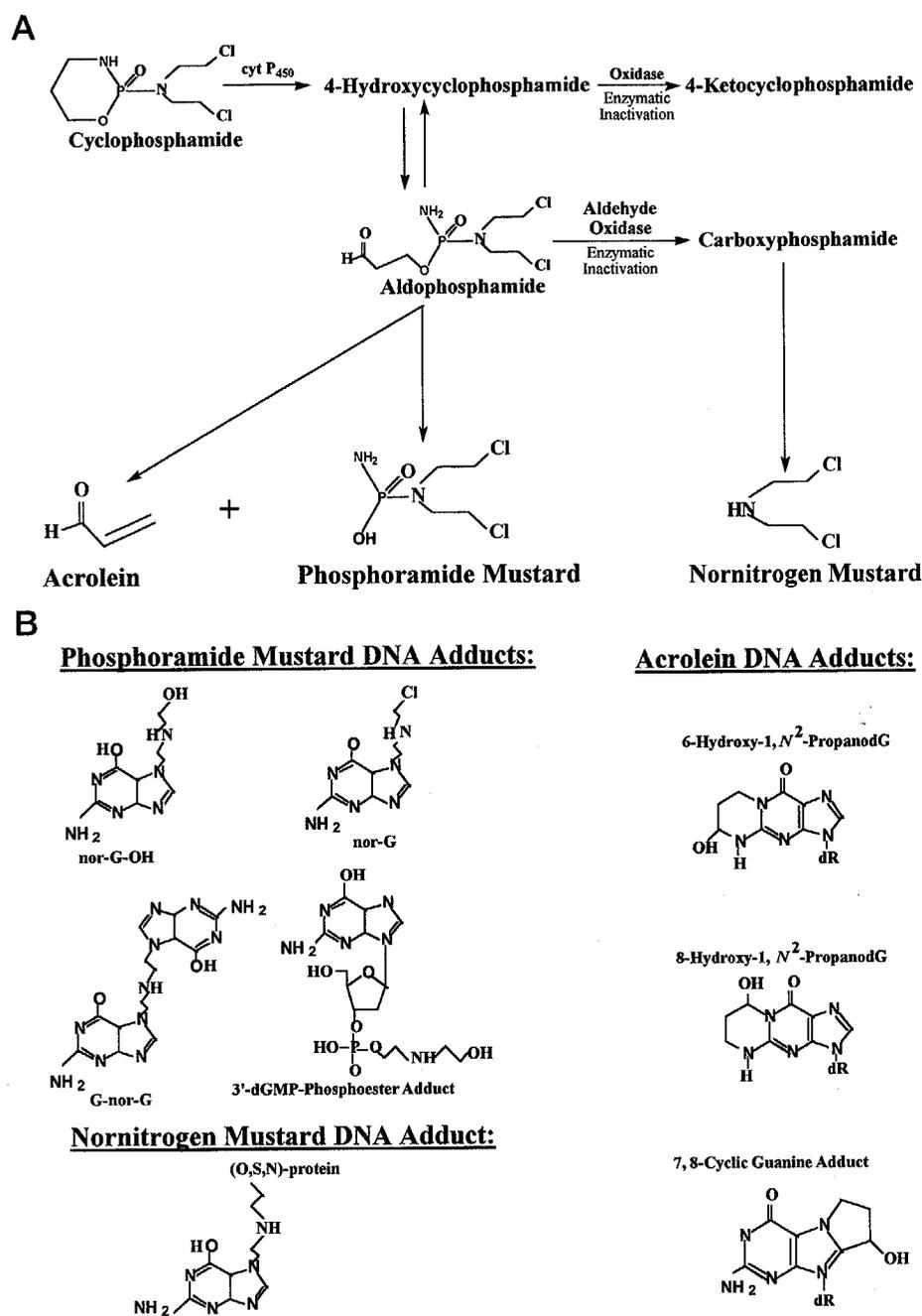


Fig. 1. A, metabolism of cyclophosphamide to phosphoramidate mustard, acrolein, and nornitrogen mustard. Cyclophosphamide is metabolized by cytochrome P450 enzymes to 4-hydroxycyclophosphamide, which equilibrates with aldophosphamide to spontaneously yield phosphoramidate mustard and acrolein. Aldophosphamide is also metabolized by aldehyde oxidase to carboxyphosphamide, which produces nornitrogen mustard. 4-Hydroxy-cyclophosphamide can be oxidized to the inactive 4-keto-cyclophosphamide. B, phosphoramidate mustard produces multiple monofunctional and bifunctional adducts with guanine, and acrolein forms exocyclic adducts. Nornitrogen mustard forms mono- and bifunctional adducts with guanine. Figure adapted from Anderson *et al.* (5) and Povirk and Shuker (7).

with cyclophosphamide (median cumulative dose, 16.6 g; range, 6–125.2 g), and some also received radiotherapy.

Genomic DNA Extraction, p53 PCR Amplification, and DNA Sequence Analysis. Tumor and nontumor tissues were microdissected from unstained, paraffin-embedded, formalin-fixed histological sections. After proteinase K digestion, genomic DNA was isolated by organic extraction as described (19). Exons 5–8 were amplified individually using nested primers, and each product was sequenced as reported previously (19); mutations were confirmed by finding the same base change in two independent PCR products.

Statistical Analyses. The overall type and distribution of mutations among the 570 bp in exons 5–8 of the p53 gene in the

cyclophosphamide-associated bladder cancers were compared with the mutational spectra observed in sporadic bladder cancers and those linked to tobacco use, schistosomiasis, and arylamine exposure using the procedure described by Cariello *et al.* (20). This method uses the Adams and Skopek algorithm to estimate *P* corresponding to an exact multivariate hypergeometric test for contingency tables (21). A random number generator simulates a large number of spectra based on the multivariate hypergeometric distribution, conditional on the numbers and locations of mutations in the two series to be compared.

The frequency of specific mutations within p53 exons was compared with the distribution reported for bladder cancers following exposure to other known carcinogens or to sporadic

Table 1 Secondary bladder cancer after cyclophosphamide therapy for non-Hodgkin's lymphoma: Patient summaries and p53 analyses

Patient no.	Age ^a (yr)/ Sex	Tobacco use ^b	Non-Hodgkin's lymphoma					Bladder cancer		p53 sequence analysis		
			NHL DX ^c (mo/yr)	NHL stage	All therapy	Begin-End (mo/yr)	Total dose of CTX ^d (g)	Total radiation dose to bladder (Gy)	Interval NHL to bladder cancer (yr)/histology	Codon:Mutation	Base change	CpG site
1	67/M	Ex-smoker	1/74	Unk	CVP	1/78-12/78	24.0	34.7	13.5/TCC	WT		
2	62/M	5 cigars/day	11/78	II	RT	11/77-12/77	13.5	33.8	5.5/TCC	WT		
					RT	12/78-1/79						
					CVP	1/79-1/79						
					CHVP	2/79-5/79						
					RT	5/79-2/80						
RT	3/80-3/80											
RT	5/80-5/80											
RT	10/80-10/80											
3	58/M	Ex-smoker, <1 PPD	6/77	II	RT	7/77-8/77	8.8	<0.1	10.0/TCC	WT		
4	64/F	None	3/69	III	CVP	1/78-6/78	34.0	6.5	14.2/Pap. TCC	WT		
RT	4/69-4/69											
5	61/M	Pipe-smoker, heavy	1/75	IV	CTX	10/69-10/71	10.3	19.5	6.1/TCC	230: ACC ^{thr} →ATC ^{ile}	G:C→A:T	No
					CVP	2/75-7/75						
					RT	1/75-1/75						
RT	8/75-9/75											
RT	10/75-11/75											
RT	12/75-1/76											
6	61/M	>2 PPD × 40 yr	12/70	III	RT	1/71-1/71	47.2	5.4	6.4/SqCC	279: GGG ^{gly} →AGG ^{arg}	G:C→A:T	No
					COPP	11/72-3/77						
					ABVD	3/77-4/77						
RT	5/76-6/76											
7	69/M	Ex-smoker, cigarette/pipe	7/78	Unk	BACOP	1/84-7/84	13.3	<0.1	7.2/Pap. TCC	WT		
					RT	1/79-1/79						
8	68/M	Ex-smoker, other tobacco	10/78	Unk	CTX	11/78-4/79	20.2	<0.1	3.9/Pap. TCC	241: TCC ^{ser} →ACC ^{thr}	A:T→T:A	NA
					COPP	4/79-5/80						
					RT	3/79-4/79						
9	65/M	Ex-smoker 1-2 PPD	3/78	I	CHOP	6/78-12/78	6.0	38.8	6.3/TCC	WT		
					RT	5/78-6/78						
10	46/M	>2 PPD	2/79	Unk	CHOP	3/80-11/80	56.0	(None)	5.9/TCC	261 ^f : AG→AA	G:C→A:T	No
					MEV-IMTX	11/80-5/82						
11	67/F	None	2/74	I	COPP	6/74-5/76	12.0	23.8	11.5/Pap. TCC	199: GGA ^{gly} →AGA ^{arg}	G:C→A:T	No
					RT	4/74-5/74						
12 ^f	61/M	Ex-pipe	7/84	IV	RT	7/84-7/84	15.3	0.9	3.4/Pap. TCC	WT		
					BACOP	7/84-1/85						
13 ^f	63/M	Cigars	1/79	III	COPP	2/79-8/79	49.6	17.3	12.3/Pap. TCC	193: CAT ^{his} →CGT ^{arg}	A:T→G:C	NA
					RT	8/79-9/79						
					COPP	2/80-4/84						
					CHOP	5/81-11/82						
14 ^f	56/F	1-2 PPD	4/81	IV	CHOP	12/78-9/79	18.0	(None)	8.7/Pap. TCC	WT		
15 ^f	38/M	1-2 PPD	12/78	IV	CHOP	12/78-9/79	125.2	(None)	10.8/TCC	WT		
					CTX	9/79-9/82						
					CHLB	9/82-3/83						
					CTX	3/83-1/85						
					VLB	1/85-12/85						
					MTX	12/85-1/90						
					CHOP	11/83-3/84						
16 ^{f,g}	52/M	2 PPD × 36 yr	10/83	II or IIIa	CHOP	11/83-3/84	8.3	(None)	6.6/Pap. TCC	196: CGA ^{arg} →CCA ^{pro}	G:C→C:G	Yes
17 ^f	67/F	None	6/81	II	CHOP	7/81-12/81	6.4	(None)	5.7/Pap. TCC	188: CTG ^{cu} →GTG ^{val}	G:C→C:G	No
18 ^f	61/M	1-2 PPD × 50 yr	8/79	IV	CHOP	8/79-12/79	30.5	(None)	12.8/Pap. TCC	135: TGC ^{cys} →TAC ^{tyr}	G:C→A:T	No
					CTX	12/79-4/82						

^a At diagnosis of non-Hodgkin's lymphoma.^b All histories refer to cigarette use unless noted otherwise.^c DX, diagnosis; ABVD, doxorubicin, bleomycin, vincristine, and dacarbazine; BACOP, cyclophosphamide, bleomycin, doxorubicin, vincristine, and prednisone; CDDP, cisplatin; CHLB, chlorambucil; CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisone; CHVP, cyclophosphamide, doxorubicin, teniposide, and prednisone; COPP, cyclophosphamide, vincristine, procarbazine, and prednisone; CTX, cyclophosphamide; CVP, cyclophosphamide, vincristine, and prednisone; IMTX, intrathecal methotrexate; MEV, cyclophosphamide, methotrexate, and vincristine; NA, not applicable; NHL, non-Hodgkin's lymphoma; Pap. TCC, papillary transitional cell carcinoma; PPD, pack(s) per day; RT, radiotherapy; SqCC, squamous cell carcinoma; TCC, transitional cell carcinoma; Unk, unknown; WT, wild type.^d Cumulative dose of cyclophosphamide.^e The mutation in Patient 10 occurred in the 5' splice site for exon 8 adjacent to the last nucleotide of codon 261.^f Case was not included in the prior analytic investigation (1) because strict eligibility criteria were not met or because the bladder cancer developed after the close of follow-up (December 31, 1989).^g Patient 16 had a second bladder cancer diagnosed 9.6 years after the NHL.

Table 2 p53 mutation spectra in human bladder cancers linked to cyclophosphamide therapy, occupational exposure to arylamine, schistosomiasis, smoking, or no known exposures (i.e., sporadic)

Exposure before bladder cancer	Mut. Freq. ^a (%)	n	P ^b	G:C→				A:T→			Del/Ins/Other	Mutations in exon 6	
				A:T (CpG)	A:T (non-CpG)	T:A	C:G	G:C	T:A	C:G		%	P ^c
Cyclophosphamide	49	9	NA	0	5	0	2	1	1	0	0	44	NA
Arylamine	47	27	0.860	0	17	3	1	4	1	0	1	17	0.300
Schistosomiasis	40	71	0.002	21	20	7	9	6	2	0	6	10	0.045
Tobacco	NA	81	0.043	9	32	9	14	3	1	5	7	10	0.044
Sporadic ^d	34	113	0.020	18	24	16	18	11	5	5	16	6	0.012

^a Mut. Freq., mutation frequency; NA, not applicable.

^b Comparison of the overall p53 mutation spectrum in cyclophosphamide-associated tumors with those observed in the indicated series. Data for sporadic, schistosomiasis, tobacco, and arylamine-associated bladder cancers were collected from the p53 mutation database (54) and compared with the cyclophosphamide spectrum by Monte Carlo analysis (Ref. 20; see "Materials and Methods" for additional description). Statistically significant ($P < 0.05$) differences were observed between the pattern of p53 mutations in cyclophosphamide-associated cases compared with sporadic, schistosomiasis, and tobacco-related tumors.

^c The cyclophosphamide-linked p53 mutations clustered in exon 6 (44%) were compared with other bladder cancer series, and the differences were statistically significant for all comparisons except arylamine (Fisher's exact test, two-tailed), with Ps denoted in this column. p53 mutations occurring in exons 5-8 in sporadic bladder cancers and those linked to schistosomiasis, tobacco, and arylamine were retrieved from the p53 mutation database (54).

^d The 113 mutations from "sporadic" tumors exclude all other groups.

cases by means of the two-tailed Fisher's exact test. An exact permutation test for trend (21) was conducted to test for a dose-response between cumulative amount of cyclophosphamide and p53 mutations. Exact binomial Ps were calculated to test whether a significant excess of mutations or adducts occurred within a selected class of nucleotides.

Results

p53 Mutations. Nine p53 mutations occurred in 8 of 18 patients with secondary bladder cancer. Patient 16 had two bladder cancers that were diagnosed 3 years apart and contained different p53 mutations; due to the time interval, disparate mutations, and established precedent (22, 23), these were considered as separate, metachronous tumors (i.e., not metastatic or recurrent lesions). The nine mutations consisted of five G:C→A:T transitions (all non-CpG sites), two G:C→C:G transversions (one CpG and one non-CpG site), one A:T→T:A transversion, and one A:T→G:C transition (Table 1). Distribution by exon was as follows: one (11%) in exon 5, 4 (45%) in exon 6, two (22%) in exon 7, and two (22%) in exon 8.

The Cyclophosphamide Mutation Spectrum Is Distinct among Bladder Cancers and Concentrates in Exon 6. The overall p53 mutation spectrum in cyclophosphamide-associated tumors differed significantly from those described in sporadic ($P = 0.020$), smoking-related ($P = 0.043$), and schistosomiasis-associated ($P = 0.002$) bladder cancer but not arylamine-linked cancers ($P = 0.86$). The predominant mutations in our series were G:C→A:T transitions occurring only at non-CpG sites (five of five), similar to mutations described for arylamine. In contrast, ratios of G:C>A:T transitions at non-CpG versus CpG sites for most other bladder cancer series varied from 1:1 (schistosomiasis) to 1:4 (tobacco).

Cyclophosphamide-associated bladder cancers differed markedly from those related to arylamine in the degree of mutational clustering in exon 6 and in the absence of multiple lesions. Four (44%) of the nine mutations in our study and three (43%) of the seven mutations at G:C bp occurred in exon 6, a disproportionate representation, given the small size of this exon (37 codons) compared with exons 5, 7, and 8 (142 codons). Moreover, the percentage of mutations in exon 6 in cyclophosphamide-associated tumors was considerably larger than the frequency (6-17%) reported for sporadic bladder cancers ($P = 0.012$) or those linked to tobacco ($P = 0.044$), schistosomiasis

(0.045), or arylamine ($P = 0.30$, see Table 2). Multiple p53 mutations, which were not observed in our series, were prominent in bladder malignancies after arylamine exposure (24).

Investigation of a Possible Dose-Response Relationship between Cyclophosphamide and p53 Mutation Rates. Mutations at G:C bp in non-CpG sites ($n = 6$) were categorized according to cyclophosphamide dose groups used in the analysis of the prior case-control study (1); 27% (3 of 11), 33% (2 of 6), and 50% (1 of 2) occurred in the <20-, 20-50-, and >50-g groups, respectively ($P = 0.40$). A similar pattern was observed when all p53 mutations were analyzed according to cumulative cyclophosphamide dose categories ($P = 0.31$).

Discussion

Cyclophosphamide Produces a Mutation Spectrum That Matches Phosphoramidate Mustard Adduction Sites. We provide the first description of p53 mutations in cyclophosphamide-related bladder cancers. The p53 mutation spectrum in these tumors is distinguished by a predominance of mutations at G:C bp, a preference for a non-CpG site, an excess of G:C>A:T transitions, and a clustering in exon 6. This unusual constellation of features differs significantly from sporadic, smoking-related, and schistosomiasis-linked bladder cancers, and the concentration of mutations in exon 6 differs markedly from bladder tumors in these series (Table 2). The character and location of mutations in cyclophosphamide-related bladder cancer match the distribution of phosphoramidate mustard adducts determined by a repetitive primer-extension assay (25). Both the mutations and the adducts have strong preferences for G:C bp at non-CpG dinucleotides, with significant excesses of p53 mutations and strong adduct sites in exon 6. Furthermore, three (43%) of seven mutations at G:C bp in our series occupied strong adduct sites. It is unlikely that this distribution occurred by chance alone ($P = 0.018$, binomial probability), because only 6.8% of G:C pairs are found at these locations. Taken together, our data suggest that phosphoramidate mustard may be an important mutagenic metabolite in cyclophosphamide-related bladder cancer.

Phosphoramidate Mustard Forms Guanine Adducts within Consensus Sequences. Phosphoramidate mustard and nonnitrogen mustard form adducts predominantly at the N⁷-position of guanine, occasionally at the N³ position of cytosine (reviewed in Refs. 5, 7, and 8), and rarely at adenine or thymidine (26, 27).

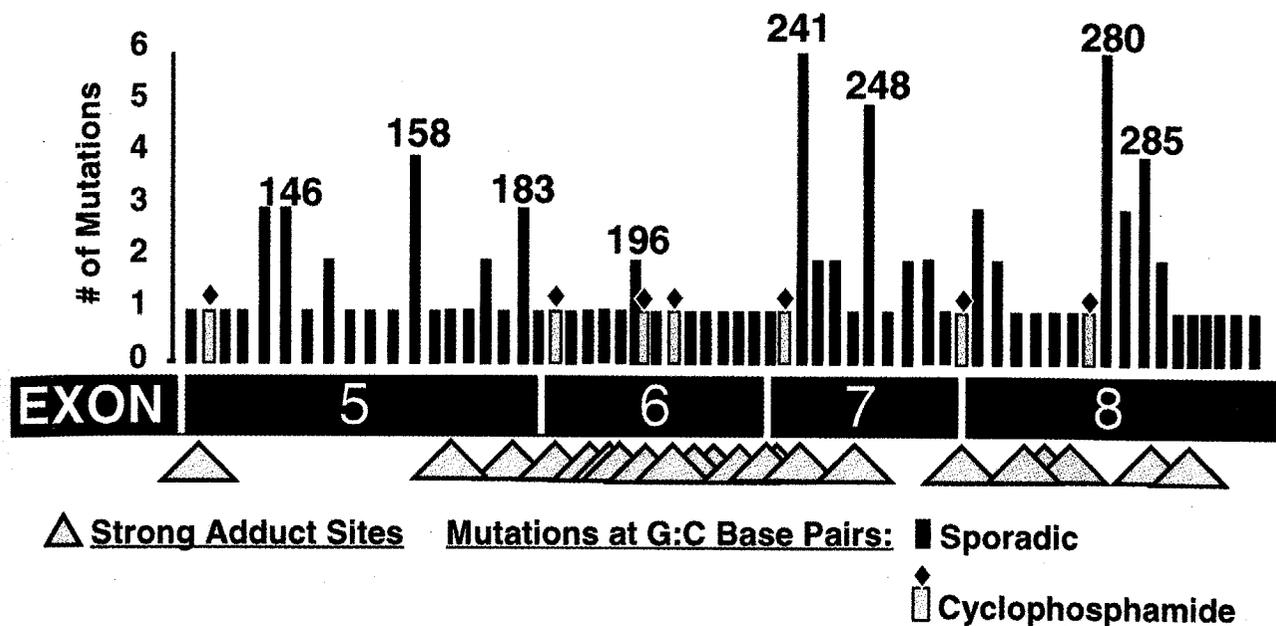


Fig. 2. Distribution of cyclophosphamide-induced *p53* mutations compared with mutations from sporadic tumors and adduction sites for phosphoramidate mustard. The *p53* coding sequences for exons 5–8 are represented by the horizontal bar. The cyclophosphamide-linked mutations (yellow columns with diamonds) and sporadic mutations (black columns) are shown. The locations of phosphoramidate mustard adducts were determined by a repetitive primer-extension analysis of human aliphoid DNA (25), and the consensus sequences for strong adducts are indicated by yellow triangles.

Consensus sequences flanking preferential guanine adduction sites were recently defined by a repetitive primer extension assay (25) used to map the location and intensity of DNA adducts in human cells. These experiments found phosphoramidate mustard adducts only at guanine residues, as reported previously (26, 27). There were variations in binding intensity among guanines, and consensus sequences for strong, intermediate, and weak adduction sites were defined; it is notable that adduction of guanines flanked by cytosine was rare (25). Because acrolein adducts are not detected by this assay (25), the adducts were produced either by phosphoramidate mustard or nornitrogen mustard. When these consensus sequences are compared with *p53* coding regions, there is a striking clustering of strong adduction sites in exon 6 ($n = 9$) compared with exon 8 ($n = 6$) or exons 5 and 7 ($n = 3$ each; Fig. 2). It is unlikely that 9 (43%) of 21 strong adduct sites occurred in exon 6 by chance ($P = 0.024$, binomial probability), because this exon contains only 19.4% of G:C sites. Nornitrogen mustard may also be a plausible mutagen, but we cannot assess the contribution of this metabolite until its associated adduct distribution has been determined.

Phosphoramidate Mustard Adducts Match Cyclophosphamide-induced *p53* Mutations. All major features of the phosphoramidate mustard adduct distribution are seen in the *p53* mutations, including a strong preference for non-CpG sites and a notable exon 6 clustering. In fact, cogent arguments link all seven mutations at G:C bp in our series to phosphoramidate mustard adducts. For example, all matched strong (3), intermediate (1), or weak (3) consensus adduction sequences and all but two of the mutation sites had at least 80% homology to the consensus adduction sequences (Ref. 25; Table 3). Five of seven mutations at G:C bp have a coding strand bias, which is characteristic of chemical carcinogens due to preferential repair of the transcribed strand (28, 29); it is notable that the two exceptions (*i.e.*, patients 5 and 16) occupy strong adduction

sites that may compensate for the rapid repair of the transcribed strand. Data for DNA repair rates within the *p53* coding sequences are incomplete, but three of the mutations (*i.e.*, patients 6, 13, and 16) occupy known slow spots for DNA repair, which is a predisposing factor for mutation (30).

DNA-adduct hotspots were previously linked with characteristic mutations in a report correlating the distribution of *p53* mutations in human lung cancer with the DNA adduct distribution of benzo(a)pyrene diol-epoxide, a carcinogen in tobacco smoke (31). Our study provides the first example describing homology of carcinogen-DNA adduct hotspots with the pattern of *p53* mutations in an iatrogenic cancer. There are no reports, to our knowledge, of the mutation spectrum generated by cyclophosphamide in animal or *in vitro* models.

Acrolein: Toxicity, Adducts, and Mutation Spectrum. Despite the established role of acrolein in hemorrhagic cystitis (14), there is insufficient evidence to classify it as a carcinogen (11). Three extended studies in animals have been negative (32, 33), including a 2-year ingestion study in rats (34). Although a recent investigation found initiating activity for bladder cancer when acrolein was administered intraperitoneally (35), complete carcinogenic activity could not be evaluated due to its extreme toxicity. Acrolein is considered mutagenic (9, 36–38), presumably due to the formation of DNA adducts; in aqueous solution, acrolein reacts with deoxyguanosine to produce two major isomers of the hydroxy-1,*N*²-propanodeoxyguanosine adduct and the 7,8-cyclic guanine adduct (Refs. 39–41; Fig. 1B). Because of the toxicity of acrolein (42), investigators have incorporated 1,*N*²-propanodeoxyguanosine² into shuttle vectors

² 1,*N*²-Propanodeoxyguanosine has been used as a model because the hydroxy-1,*N*²-propanodeoxyguanosine adduct is unstable under conditions used for oligodeoxynucleotide synthesis.

Table 3 Correlation of mutations, adduct sites, and DNA repair rates

Patient no. ^a	<i>p53</i> mutation				<i>p53</i> WT sequence ^c	Phosphoramidate mustard adducts ^d		DNA ^e repair rate
	Exon	Codon	Base change	CSB ^b		Site ^c	Intensity	
18	5	135	G:C→A:T	Yes	ttGcc ^f	ttGca	Weak	—
16	6	188	G:C→C:G	No	[caGac] ^{f,g}	caGac	Strong	—
13	6	193	A:T→G:C	Yes	NA	NA	NA	Slow ^h
16	6	196	G:C→C:G	Yes	ccGag	acGaa	Weak ⁱ	Slow
11	6	199	G:C→A:T	Yes	aaGgaaat	aaGgaGtt	Intermediate	—
5	7	230	G:C→A:T	No	[tgGta] ^g	aaGgtt	Strong	—
8	7	241	A:T→T:A	Yes	NA	NA	NA	—
10	8	Splice site	G:C→A:T	Yes	taGtg	taGtt	Weak	—
6	8	279	G:C→A:T	Yes	ctGgg	ctGag	Strong	Slow ^j

^a Ordered sequentially by codon number.

^b CSB, coding strand bias; NA, not applicable.

^c Sequence is given in the 5'→3' direction, and the capital "G" represents the sites of mutation or adduction.

^d 4-Hydroperoxycyclophosphamide (4-HC) adduction sites were taken from Bublely *et al.* (25); the 4-HC adduction sites are the same as those for phosphoramidate mustard (G. J. Bublely, personal communication).

^e DNA repair rates for mutated codons were reported by Tornaletti and Pfeifer (30). —, not available.

^f Mutations at codons 135 and 188 both match the GNC sequence pattern for interstrand cross-linking, where "N" is any nucleotide. For codon 135, the target guanine on the opposite strand has 80% homology with an intermediate adduction site; therefore, this location is favorable for mutation because it has both intermediate and weak adduction sites in a GNC pattern.

^g Brackets indicate the sequence of the non-coding strand which contains the guanine residue, the presumed site of adduction.

^h Although the mutated nucleotide is not a target for phosphoramidate mustard adduction, codon 193 is a slow spot for DNA repair.

ⁱ The codon 196 mutation in patient 16 has only 60% homology with a weak adduction site, but that could be plausibly compensated by slow repair (30).

^j Codon 279 is a potential mutation hotspot since it is a strong adduction site, and a slow spot for DNA repair is nearby in codon 278 (30).

(reviewed in Refs. 39 and 43) to evaluate acrolein-associated mutations. In both bacteria and mammalian cells, the predominant (75–90%) bp substitution is G:C→T:A transversions (44, 45), followed by G:C→A:T mutations (10–25% of total mutations). Although the absence of G:C→T:A transversions, the fingerprint of acrolein (44–46), in our series suggests a minimal role for this metabolite in cyclophosphamide-associated bladder cancer, further study in a larger number of cases is needed.

Summary and Implications. Our report provides the first correlation between the location of carcinogen-DNA adducts and a characteristic spectrum of *p53* mutations in cyclophosphamide-associated bladder cancer. These findings support a new paradigm (31, 47) for chemical carcinogenesis in which the distribution of DNA adducts can be linked with an observed pattern of mutations. Further descriptions of specific configurations of carcinogen-DNA adducts will facilitate the interpretation of complex *p53* mutation spectra, providing new insights into cancer etiology. Our findings suggest that phosphoramidate mustard may be the primary mutagenic metabolite of cyclophosphamide in iatrogenic bladder cancer. If confirmed in a larger series, these new results imply that chemoprotectant agents that bind nitrogen mustards (48) might be considered to ameliorate the risk of cyclophosphamide-associated bladder cancer. Amifostine, for example, has been shown to decrease mutagenesis by nitrogen mustard (49, 50), presumably by reducing mustard-induced DNA cross-links (51). In contrast, although available sulfhydryl compounds may prevent cyclophosphamide-related hemorrhagic cystitis by binding acrolein, their inability to enter most cell types likely makes them ineffective in blocking phosphoramidate mustard mutagenicity (reviewed in Ref. 52).

Further study of the pathogenesis of bladder cancer following well-characterized exposures, such as cyclophosphamide, tobacco, and schistosomiasis, will continue to provide valuable insights into underlying molecular mechanisms. In addition, because bladder cancer is now the fifth most common malignancy in the United States, with an estimated 54,500 cases expected to occur in 1997 (53), further understanding of these tumors is important for the development of prevention strategies.

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