

Global and Gene-specific Epigenetic Patterns in Human Bladder Cancer Genomes Are Relatively Stable *in Vivo* and *in Vitro* over Time¹

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ABSTRACT

We used a methylation-sensitive arbitrarily primed PCR technique to analyze, in a nonselective manner, methylation alterations at GC-rich regions of the genome in metachronous tumors and their derived cell lines from two patients with transitional cell carcinoma of the bladder. The methylation status of the majority of evaluable sequences (83%) remained unchanged in the tumors from both patients relative to a panel of normal urothelium samples obtained from individuals free of bladder disease, in which we measured <1% interindividual variation. The 17% of methylation alterations represents sequences altered in either a cancer-specific (3%), tumor-specific (1%), or patient-specific (13%) manner. The proportion of the altered sequences analyzed that were CpG islands corresponds to ~7000 CpG islands altered in the genome. Surprisingly, few additional changes in methylation patterns were observed in cell lines derived from the tumors; however, all of the cell lines showed altered methylation in a common set of 3% of evaluable sequences. Three genes known to be aberrantly methylated in bladder cancer (*p16*, *p15*, and *PAX6*) were studied in detail by methylation-sensitive single nucleotide primer extension and showed increased methylation in culture at pre-existing methylated sites for all of the exons but no *de novo* methylation in culture for the promoters in any cell line. Therefore, our investigation provides the first serial as well as parallel quantitation of the global epigenetic stability in two independent bladder cancer genomes over the course of progression and in culture. In addition, our investigation also provides the first direct comparison of the epigenetic and genetic patterns on the global scale, showing the epigenetic pattern to be relatively stable *in vivo* and *in vitro* over time within an individual.

INTRODUCTION

Analysis of cancer genomes has traditionally focused on one of three mechanisms for tumor suppressor inactivation: gain/loss of genetic information; mutation of existing genetic information; or alteration of epigenetic patterns by hypermethylation (gain of methylation) or hypomethylation (loss of methylation). Whereas there is widespread evidence to suggest that cancer genomes are genetically (1, 2) and epigenetically (3–5) unstable, no studies have examined the frequencies of occurrence of both abnormalities in a given human cancer on a global scale. This lack of data extends to studies in the same patient over the course of progression, because much of the current knowledge is based on “snapshot” analysis of different disease stages in different individuals.

Whereas there is a significant amount of information on the genetic status of several cancers including that of the bladder (6–9), much less is known about the epigenetic status or, more specifically, the DNA methylation pattern. Modification of CpG dinucleotides in gene promoters by methylation has been shown to inhibit transcription initiation (10–12) and lead to chromatin condensation (13). About 1% of the genome has the expected frequency of the CpG dinucleotides (14),

and these “islands” are defined by having a GC content >0.5, an O/E for the frequency of occurrence of CpG sites >0.6, and a minimum length of 200 bp (15). There are ~45,000 CpG islands in the human genome (14), which are typically unmethylated in somatic cells (10) and frequently but not always associated with the promoters of genes (10, 15–17).

Costello *et al.* (3) recently showed that alterations in methylation at CpG islands, as detected by restriction landmark genomic scanning (RLGS) (18) in 98 primary human tumors, were nonrandom and tumor-type specific. Muto *et al.* (19) demonstrated that 77% of normal urothelium samples from bladder cancer patients had either loss of heterozygosity at 1 of 10 microsatellite sites or methylation of the *p16* 5' region. This indicated that alterations had occurred in these patients early in tumorigenesis and before pathological detection. Even so, all of the data published to date represent static comparisons, and there have been no longitudinal studies on the global level that have looked at epigenetic variability inter- and intraindividually.

In this study, we investigated the epigenetic stability of genomes from two patients with transitional cell carcinoma of the bladder for which genetic information was also available from a previous study (20). Two metachronous tumors were obtained from each patient, in one case separated by 6 months and in the other case by 11 months, and cell lines were derived from each tumor for which analysis was performed at low and high passage (see Fig. 6 for the temporal and spatial relationships of the samples). These were compared with a panel of normal urothelium samples derived from patients free of bladder disease using global MS.AP-PCR³ and gene-specific MS.S-NuPE approaches. We were able to distinguish between alterations in methylation that were shared by all of the tumors and were therefore possibly common in bladder cancer (cancer-specific), those that were shared by only one tumor of each patient (tumor-specific), those that were unique to the tumor of a given patient (patient-specific), and those that occurred in culture. The data allowed us to determine DNA methylation patterns *in vivo*, for alterations in the two tumors, and *in vitro*, for progression from low- to high-passage cell lines.

The importance of our findings is 2-fold. This is the first concurrent serial and parallel quantitative estimation of the global epigenetic alterations in bladder cancer genomes. This is also the first direct global comparison of the epigenetic and genetic patterns showing the epigenetic pattern to be relatively stable *in vivo* and *in vitro* over time within an individual.

MATERIALS AND METHODS

Global Methylation Analysis: MS.AP-PCR

Normal urothelium was collected from five individuals free of bladder disease, whereas metachronous transitional cell carcinoma tumor tissues were collected from two bladder cancer patients at the Norris Comprehensive Cancer Center. In the case of the first bladder cancer patient (A), the first (A1) and second (A2) tumors were collected 6 months apart, whereas in the case of the second bladder cancer patient (B), the first (B1) and second (B2) tumors

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³ The abbreviations used are: MS.AP-PCR, methylation-sensitive arbitrarily primed PCR; MS.SNuPE, methylation-sensitive single nucleotide primer extension; O/E, observed/expected ratio.

were collected 11 months apart. Cell lines were derived and propagated from the tumor tissues as described previously (8) using Keratinocyte Medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated FCS (Omega Scientific Inc., Tarzana, CA) and 100 units/ml penicillin-streptomycin (Life Technologies, Inc., Rockville, MD). The sample set therefore comprised of: normal urothelium numbers 1–5; patient A with WBC, tumor A1, LD583 (cell line A1 derived from tumor A1) low and high passage, tumor A2, and LD611 (cell line derived from tumor A2) low and high passage; and patient B with WBC, tumor B1, LD660 (cell line derived from tumor B1) low and high passage, tumor B2, and LD692 (cell line derived from tumor B2) low and high passage. A brief of history of diagnosis and treatment for each patient has been published previously (20).

Genomic DNA was isolated using a simplified procedure developed previously (21, 22). After spectrophotometric determination of the concentration, 2 μ g of DNA was digested separately in a total volume of 40 μ l with either 20 units of *RsaI*, 20 units of *RsaI* and *HpaII*, or 10 units each of *RsaI* and *MspI* (Roche Molecular Biochemicals, Indianapolis, IN) at 37°C for 16 h. Another 2 units of each enzyme were added for an additional 1 h at 37°C to ensure complete digestion with the methylation-sensitive restriction endonucleases.

The arbitrarily primed PCR was conducted following the basic protocol described by Gonzalzo *et al.* (23). For each sample, 2 μ l of restriction enzyme-digested DNA equivalent to 100 ng was amplified using a combination of two or three randomly designed GC-rich primers (see Table 1) based on the principles of Welsh and McClelland (24). Conditions for amplification were: a primary denaturation step at 95°C for 5 min; then 95°C for 2 min, 40°C for 1 min, and 72°C for 2 min \times 35 cycles; and a final extension at 72°C for 8 min. The PCR mixture contained 1 \times buffer [10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, and 0.1% gelatin/ml], 200 μ M each of the four deoxynucleotide triphosphates (Roche Molecular Biochemicals), 0.5 μ M of each primer, 5% DMSO (Sigma-Aldrich), 1 unit of Taq polymerase (Roche Molecular Biochemicals), and 2 μ Ci of α^{32} P-labeled dATP (NEN Life Sciences Products Inc., Boston, MA) in a final volume of 25 μ l.

Table 1 Primers and primer sets for MSAP-PCR

A. Primers	
Primer	Primer sequence
G1	5'-GCG-CCG-ACG-T-3'
G2	5'-CGG-GAC-GCG-A-3'
G3	5'-CCG-CGA-TCG-C-3'
G4	5'-TGG-CCG-CCG-A-3'
G5	5'-TGC-GAC-GCC-G-3'
GC1	5'-GGG-CCG-CGG-C-3'
GC2	5'-CCC-CGC-GGG-G-3'
GC3	5'-CGC-GGG-GGC-G-3'
GC4	5'-GCG-CGC-CGC-G-3'
GC5	5'-GCG-GGG-CGG-C-3'
TIM1	5'-AGC-GGC-CGC-G-3'
TIM7	5'-GAG-GTG-CGC-G-3'
TIM10	5'-AGG-GGA-CGC-G-3'
TIM11	5'-GAG-AGG-CGC-G-3'
TIM12	5'-GCC-CCC-GCG-A-3'
TIM13	5'-CGG-GGC-GCG-A-3'
TIM17	5'-GGG-GAC-GCG-A-3'
TIM18	5'-ACC-CCA-CCC-G-3'
B. Primer sets	
Primer Set No.	Primer combinations
1	G2, G4, G5
2	GC1, GC4
3	GC1, GC5
4	GC2, GC5
5	TIM1, TIM7
6	TIM1, TIM18
7	TIM11, TIM12, TIM13
8	TIM13, TIM17
9	TIM1, TIM13
10	G1, G3, G4
11	G1, G4, G5
12	G1, G7, G8
13	G1, G6, G7
14	G1, G2, G3
15	G4, GC4, TIM10
16	G8, GC3, TIM17

The reaction mixture was then diluted with 8 μ l of formamide dye-loading buffer [95% formamide (EM Industries Inc., Hawthorne, NY), 20 mM EDTA (pH 8.0), 0.05% bromophenol blue (Bio-Rad Laboratories Inc., Hercules, CA), and 0.05% xylene cyanol FF (Sigma-Aldrich)], denatured at 90°C for 3 min and then immediately cooled on ice. For each sample, 2.5 μ l was loaded onto a high-resolution 5% polyacrylamide sequencing gel under denaturing conditions (7 M urea) for approximately 3–3.5 h at 121 W to maintain a running temperature of \sim 55°C. The gel was then dried at 80°C for 1 h and exposed to autoradiographic film overnight with an intensifying screen at -70°C .

Bands were scored visually by comparing the pattern among the three sets of digestions. Candidate bands were excised from the gel using the film as a marker and placed in a microfuge tube containing 50 μ l of sterile H₂O. The sample was then heated at 80°C for 10 min and vortexed to ensure dissolution of the DNA from the gel/paper slice. Two μ l of eluate was then used in a PCR with the same primers used in the original arbitrarily primed PCR, to amplify the given band and generate a sufficient template for plasmid cloning and sequencing. Conditions for amplification were: a primary denaturation step at 95°C for 2 min; then 95°C for 1 min, 49°C for 45 s, and 72°C for 1 min \times 35 cycles; and a final extension at 72°C for 10 min. The PCR mixture was the same as before except that no radiolabeled nucleotide was used, and the enzyme was a 1:1 combination of Taq polymerase (1 unit) and Taq Antibody (0.275 μ g; Clontech Laboratories Inc., Palo Alto, CA).

The PCR products were resolved on a 2% (w/v) agarose gel (Life Technologies, Inc.) with 0.5 μ g/ml ethidium bromide (Sigma-Aldrich) in 1 \times TAE buffer [0.04 M Tris-base, 0.004 M acetic acid, and 0.001 M EDTA (pH 8.0)] at 100 V to ensure appropriate size and then isolated using the Gel Extraction kit (Qiagen Inc., Valencia, CA) with a final volume of 30 μ l in sterile H₂O. This product was immediately cloned into a plasmid (pCR) vector using the TA cloning kit (Invitrogen Corporation, Carlsbad, CA). Five colonies for each sample were selected and dissolved in 500 μ l of sterile H₂O assisted with vortexing. Ten μ l of each suspension were then amplified by another round of PCR under these conditions: a primary denaturation step at 95°C for 2 min; then 95°C for 1 min, 58°C for 45 s, and 72°C for 1 min \times 35 cycles; and a final extension at 72°C for 4 min. The PCR mixture contained 1 \times buffer [10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, and 0.1% gelatin/ml], 200 μ M each of the four deoxynucleotide triphosphates, 0.5 μ M of each primer, 5% DMSO, and a 1:1 combination of Taq polymerase (1 unit) and Taq Antibody (0.275 μ g) in a final volume of 25 μ l.

The amplified product was divided into two aliquots, only one of which was further digested with 5 units of *HpaII* at 37°C for 1 h, and then both were run on a 2% agarose gel with 0.5 μ g/ml ethidium bromide in 1 \times TAE buffer at 100 V. This ensured that the cloned sequences contained the methylation-sensitive site, which was an internal experimental control. The sequence was isolated using the Gel Extraction kit and resuspended in 30 μ l of sterile H₂O.

Each sequence was then sent to the University of Southern California/Norris Comprehensive Cancer Center Microchemical Core Facility for automated DNA sequencing by a dye-based Applied Biosystems procedure where 3'-fluorescence-labeled dideoxynucleotide triphosphates are incorporated into DNA extension products (asymmetric PCR) and then excited during electrophoresis by laser light. Each sample contained 0.15 μ g of PCR product and 4.8 pmol of one vector-flanking primer (5' or 3') in a final volume of 18 μ l.

The profile of the sequence was then determined by two calculated values, GC content and O/E (15), defined in Fig. 3. After this, the sequence was inputted to the standard nucleotide-nucleotide BLAST databases [Nonredundant Sequence = GenBank + EMBL + DDBJ + PDB + Phase-3 finished human genomic sequences; Expressed Sequence Tag = GenBank + EMBL + DDBJ from EST Divisions; and High Throughput Genome Sequence = Phase 0/1/2 unfinished human genomic sequences databases]⁴ (25), the Human Genome Database (working draft human genomic sequences),⁵ and the RepeatMasker Web Server Sequencing⁶ to determine whether the sequences were part of known regions. In some cases, the sequences were identified as having putative promoter elements⁷ and/or CpG island DNA genomic *MseI* sequences (as originally submitted by A.P. Bird *et al.* c/o United Kingdom MRC Human Genome Mapping Project Resource Center).

⁴ <http://www.ncbi.nlm.nih.gov/blast>.

⁵ <http://www.ncbi.nlm.nih.gov/genome/seq>.

⁶ <http://ftp.genome.washington.edu/cgi-bin/RepeatMasker>.

⁷ <http://dot.imgen.bcm.tmc.edu:9331/seq-search/gene-search.html>.

To describe briefly the derivation of our estimate for CpG islands altered in the genome as a whole (reported in "Results"), we found that 26/60 of the sequenced bands were CpG islands and 16/60 had an altered methylation pattern. We found that 6/16 (38%) of the altered sequences were CpG islands and calculated that 6/26 (23%) of the total CpG islands in the sequenced set were altered. Going back to the larger set of sequences analyzed, our analysis showed that 37/214 (17%) were altered in either a cancer-, tumor-, or patient-specific manner (Fig. 2). But as stated already, 16/60 (27%) of the sequenced set showed an altered methylation pattern. This discrepancy in the percentages of altered sequences (17% versus 27%) allowed us to realize that there was indeed a bias in the subset of bands selected for sequencing, although it was toward those with an altered methylation pattern instead of those that were CpG islands.

To correct for this bias, we calculated the proportion of CpG islands in the remaining bands that were not sequenced using the ratio of CpG islands in the sequenced subset. Then, by summing that number of sequences we could estimate that there were ~94 CpG islands from the total 214 sequences analyzed and 14 of these that had an altered methylation pattern. Applying this proportion, 14/94, to the 45,000 CpG islands in the genome yields 6,750 or 7,000 (because there is only one significant digit in the approximation) CpG islands that are altered. Only a fraction of these sequences ($7/14 = 50\%$ of $7,000 = 3,500$), therefore, are CpG islands altered in a cancer-specific manner in the genome.

Gene-specific Methylation Analysis: MS.SNuPE

Three genes were analyzed by MS.SNuPE at their promoter regions and internal exons: *p16*, *p15*, and *PAX6* as described previously (26). Genomic DNA (2 μ g) was first treated with sodium bisulfite based on a protocol outlined by Frommer *et al.* (27) and resuspended in a total of 40 μ l of sterile H₂O.

***p15* Promoter.** Converted DNA (4 μ l) was used to amplify the promoter region in a PCR as described previously (28).

***p15* Exon 2.** Converted DNA (4 μ l) was used to amplify the exon 2 region in a PCR with these conditions: hotstart with a primary denaturation step at 95°C for 2 min; then 95°C for 1 min, 58°C for 45 s, and 72°C for 1 min 15 s \times 40 cycles; and a final extension at 72°C for 4 min. The PCR mixture contained 1 \times buffer [20 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM DTT, 0.1 M EDTA, 0.5% Tween 20 (v/v), 0.5% NP40 (v/v), and 50% gelatin/ml], 160 μ M each of the four deoxynucleotide triphosphates, 0.5 μ M of each primer, 2% DMSO, and 1.25 units Taq polymerase with 0.275 μ g Taq Antibody in a total volume of 25 μ l. The primers used were sense 5'-GGT-GAG-GGG-GTT-TTA-TAT-AAG-3' and antisense 5'-GAA-GGT-TTT-TTA-GGT-GAG-G-3'.

For the SNuPE reaction, 4 μ l of PCR product was subject to a single nucleotide primer extension through one cycle of 95°C for 2 min, 50°C for 2 min, and 72°C for 1 min. The SNuPE mixture contained 1 \times buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, and 0.001% gelatin], 1 μ M primers, 0.4 units Taq polymerase and 0.088 μ g Taq antibody (1:1), and 1 μ Ci of either α^{32} P-dCTP or α^{32} P-TTP in a total volume of 10 μ l. The primers used were (22mer) 5'-TAT-TAG-AGG-TAG-TAA-TTA-TGT-T-3', (17mer) 5'-GTT-ATG-ATG-ATG-GGT-AG-3', and (14mer) 5'-TGG-TGG-TGT-TGT-AT-3'.

***p16* Promoter.** Converted DNA (4 μ l) was used to amplify the promoter region in a PCR as described previously (29, 30).

***p16* Exon 2.** Converted DNA (4 μ l) was used to amplify the exon 2 region in a PCR as described previously (31).

***PAX6* Promoter.** Converted DNA (2 μ l) was used to amplify the promoter region in a PCR as described previously (32).

***PAX6* Exon 5.** Converted DNA (2 μ l) was used to amplify the exon 5 region in a PCR as described previously (33).

For all of the SNuPE samples, 6 μ l of formamide dye-loading buffer was added to each, and 2.5 μ l was loaded onto a high-resolution 15% polyacrylamide gel under denaturing conditions (7 M urea), which was run at 121 W for ~1 h to maintain a running temperature of ~55°C. The gel was then dried at 80°C for 1 h and exposed to a phosphorimager screen overnight. The exposure was scanned (PhosphorImager 445-SI Version 4.0; Molecular Dynamics, Sunnyvale, CA) and the degree of methylation quantitated using the ImageQuant version 5.0 software (Molecular Dynamics), correcting each data point

for background lane noise. The following calculation yielded percentage of methylation: % C = volume C/(volume C + volume T) \times 100.

RESULTS

Comparing Methylation Patterns by MS.AP-PCR. The MS.AP-PCR method, which was developed in our laboratory, provides a rapid and semiquantitative approach to mapping methylation changes in human cancers (26, 27, 33). In this study the technique was applied to two patients, who each had two separate tumors to assess the variation in methylation patterns in a serial as well as parallel manner among normal urothelium, individual tumors, and cell lines at low and high passage (see Fig. 6). The method also allowed us to determine how frequently methylation changes occur in a cancer-specific way (occurring in all four tumors), a tumor-specific way (occurring in only the first or second tumor of each patient), or a patient-specific way (occurring in one or both tumors of one patient but not in those of the other).

An example of an MS.AP-PCR gel is given in Fig. 1. The method relies on the predigestion of DNA with an enzyme to reduce the size of genomic sequences (in this case *RsaI*) before double digestion with either an enzyme sensitive to the methylation of the internal cytosine in the context of the sequence CCGG (*HpaII*) or digestion with an enzyme insensitive to the methylation of the internal cytosine residue in the same sequence (*MspI*). The three different digestions are then subjected to an arbitrarily primed PCR reaction with two or three GC-rich primers at a time for a total of 16 unique primer sets. The amplified regions are resolved on a 15% denaturing polyacrylamide gel and scored in one of four ways: (a) unevaluable, where a band is present in the *RsaI*, the *RsaI* + *HpaII*, and the *RsaI* + *MspI* digestions indicating the lack of an internal CCGG site; (b) methylated, where a

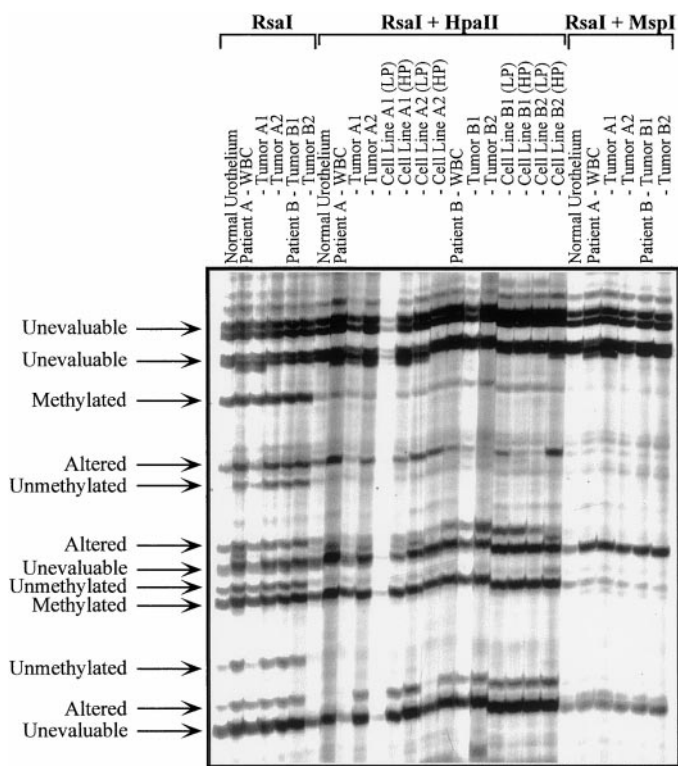


Fig. 1. Example gel for MS.AP-PCR. Portion of an MS.AP-PCR gel is shown to provide examples of scoring for altered, unmethylated in all samples, and methylated in all samples bands (referred to in Fig. 2). The samples are run in groups by digest: *RsaI*, *RsaI* + *HpaII*, and *RsaI* + *MspI*. The cell lines are labeled as low passage (LP) or high passage (HP).

band is present in the *RsaI* and *RsaI* + *HpaII* but absent or with much reduced intensity in the *RsaI* + *MspI* digestions; (c) unmethylated, where a band is present in the *RsaI* digestion only; and (d) altered, where a band is present in the *RsaI*, variably present in the *RsaI* + *HpaII*, and not present in the *MspI* digestion. Previous work has shown that if the bands are excised from the gel, cloned, sequenced, and subjected to quantitative methylation assays (MS.S-NuPE), the resulting level of methylation at the individual CCGG sites corresponds to the visual pattern seen by MS.AP-PCR (33).

A total of 214 sequences that were methylated in all of the samples, unmethylated in all of the samples, or altered in the tumors and/or cell lines in methylation pattern in the tumors and/or cell lines were scored for the two tumors from each of the patients as compared with normal urothelium. Whereas we were not able to obtain normal urothelium from the patients in this study, in separate experiments we have analyzed methylation changes among five different urothelium samples obtained from individuals without bladder cancer and observed very little variation between them (<1%; data not shown). This agrees with Behn-Krappa *et al.* (34) who found that the patterns of methylation in the WBCs of various individuals were indistinguishable among a set of human DNA sequences. Our finding also demonstrates the importance of using normal controls from individuals free of the tissue-specific disease, because alterations are now known to occur in the adjacent as well as target tissue before pathological detection (19). Therefore, the tumors were compared with this normal urothelium panel to determine how frequently methylation changes occurred during carcinogenesis of the bladder. Inevaluable sequences were not relevant to the analysis, and were subsequently not scored, because they yielded no information on the methylation pattern.

A summary of the methylation patterns is provided in Fig. 2. Results obtained from patient A showed that 89 sequences that were unmethylated in normal urothelium were unchanged in the tumors, whereas 106 of the total 214 evaluable sequences remained methylated in all of the samples (Fig. 2A). Thus, in patient A, 91% of the sequences did not change their methylation status as assessed by MS.AP-PCR. Hypermethylation events relative to normal urothelium occurred in 8 sequences and were observed in both tumors suggesting that the changes were either cancer- or patient-specific, whereas a total of 11 sequences were found to be either hyper- or hypomethylated in a tumor-specific way. Results obtained for patient B using the identical primer sets showed 83 sequences unmethylated in all of the samples and 101 sequences methylated in all of the samples, representing 86% of the total sequences scored. Of the 30 altered sequences, 19 sequences were methylated and 5 were unmethylated in both tumors, again representing potential cancer- or patient-specific changes. Of the remaining 6 sequences that were altered in a tumor-specific way, only 1 sequence represented a hypermethylation event.

Fig. 2B shows the methylation changes common to both patients, an analysis which was possible because of the highly reproducible banding pattern by each primer set in the MS.AP-PCR assay. The methylation patterns were compared on a band-by-band basis so that the 214 sequences analyzed were identical for both patients, allowing for a more accurate estimation than simply totaling or averaging the individual results. In this case, the number of unchanged regions in all of the tumors was 83% (77 of 214 sequences). There was a considerable overlap of 3% (7 of 214 sequences) of evaluable sequences that underwent methylation changes in all four tumors, which represented cancer-specific changes. This suggested that alterations in these sequences may play a key role in the genesis of bladder cancer. Also of importance is that the changes that were specific to an individual patient could be identified in this analysis and represented 13% (29 of 214) of evaluable sequences.

We next examined the sequence characteristics of 60 of the 214

A.						
PATTERN	NORMAL UROTHELIUM	TUMOR 1	TUMOR 2	PATIENT A	PATIENT B	
Unchanged	○	○	○	89	83	
	●	●	●	106	101	
Cancer or Patient-Specific	○	●	●	8	19	
	●	○	○	-	5	
Tumor-Specific	○	○	●	5	1	
	○	●	○	1	-	
	●	○	●	5	2	
	●	●	○	-	3	
TOTAL				214	214	

B.						
PATTERN	NORMAL UROTHELIUM	TUMOR A1	TUMOR A2	TUMOR B1	TUMOR B2	COMMON PATTERNS
Unchanged	○	○	○	○	○	81
	●	●	●	●	●	96
Cancer-Specific	○	●	●	●	●	7
	●	○	○	○	○	-
Tumor-Specific	○	○	●	○	●	1
	○	●	○	●	○	-
	●	○	●	○	●	-
	●	●	○	●	○	-
Patient-Specific	○	○	○	●	●	9
	○	○	○	●	●	3
	○	●	○	○	○	1
	○	●	●	○	○	1
	●	○	●	●	●	5
	●	●	●	○	○	4
	●	●	●	○	●	3
●	●	●	●	○	3	
TOTAL						214

Fig. 2. Results of MS.AP-PCR analyses for 214 sequences. A, the 214 sequences were scored as either methylated (●) or unmethylated (○), comparing Tumor 1 and 2 to the Normal Urothelium panel for patients A and B independently. They were classified by number of sequences into one of four categories: unchanged, cancer-specific (methylation change occurring in all four tumors), tumor-specific (methylation change occurring in only the first or second tumor of each patient), and patient-specific (methylation change occurring in one or both tumors of one patient but not the other). -, no sequences were observed for the respective category. B, common patterns analysis refers to a comparison of the methylation pattern of all 214 sequences between the normal urothelium and both tumors of both patients simultaneously. It was only in this situation that we could distinguish between cancer- or patient-specific changes.

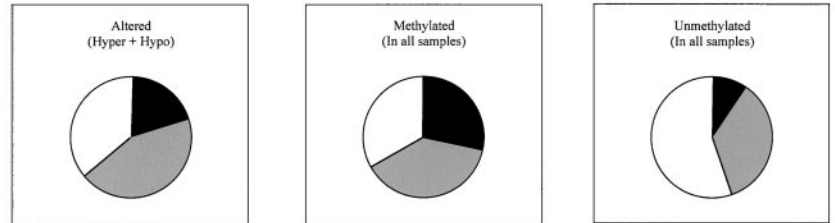
evaluated sequences and divided them into three categories depending on whether they were located within (or contained) CpG islands, non-CpG islands, or repetitive elements [which includes short interspersed nuclear elements (SINES) (Alu and mammalian-wide interspersed repeats (MIR), long interspersed nuclear elements (LINES) (LINE 1 and LINE 2), and long terminal repeats (LTR) but not simple repeats or microsatellite repeats; Fig. 3]. The size range of the sequences was 136–618 bp with only 2 sequences falling below 200 bp (at 179 and 136 bp) that were included in the analysis. Sequences are available on request.

Sequences with an altered methylation pattern were almost evenly distributed between CpG islands and non-CpG islands with fewer changes occurring in sequences located within repetitive elements (6, 7, and 3 sequences, respectively). The sequences that were methylated in all of the samples were relatively evenly distributed between the three sequence classes (7, 8, and 6 sequences), whereas a higher percentage of sequences that were unmethylated in all of the samples were located in non-CpG islands with considerably fewer falling into the repetitive element category (13, 8, and 2 sequences). A preponderance of unmethylated samples was therefore located in CpG islands, which was expected because these regions are known to be free of methylation in somatic tissues (16). They also showed that alterations in methylation occurred in all sequence classes of DNA and that, on average, the sequences identified were relatively GC-rich, with a GC content of 0.51 and an O/E of 0.64.

Table 2 shows the details of 32 sequences that matched to a

	ALTERED PATTERN		METHYLATED (In all samples)	UNMETHYLATED (In all samples)	TOTAL AVG
	Hypermethylated	Hypomethylated			
CpG ISLANDS	3	3	7	13	26
GC Content	0.56	0.55	0.52	0.55	0.54
Obs/Exp Ratio	0.73	0.78	0.78	0.72	0.74
NON-CpG ISLANDS	2	5	8	8	23
GC Content	0.43	0.49	0.51	0.50	0.50
Obs/Exp Ratio	0.92	0.62	0.58	0.46	0.58
REPETITIVE ELEMENTS	0	3	6	2	11
GC Content	-	0.47	0.50	0.52	0.50
Obs/Exp Ratio	-	0.58	0.53	0.61	0.56
TOTAL BANDS	5	11	21	23	60
Average GC Content	0.51	0.50	0.51	0.52	0.51
Average Obs/Exp Ratio	0.81	0.65	0.63	0.60	0.64
	Hypermethylated + Hypomethylated				
	16				
	0.50				
	0.65				

Fig. 3. Sequence profile of 60 sequences identified by MS.AP-PCR. Each of the 60 sequences sequenced fit into one of three categories with respect to the methylation pattern comparing normal urothelium to the tumors and cell lines: altered (hyper- or hypomethylated), methylated in all samples, or unmethylated in all samples. The resulting sequences were then divided into three types: CpG islands, repetitive elements, and non-CpG islands. For each of these divisions, the number of sequences, GC Content [(number of C bases + number of G bases)/sequence length], and Obs/Exp or O/E {[number of CpG sites/(number of C bases × number of G bases)] × sequence length} are listed and then averaged with respect to the category, type, and overall. Note that repetitive elements include SINEs (Alu, MIR), LINEs (LINE1, LINE 2), and long terminal repeat elements but not simple repeats or microsatellite repeats.



chromosome in the genome database. The sequences are listed in three groups based on whether the methylation pattern was methylated in all of the samples, unmethylated in all of the samples, or altered in the tumors and/or cell lines. The details included are GC content, O/E, chromosome match, ID or Accession No., clone name, sequence status, and region of base matches (with the percentage match). These sequences were part of the set of 60 analyzed in Fig. 3.

Because the bands we chose to sequence were not randomly selected, we based the validity of an extrapolation to the genome as a whole on the fact that we found the percentage of CpG islands in the whole sequenced band set ($26/60 = 43\%$) to be similar to the percentage of CpG islands in the altered sequenced band set only ($6/16 = 38\%$). That is to say, whereas we knowingly selected the sequences with methylation patterns as methylated in all of the samples, unmethylated in all of the samples, or altered, we did so blindly in regard to which of these sequences were actually CpG islands. Thus, we were able to calculate that ~ 7000 CpG islands in the genome are altered in bladder cancer with ~ 3500 altered in a cancer-specific manner (See “Materials and Methods” for an explanation of the derivation of this estimate).

Few Additional Methylation Changes Occur in Cell Culture. It is well known that normal tissues undergo considerable changes in methylation patterns in processes associated with adaptation to culture (13, 35). The four tumors studied in this report easily adapted to culture so that we were able to determine how methylation patterns of bladder cancers as opposed to normal tissues evolved *in vitro*. Low and high passages were analyzed for each cell line and compared with the respective tumor by MS.AP-PCR. The results of these studies (data not shown) indicated that an additional 10% (21 of 214 sequences) of methylation changes occurred with the culturing of tumors 1 and 2 from patient A, whereas 6% (13 of 214 sequences) of methylation changes occurred in the case of patient B. The details of this analysis (the distribution of hyper- versus hypomethylated sequences) are shown in Fig. 6, which also contains data published previously on the genetic changes present in the cell lines (20).

When the results for the cell lines were compared with the normal urothelium and tumors concurrently, there were only 3% (7 of 214 sequences) of cell line-specific methylation changes in common be-

tween the patients (Fig. 4). The majority of changes (28/35) involved alterations in the methylation pattern of sequences in one patient but not the other and were therefore labeled “discrepant.” This result was surprising, because it had been anticipated that the methylation changes in culture would be much more extensive. However, the data clearly show that there is no convergence toward a relatively uniform methylation pattern *in vitro* because of a subset of sequences that is susceptible to proliferation-based hypermethylation.

We subsequently used the quantitative MS.SNuPE method, devised in this laboratory (26), to determine how methylation changed in culture at defined CpG sites in regions of DNA known to be abnormally methylated in human cancers. The levels of methylation within the *p16*, *p15*, and *PAX6* genes in the promoters and one exon of each were therefore assessed at three individual sites within each of the tumors and late passage cell lines. Fig. 5 shows that in patient B the levels of methylation in the exonic regions of all three genes increased as a function of time in culture for all of the specimens examined, although there were no discernable alterations in the level of methylation of the respective promoters, which remained unmethylated. We were unable to measure the methylation of the *p15* and *p16* genes in patient A, because this region had been homozygously deleted within the tumor (8). However, for patient A, the *PAX6* gene showed no methylation of the promoter region, although methylation of the exonic region approached 100% in those lines that had been cultured for an extended period of time. Thus, we observed no *de novo* methylation of regions that were unmethylated in the tumor but rather an apparent enhancement of existing methylation patterns within these three loci.

DISCUSSION

Methylation Patterns *in Vivo*. Our study provides the first quantitative estimation of genome-wide methylation changes that occurred in two patients with transitional cell carcinoma of the bladder, comparing metachronous tumors within a patient and between patients. This provided an epigenotype from which cancer-specific, tumor-specific, and patient-specific alterations in methylation could be identified. The MS.AP-PCR method used in this study scans the genomes

Table 2 Details of MS.AP-PCR sequences that matched to chromosomes

Band	Methylation pattern						GC ^a	O/E	CHR	ID or accession no.	Clone name	Status	Base matches to human genome sequence
	NU	T1	T2	EP	LP	LP							
M1-6B	●	●	●	●	●	●	0.62	0.82	3	AC064795	RP11-785G3	WDS	52405–52083 (100%)
M4-1D	●	●	●	●	●	●	0.63	1.01	3	NT_005678.1		WDS	1013324–1013002 (100%)
M12-11A	●	●	●	●	●	●	0.62	0.87	10q26.3	AC006171	LA10NC01_15_3_11	SIP	33600–33430 (100%)
										Putative promoter element			
									1	AC026931	RP11-180L13	WDS	12846–12557 (100%)
									4q22–24	AP002822	191E02	SIP	65013–65302 (100%)
									4	NT_022760.1		WDS	575424–575131 (95%)
M2-3C	●	●	●	●	●	●	0.61	0.31	18	AC013563	RP11-315M18	WDS	40759–40979 (100%), 41003–41174 (99%)
									18	AC018445	RP11-196B3	WDS	133492–133272 (100%), 133248–133077
									18	AC044917	RP11-467N12	WDS	49964–50184 (100%), 50208–50379 (99%)
									18	AP001908	RP11-820P14	WDS	29221–29001 (100%), 28977–28806 (100%)
									18	NT_010879.1		WDS	264292–264707 (100%), 37561–37146 (99%)
M2-6C	●	●	●	●	●	●	0.48	0.37	5	AC025455	CTD-2195L15	WDS	53113–53492 (100%)
									5	AC018420	RP11-18K15	WDS	34692–34313 (100%)
M7-4D	●	●	●	●	●	●	0.40	1.15	13	AL139035	RP11-214F16	SIP	102482–102743 (100%)
									13	AL359748	RP11-158C4	SIP	146082–146343 (100%)
									13	NT_024511.1		WDS	21275–21014 (100%)
M8-2C	●	●	●	●	●	●	0.44	0.49		CpG island DNA genomic MseI fragment			
									?	AC016405	RP11-44N11	WDS	14620–14872 (100%)
									8	AC084708	RP11-784F18	LPSS	40021–39912 (97%)
									8	NT_008295.1		WDS	146641–146893 (100%)
M12-8E	●	●	●	●	●	●	0.62	0.38		Putative promoter element			
									16	AC009093	RP11-426C22	WDS	90009–90243 (100%), 89770–89949 (100%), 89890–89949 (100%)
									16	AC007615	RP11-528K16	WDS	120026–120260 (100%), 119787–119966 (100%), 119907–119966 (100%)
									16	AC009130	RP11-501P17	WDS	166904–166670 (100%), 167144–166966 (99%), 167024–166966 (100%)
									16	NT_024782.1		WDS	190714–190948 (100%), 190475–190654 (100%), 190595–190654 (100%)
M12-13E	●	●	●	●	●	●	0.51	0.59	17	AC012333	RP11-881D9	SIP	84795–84527 (99%)
									17	NT_024874.1		WDS	512484–512216 (99%)
M9-4D	●	●	●	●	●	●	0.45	0.61	1	NT_004706.1		WDS	455281–455639 (85%), 471546–471829 (84%)
M10-3D	●	●	●	●	●	●	0.57	0.49	16q24.3	AC074302	RP4-597G12	WDS	45422–45770 (87%)
									16	NT_010542.1		WDS	47878–47530 (87%)
U1-2A	○	○	○	○	○	○	0.54	0.74		Putative promoter element			
									4	AC058794	RP11-41F9	WDS	53026–52581 (100%)
									4	AC040966	RP11-182A9	WDS	94615–94170 (100%)
									4	AC024664	RP11-597P9	WDS	148013–148458 (100%)
									4	NT_006169.1		WDS	958272–957827 (100%)
U3-2C	○	○	○	○	○	○	0.54	0.86		CpG island DNA genomic MseI fragment			
									6	AL357077	RP11-579C5	WDS	142077–141694 (100%), 65490–65750 (93%)
									6	NT_007291.1		WDS	413199–413582 (100%), 384774–385034 (93%)
U4-2A	○	○	○	○	○	○	0.55	0.72	10	AL135794	RP11-324L3	SIP	29654–29327 (100%)
									10	NT_008874.1		WDS	450794–451121 (100%)
U5-5A	○	○	○	○	○	○	0.58	0.79	5	AC025758	CTD-2235A13	WDS	42064–42471 (100%)
									5	AC008810	CTD-2096I23	WDS	107002–106595 (100%)
									5	NT_023179.1		WDS	193492–193085 (100%)
U7-3E	○	○	○	○	○	○	0.59	0.91	11q13	AP000449	XXp1-24A2	WDS	4601–4261 (100%)
									11	AC051660	RP11-399J13	WDS	138469–138809 (99%)
									11	AP003068	RP11-399J13	WDS	19806–19466 (99%)
									11	AJ02553	1118I22	SIP	37952–38293 (99%)
									11	NT_009379.1		WDS	2540070–2540410 (99%)
U8-3C	○	○	○	○	○	○	0.60	0.69	12	AC025031	RP11-474P2	WDS	158798–159216 (100%)
									12	AC021353	RP11-24021	WDS	128713–128295 (100%), 127188–127688 (89%)
									12	NT_009781.1		WDS	830781–831199 (100%)
U9-5C	○	○	○	○	○	○	0.62	0.86		CpG island DNA genomic MseI fragment			
									13	AL161775	RP11-264M3	SIP	79089–78590 (99%)
									13	NT_024504.1		WDS	188935–189434 (100%)
U4-1B	○	○	○	○	○	○	0.62	0.32	17	AC069487	RP11-651P17	WDS	144952–145391 (100%)
									17	NT_024969.1		WDS	15694–16133 (100%)
									22	NT_011525.1		WDS	398572–398133 (100%)
U6-1A	○	○	○	○	○	○	0.61	0.41	17	AC019152	RP11-812N9	WDS	41199–40757 (100%)
									17	AC019151	RP11-811N11	SIP	80939–80497 (99%)
									17	NT_010755.1		WDS	952636–952194 (100%)
U10-2C	○	○	○	○	○	○	0.64	0.36	7	NT_007707.1		WDS	138351–138773 (99%)
U13-6A	○	○	○	○	○	○	0.64	0.49		Putative promoter element			
									19	NT_011268.1		WDS	145167–144665 (100%)
U9-6D	○	○	○	○	○	○	0.63	0.39	12q	AC079408	RP11-60114	WDS	3818–3386 (97%)
									12	NT_024388.1		WDS	91318–91750 (97%)
U7-4A	○	○	○	○	○	○	0.54	0.44	2	AC027597	RP11-394A2	WDS	79034–78733 (98%)
									2	NT_005062.1		WDS	53463–53764 (98%)

^a GC = GC content; CHR = chromosome; WDS = working draft sequence; SIP = sequencing in progress; LPSS = low-pass sequencing sample.

Table 2 *Continued*

Band	Methylation pattern				GC ^a	O/E	CHR	ID or accession no.	Clone name	Status	Base matches to human genome sequence
	CL1 NU	CL1 T1	CL2 EP	CL2 LP							
U2-8E	○●●●●●●●	0.59	0.72				CASPR (contactin associated protein)				
							? AC016279	RP11-20N1	LPSS	64837-64988 (100%)	
							2 AC027146	RP11-123N2	WDS	141426-141577 (100%)	
							17 NT_010771.1		WDS	56096-56247 (100%)	
M1-8C	●●●○●●●○	0.67	1.07				11 AC025972	RP11-6K5	WDS	159539-159352 (100%)	
							11 NT_009003.1		WDS	943117-943304 (100%)	
										33610-33360 (100%), 1178636-1178194 (99%)	
M12-4E	●●●○●●●○	0.61	0.85	10			AC006171	LA10NC01_15_E_11	SIP		
M12-10a	●●●○●●●○	0.52	0.80	7			NT_007784.1		WDS	385976-386269 (100%)	
U12-1A	○●●●●●●○	0.58	0.79				CpG island DNA genomic MseI fragment				
							2 AC062014	RP11-477F18	WDS	78533-78236 (100%)	
							2 AC080187	RP11-554I7	WDS	126709-127002 (94%)	
							2 NT_019306.1		WDS	265456-265753 (100%)	
U1-5A	○●●●●●●●	0.45	0.92	1			AC025691	RP11-12H8	WDS	28157-27780 (100%)	
							1 AL356853	RP11-430G17	SIP	140689-140312 (100%)	
							1 NT_021923.1		WDS	238677-239054 (100%)	
M1-4P	●●●○●●●○	0.49	0.28	7			AC021218	RP11-52501	WDS	72699-72339 (100%)	
							7 NT_007905.1		WDS	58550-58190 (100%)	
M2-8M	●○●●●○●○	0.72	0.56				Putative promoter element				
							6 AL356123	RP11-351J23	SIP	32838-33023 (100%)	
							6 NT_007620.1		WDS	28379-28564 (100%), 174162-174347 (100%)	

^a GC = GC content; CHR = chromosome; WDS = working draft sequence; SIP = sequencing in progress; LPSS = low-pass sequencing sample.

globally and depends on methylation-sensitive digestion coupled with a “randomly” primed PCR. The only known bias of this method is the preference for GC-rich sequences based on the primers being composed of only C and G bases in random order and in different combinations. Other recently published studies have used a variety of alternative global analysis methods, including restriction landmark genomic scanning (3), differential methylation hybridization (36), methylated CpG island amplification (37), and methylation-sensitive representational difference analysis (38). The MS.AP-PCR method was selected because it is a quick, simple, and robust method that has been validated in our laboratory and used in previous studies for similar analyses (33).

It has been shown previously that methylation patterns are tissue-specific and show little interindividual variation (34). Because our study focused on a sample set derived from the bladder, we obtained a panel of five normal urothelium controls from individuals known to be pathologically free of bladder disease. Of the 214 examined regions, we found <1% variation between five individuals (data not shown). This was critical, because it substantiated earlier work that showed that variations in the methylation pattern between individuals are minimal (34) and allowed a more accurate quantitation of the differences in methylation pattern measured in this study by giving us a margin of error of <1% when comparing data between patients.

Our data indicate that there were 17% (37/214) differences in methylation pattern of the bladder cancer genomes of the two patients,

of which 14 were located in CpG islands. Through the calculations explained in “Results,” this corresponds with ~7,000 of the estimated 45,000 CpG islands in the human genome (14). About 3,500 of these CpG islands represent bladder cancer-specific changes, because these sequences were altered in the genomes of both tumors from both patients. Costello *et al.* (3) suggested that 0–4,500 of 45,000 CpG islands (with an average of 600 actually related to the cancer) in the genome were aberrantly methylated between the tumors and matched normal controls of 98 primary human tumors from six types of cancer not including those of the bladder. By comparison, we estimated that 7,000 *versus* 0–4,500 CpG islands are altered in the genome, and 3,500 *versus* 600 CpG islands are altered in a cancer-specific manner. Costello *et al.* (3) compared separate tumors in independent patients from a variety of malignancies and then provided an average of all of them, which may explain why our values are somewhat different from theirs, as our study focused on a smaller sample set and only bladder cancer.

The nature of the sample set in this study allows us to also address the effect of chemotherapy on global methylation levels. Because most patients receive some treatment after removal of a tumor(s), it is a complication that is poorly understood but biologically relevant. The two patients analyzed here both received an adjuvant methotrexate vinblastine Adriamycin cisplatin (MVAC) cycle after surgical resection of the first tumor. For patient A, additional chemotherapy regimens were applied when the second tumor was noted. However, the

PATTERN		TUMOR	CELL LINE	PATIENT A	PATIENT B	COMMON PATTERNS
Unchanged	Unmethylated	○	○	104	116	98
	Methylated	●	●	89	85	81
Altered	Hypomethylated	●	○	11	8	6
	Hypermethylated	○	●	10	5	1
Discrepant	Changed in Patient A, Not in Patient B	-	-	-	-	18
	Changed in Patient B, Not in Patient A	-	-	-	-	10
TOTAL				214	214	214

Fig. 4. Methylation patterns in cell lines. The methylation patterns in cell lines were compiled for each patient individually and then analyzed for those in common by comparing low and high passage cell lines (*Cell Line*) to the respective tumor (*Tumor*) from which it had been cultured. The results are classified as either unchanged (*Unmethylated* or *Methylated* in all of the samples), altered (*Hypomethylated* or *Hypermethylated* in the cell line samples), or discrepant (where the methylation pattern was unchanged in one patient and altered in the other patient). As in Fig. 2, methylated is indicated by ●, whereas unmethylated is indicated by ○. -, no sequences were observed for the respective methylation pattern or no consistent methylation pattern found between the patients.

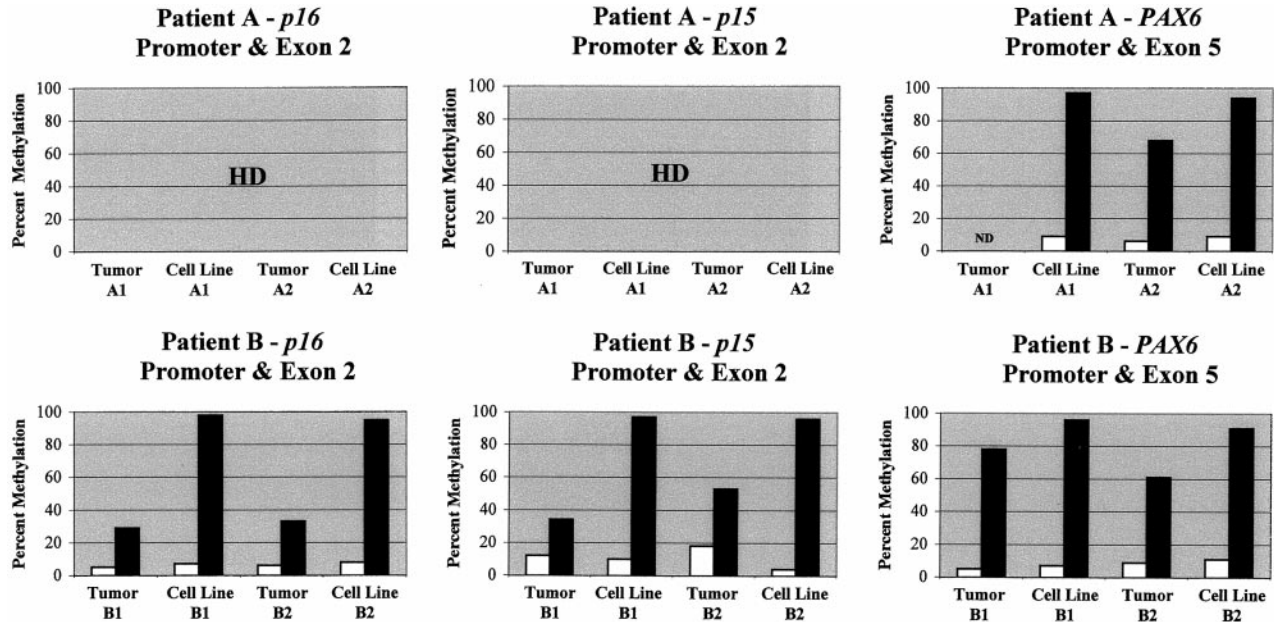


Fig. 5. Results of MS.SNuPE analyses for three genes. *p16*, *p15*, and *PAX6* were analyzed by MS.SNuPE assay in the tumors (Tumor 1/2) and cell lines (Cell Line 1/2) to quantitate the methylation at three individual CpG sites each within the promoter and exon of each gene. The results are shown as percentage methylation averaged for the three sites, which were all done in duplicate. □, values for promoter; ■, values for exons. There is no data for patient A with respect to *p16* and *p15* because there is a homozygous deletion (*HD*) in the region of these genes (8). Note that cell lines are all high passage.

patient failed to respond, and the second tumor was then surgically removed. For patient B, the recurrence of transitional cell carcinoma was followed by expiration shortly after the second tumor was removed but before follow-up chemotherapy was implemented. The data, then, supports the notion that for these patients chemotherapy did not seem to result in widespread epigenetic changes, although it remains impossible to distinguish between which methylation changes that did occur were attributable to the initial chemotherapy, disease progression, and/or aging.

By focusing on patients from which we had obtained metachronous tumors of one type of cancer, the estimation of our study allowed differentiation in the number of alterations between normal and tumor (4% and 11%, patient A and B), between patients (13%), and between tumors (5% and 3%, patient A and B). In addition, our data reflect possible alterations associated with progression, although the precise temporal order for the evolution of the metachronous tumors in patient B remains uncertain because they are both metastatic. To our knowledge, this has never been done before, and we conclude that tumor genomes within individual patients have more in common than those observed between different patients.

Methylation Patterns *in Vitro*. The extent of methylation changes attributable to the process of culturing tumor cells as a function of time was also of interest to us, because many studies use cell lines to investigate the range of epigenetic changes present in cancers. It was shown previously that normal cells undergo a large number of methylation changes in response to being cultured (13, 35), and this has brought into question the validity of using tumor cell lines as models of methylation pattern alterations in cancer. Relative to the tumors, patient A showed 10% additional differences in methylation pattern in the respective cell lines, and patient B showed 6%, whereas only 3% of these changes were in common between the patients (data not shown). This supports the notion that the majority of the methylation changes occurred in the tumor, with few additional alterations accumulating in the cell line as culture artifacts. The observation is also interesting, because patient A had less methylation changes than patient B when comparing the normal urothelium panel and the

tumors, specifically, 4% for patient A and 11% for patient B (Fig. 6). The overall variation between patients therefore was ~2-fold larger than the overall variation attributable to the culture process, 7% (11–4%) *versus* 4% (10–6%), respectively. These data provide for the first time a quantitation of the level of methylation in culture of human bladder tumors and argues that cell lines can be used with caution as relevant models for cancer epigenetic studies.

A recent report by Ross *et al.* (39) supports this finding in concept through a study of the gene expression of ~8,000 unique genes in 60 cancer cell lines using cDNA microarrays. Their results showed a consistent relationship between the gene expression patterns in the cell lines and the tissue of origin. The authors were able to determine specific features as “related to physiological properties of the cell lines” and concluded that for the most part “neither physiological nor experimental adaptation for growth in culture was sufficient to overwrite the gene expression patterns established during differentiation *in vivo*.”

Another observation related to this issue is that increased methylation was observed in the cell lines at sites with preexisting methylation in the genes examined in the tumors. The sites that became methylated correspond to the internal exons and not to the respective promoters of genes shown to become methylated with an increased frequency in tumors and in culture (*p16*, *p15*, and *PAX6*; Refs. 28–33). Methylation patterns seen in cultured tumors may, therefore, be a result of an enhancement of existing patterns as opposed to extensive *de novo* methylation of previously unmethylated CpG islands.

Several observations can be made when comparing these data to that of a previous study (20) in regard to the epigenetic *versus* genetic stability (Fig. 6). The majority of epigenetic alterations that occurred in the tumors (*in vivo*) were hypermethylation events (a total of 14 hyper- *versus* 5 hypo- and 20 *versus* 10 in patient A and B, respectively), whereas the majority of epigenetic alterations that occurred in the cell lines (*in vitro*) were hypomethylation events (a total of 22 hypo- *versus* 16 hyper- and 13 *versus* 6 in patient A and B, respectively). For all of the four tumors, the extent of additional epigenetic changes was greater when putting the tumor in culture than with

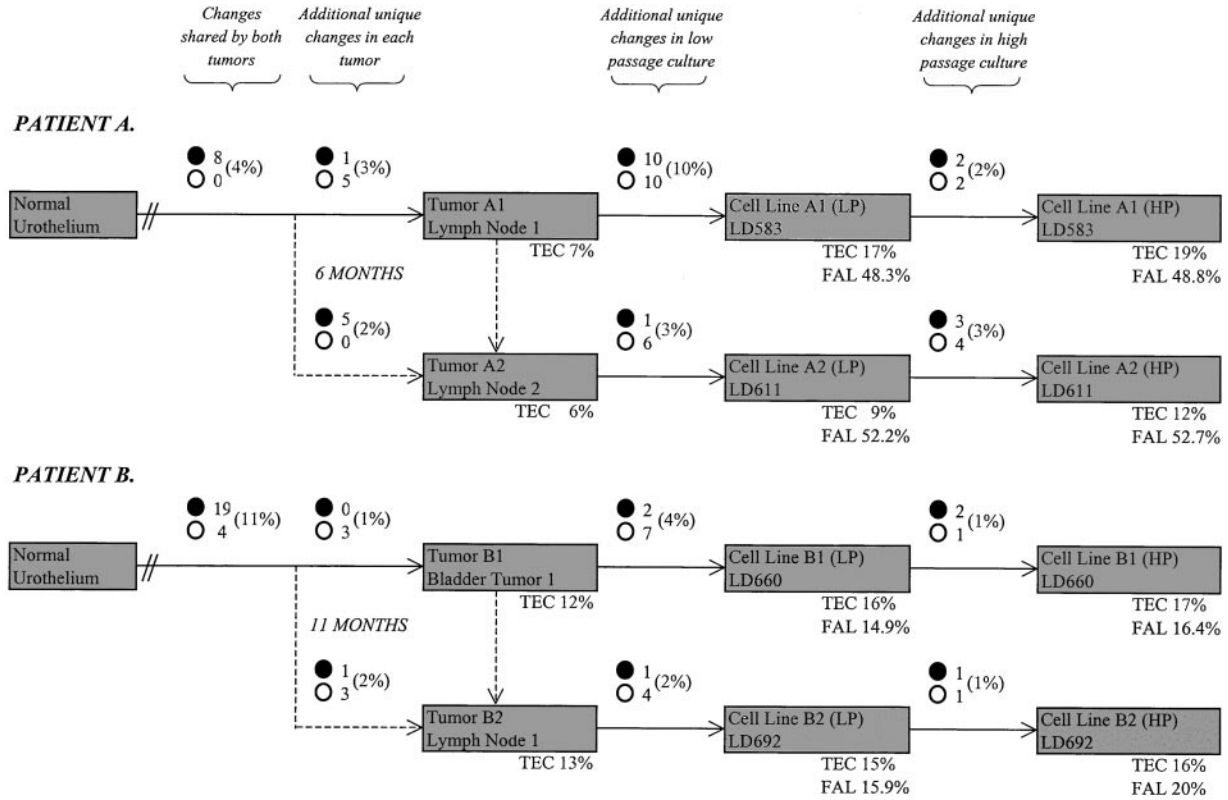


Fig. 6. Summary of epigenetic and genetic analyses of bladder cancer genomes. For each patient, a diagrammatic representation is given of the spatial-temporal relationships between the samples analyzed. Note that Normal Urothelium panel represents an average of five other individuals free of bladder disease and therefore does not include the patients (-/-). Values above arrows, number of additional sequences showing hypermethylation (●) or hypomethylation (○) alterations compared with normal urothelium, and the percentage these sequences represent of the total sequences scored (%). The values below the boxes represent total epigenetic changes (TEC: sum of methylation alterations from the Normal Urothelium panel through the respective point) and fractional allelic loss (FAL: percentage of microsatellite loss of heterozygosity; Ref. 20). Time elapsed between the first and second tumor collection is also provided (in months).

subsequent passage. Interestingly, the extent of genetic alterations (measured as fractional allelic loss) was of the same order of magnitude in both patients as the epigenetic alterations (measured as total epigenetic changes), although patient A showed a 2–3-fold difference between these values. The fractional allelic loss between the patients ranged from approximately 15–53%, whereas the total epigenetic changes between the patients ranged from only approximately 9–18%.

We are currently attempting to additionally describe the regions where differences in methylation pattern were observed in a cancer-specific manner and for which no known matches were found in the sequence databases. In addition, we are conducting studies to determine the role of known genes that became altered in our samples. Whereas our investigation was limited to only two individuals with transitional cell carcinoma of the bladder, our analysis of the epigenetic pattern provides a quantitative profile on the global scale of cancer genomes *in vivo* and *in vitro* over time as well as a context for relative stability compared with the genetic pattern.

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