Allelic Imbalances in Human Bladder Cancer: Genome-Wide Detection With High-Density Single-Nucleotide Polymorphism Arrays

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Background: **Bladder cancer is characterized by genomic instability. In this study, we investigated whether genome-wide screening using single-nucleotide polymorphism (SNP) arrays could detect allelic imbalance (loss or gain of at least one allele) in bladder cancers.** ***Methods:*** **For microarray analysis, DNA was isolated from microdissected bladder tumors and leukocytes from 11 patients. The stage T1 tumor (connective tissue invasive) and the subsequent stage T2–4 tumor (muscle invasive) were available from eight of these patients, and only the first muscle-invasive stage T2–4 tumor was available from three of the 11 patients. The microarray contained 1494 biallelic polymorphic sequences. For microsatellite analyses, DNA was isolated from tumors and leukocytes of nine patients with primary T2–4 tumors and 13 patients with Ta (noninvasive) tumors. All statistical tests were two-sided.** ***Results:*** **We assigned a genotype to 1204 loci, 343 of which were heterozygous. Allelic imbalance was detected in known areas of imbalance on chromosomes 6, 8, 9, 11, and 17, and a new area of imbalance was detected on the p arm of chromosome 6. Microsatellite analysis of nine other T2–4 tumors and 13 Ta tumors showed that allelic imbalance was more frequent in T2–4 tumors than in Ta tumors ($P<.001$). We detected 8.5 allelic imbalances (median) in 348 informative loci in T1 tumors and 28 allelic imbalances (median) in 329 informative loci in T2–4 tumors. When pairs of T1 and T2–4 tumors were analyzed from eight patients, 68% of imbalances detected in T1 tumors (146 imbalances) occurred in the subsequent T2–4 tumors (99 imbalances). Homozygous TP53 mutations were more often associated ($P = .005$) with high allelic imbalance than with low allelic imbalance.** ***Conclusion:*** **SNP arrays are feasible for high-throughput, genome-wide scanning for allelic imbalances in bladder cancer. [J Natl Cancer Inst 2002;94:216–23]**

The initiation and progression of human cancer are generally characterized by a combination of discrete mutations and chromosomal alterations. A common type of chromosomal alteration is loss of heterozygosity, which, in combination with the mutational inactivation of the remaining allele, is considered to be a basic mechanism for the inactivation of tumor suppressor genes *(1,2).* High-density mapping of genetic losses should reveal potential tumor suppressor loci and might be useful for clinical classification of individual tumors. Thus, high-throughput methods, such as comparative genomic hybridization arrays *(3)* and single-nucleotide polymorphism (SNP) arrays *(4,5),* have been introduced recently for genome-wide screening for chromosomal imbalance. SNPs may occur at more than 2 million sites in the genome *(6);* this fact makes it possible to place SNPs at high density along the genome. First-generation and second-generation SNP arrays fabricated by high-density photolithography have identified allelic imbalance (loss or gain of one allele) in esophageal adenocarcinomas and in small-cell lung carcinomas with a high reproducibility and resolution *(4,5).*

One of the most important features of urothelial cancers of the bladder and upper urinary tract is metachronous or synchronous multifocal occurrence with high frequency; another characteristic of bladder cancer is genomic instability. Approximately 30% of patients with bladder tumors have T1 tumors (connective tissue invasive) on initial presentation, and these patients have a 30% risk of developing muscle-invasive disease *(7).* Recent comparative genomic hybridization studies indicate that certain chromosomal areas are frequently altered during the progression of bladder cancer *(8–10).*

In this article, we examine whether SNP arrays can detect genome-wide allelic imbalances in bladder cancer. Allelic imbalance is defined as the loss or gain of one allele in the tumor genome compared with the individual's normal genome. We also characterize alterations detected in individual patients who had a stage T1 tumor and later a stage T2–4 tumor (muscle invasive) and identify new areas with common allelic imbalances that could harbor potential tumor suppressors.

Materials and Methods

Patients

Patients were selected from a clinical data and tissue bank holding records from 1167 patients with bladder cancer who had been followed prospectively for more than 3 years, on average. For SNP array analyses, we identified all patients with bladder cancer who had a superficially invasive stage T1 tumor and then later had a muscle-invasive stage T2–4 tumor. (Patients who had a T2–4 tumor before a T1 tumor were not included in this study.) With these criteria, we identified a total of 29 patients. For 14 of the 29 patients, we had frozen tissue from both tumors (11 patients) or from only the first T2–4 tumor after a T1 tumor (three patients). However, tumors from three patients could not be used because they contained too few tumor cells for microdissection. Thus, we studied eight patients with a T1 tumor and a subsequent T2–4 tumor and three patients with only the first T2–4 tumor after a T1 tumor and examined a total of 19 tumors.

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See "Notes" following "References."

Leukocyte DNA from all 11 patients was isolated and used as reference.

For microsatellite analyses of the p arm of chromosome 6, we used tumors from two groups of patients, one group of nine patients with primary T2–4 (muscle-invasive) tumors and another group of 13 patients with at least three recurrences of noninvasive (Ta) tumors but no higher stage tumors during the observation period. All tumors were transitional-cell tumors. Patients were selected retrospectively in the database until each group contained approximately 10 patients who fulfilled the clinical criteria above and who had tumors large enough for microsatellite analysis. None of the patients had received prior radiotherapy or chemotherapy, and none of the DNA from these patients was used for SNP analysis. As above, leukocyte DNA from all 22 patients was used as reference. The local scientific-ethical committee approved the study, and all patients gave their written informed consent. The identities of all patients involved in this study were made anonymous.

Material

Tumors were obtained fresh from surgery, immediately frozen, and stored at –80 °C. Before DNA extraction, tumors for SNP analyses were cut into sections of 10–20 μm and were stained with hematoxylin. Areas with a high content of tumor cells were microdissected, and DNA was extracted from the purified tumor tissue. Blood was obtained from the tumor patients at their first visit to the hospital. DNA was extracted from tumor tissue and from blood by the use of a Puregene DNA extraction kit (Gentra Systems, Minneapolis, MN) by following the manufacturer's instructions. Tumor stages are listed according to the guidelines from the International Union Against Cancer *(11)*.

SNP Chip Analyses

Corresponding normal and tumor DNA samples were assayed as described in the human SNP (HuSNP) protocol supplied by Affymetrix, Inc., Santa Clara, CA. Each microarray contained 1494 biallelic polymorphic sequences randomly distributed throughout the genome. Twenty-one pools of primer pairs, supplied by Affymetrix, Inc., were used to amplify each DNA sample in a 12.5-μL reaction mixture for each pool that contained 50–100 primer pairs at 50 n*M*, 5 ng of the DNA to be examined, 5 m*M* $MgCl_2$, all four deoxyribonucleoside triphosphates (dNTPs; each at 0.5 m*M*), 1.25 U of AmpliTaq® Gold (PE Biosystems, Foster City, CA), and the supplied buffer. Samples were denatured for 5 minutes at 95 °C; amplified for 30 cycles at 95 °C for 30 seconds, 52 °C increased by 0.2 °C per cycle for 55 seconds, and 72 °C for 30 seconds; amplified for five cycles at 95 °C for 30 seconds, 58 °C for 55 seconds, and 72 °C for 30 seconds; and then extended at 72 °C for 7 minutes. Each pool was diluted 1 : 1000 in double-distilled H_2O, and then 2.5 μL of this dilution was transferred to a new plate and amplified in a 25-μL reaction mixture containing 0.8 μ*M* biotinylated T7 primers, 0.8 μ*M* biotinylated T3 primers, 4 m*M* $MgCl_2$, all four dNTPs (each at 0.4 m*M*), 2.5 U of *Taq* polymerase, and the supplied buffer for 8 minutes at 95 °C. These products were further amplified for 40 cycles at 95 °C for 30 seconds, 55 °C for 90 seconds, and 72 °C for 30 seconds and then extended at 72 °C for 7 minutes. After this reaction, 1.5 μL from selected pools was tested for amplification by electrophoresis in a 4% agarose gel. The remaining products of all 21 polymerase chain reactions (PCRs) were combined, mixed, and loaded on a Microcon-10 spin column (Amicon Bioseparations, Bedford, MA). Samples were concentrated by centrifuging the column for 20 minutes at 13 000*g*, followed by a 3-minute recovery at 3000*g*. All volumes were adjusted with distilled water to 60 μL.

Hybridization, Washing, and Staining

For hybridization, 30 μL of a sample was added to 135 μL containing 3 *M* tetramethylammonium chloride, 2 n*M* control oligonucleotide B1 (supplied by Affymetrix, Inc.), 5× Denhardt's solution (1× Denhardt's solution = bovine serum albumin [0.2 g/L], Ficoll [0.2 g/L], and polyvinylpyrrolidone [0.2 g/L]), herring sperm DNA (100 μg/mL), 5 m*M* EDTA (pH 8.0), 10 m*M* Tris–HCl (pH 7.8), and 0.01% Tween 20, and the mixture was denatured for 5–10 minutes at 95 °C. After 2 minutes on ice, samples were applied to HuSNP chips and hybridized in a hybridization oven (Affymetrix, Inc.) for 16 hours at 45 °C on rollers rotating at 60 rpm. Each chip then was washed and stained on the Fluidics station (Affymetrix, Inc.) as follows: Chips were washed twice with 6× SSPE (1× SSPE = 0.15 *M* NaCl, 0.01 *M* sodium phosphate, and 0.001 *M* EDTA) containing 0.01% Triton X-100 (BioWhittaker, Walkersville, MD) at 25 °C and then were washed five times with 4× SSPE containing 0.01% Triton X-100 at 35 °C. Chips were stained for 30 minutes at 25 °C in 500 μL of streptavidin–phycoerythrin (50 μg/mL) and biotinylated anti-streptavidin antibody (0.25 mg/mL) in a solution of 6× SSPE, 1× Denhardt's solution, and 0.01% Tween 20. After four washes with 6× SSPE containing 0.01% Triton X-100 at 25 °C, 6× SSPE containing 0.01% Triton X-100 was applied to the chip, and the chip was scanned to obtain the genotype.

Scanning and Genotype Generation

HuSNP probe arrays were scanned with an HP Gene Array scanner (Hewlett-Packard, Palo Alto, CA) according to the HuSNP Mapping Assay Manual (product 700244; Affymetrix, Inc.). Typical images are shown in Fig. 1, A. Genotype assignments (i.e., calls) were made automatically from the collected hybridization signal intensities by GeneChip 3.1 software (Affymetrix, Inc.). Each allele (A or B) of an SNP was represented by four or five complementary 20-nucleotide-long probes. The SNP was at a different position in each probe. Each probe, in turn, was paired with a probe of the same sequence except for a central mismatch at or near the SNP position. These mismatched probes helped us to factor cross-hybridization out of the data analyses. A series of data quality and pattern recognition tests must be passed before the software can make a genotype call. The pattern recognition component of the software relies on the relative allele signal determined for each SNP and is further described in the HuSNP Mapping Assay Technical Note, available from Affymetrix, Inc. (product 700318). This analysis can provide six possible calls: no signal, AA, BB, AB, AB_A (i.e., AB or AA), and AB_B (i.e., AB or BB). We considered no signal, AB_A, and AB_B calls to be noninformative. For all calculated results in this article, we used the calls generated from the software of Affymetrix, Inc. Allelic imbalance is defined as call AB in DNA isolated from blood and call AA or BB in corresponding DNA from the tumor, indicating the loss of one allele or the amplification of the other allele. Fig. 2, B, shows $|\hat{p}_{\text{tumor}} - \hat{p}_{\text{blood}}|$ values for chromosomes 6 and 9 in T1 tumors and T2–4 tumors. We used $\hat{p}$ values *(4)* (where $\hat{p}$ is

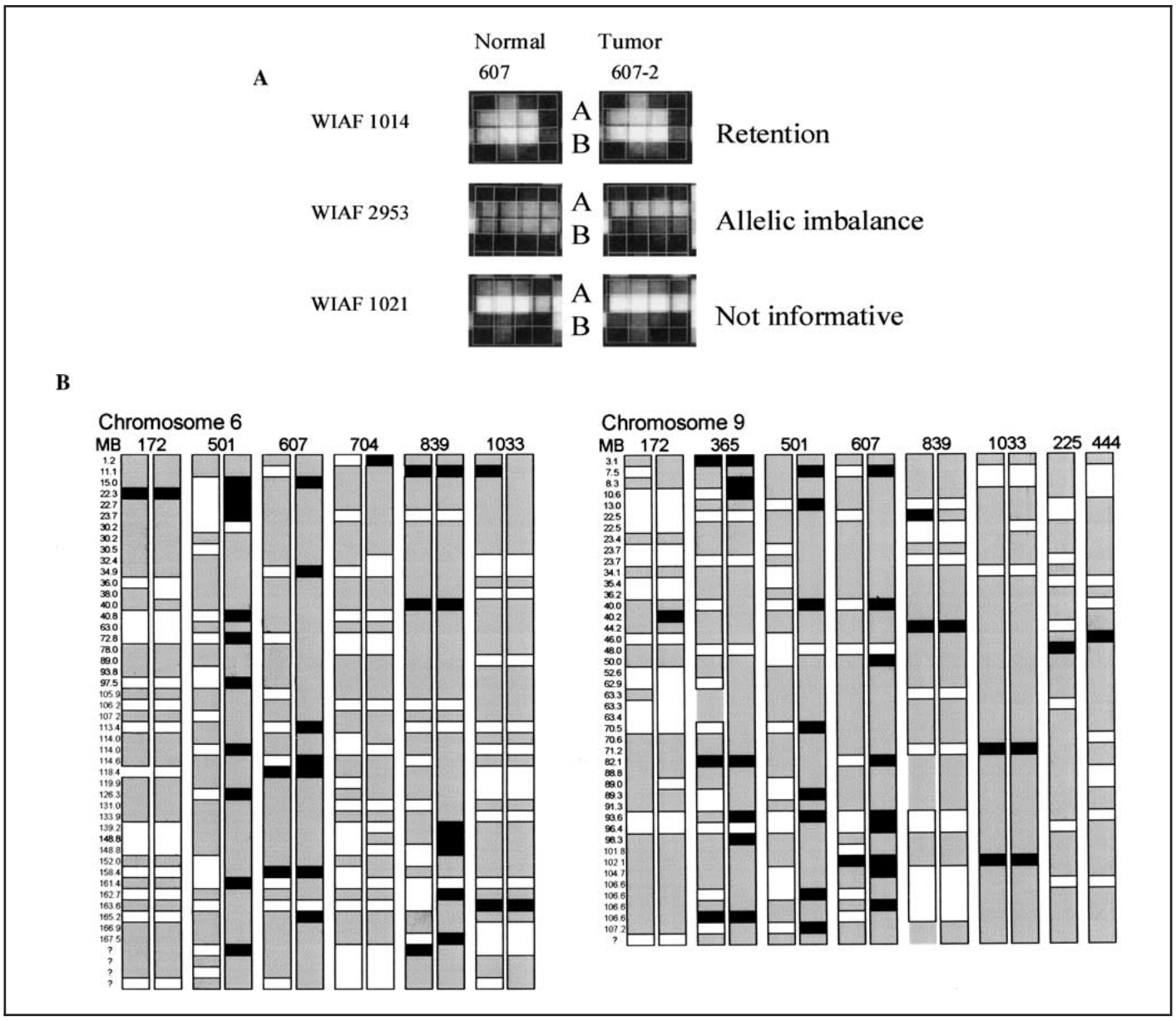

Fig. 1. Single-nucleotide polymorphism (SNP) data detected on arrays and sorted by chromosome. **A)** Representative images of SNP arrays show fluorescent signals at three different SNPs identified by their WIAF numbers (WIAF is the name of a collaboration of Whitehead Institute and Affymetrix, Inc., and all of the SNPs at the array are assigned a WIAF number). **Left:** DNA from the blood of patient 607. **Right:** DNA from the muscle-invasive tumor (patient 607, visit 2). White = high fluorescence. Alleles A and B are present in normal DNA hybridizing to WIAFs 1014 and 2953, and only allele A is present at WIAF 1021. Data show retention of heterozygosity at WIAF 1014 and allelic imbalance at WIAF 2953, whereas data show homozygosity at WIAF 1021, which is noninformative regarding allelic imbalance. **B)** Allelic imbalances at chromosomes 6 and 9 are calculated by software from Affymetrix, Inc. For patients with two tumors, the **left column** illustrates the T1 tumor and the **right column** illustrates the T2–4 tumors. **Black** = loci showing allelic imbalance; **dark gray** = noninformative loci; **light gray** = loci with retention of heterozygosity. MB = megabase(s).

a numerical value between 0 and 1 that represents the heterozygosity of an individual SNP) obtained from the HuSNP™ LOH Utility version 1.0 software supplied by Affymetrix, Inc., for quantifying the relative allele signals and informative markers (of genotype AB in the blood sample). The absolute difference between the $\hat{p}$ values from tumor samples and from the corresponding blood samples gave the values of relative imbalance. The values are plotted for each chromosome, according to the position of the SNPs, by use of the mapping described below. To combine the values from each chromosome, we used the formula

$$\sqrt{s_1^2 + s_2^2 + \ldots\ldots s_n^2}$$

(where $s = [\hat{p}_{\text{tumor}} - \hat{p}_{\text{blood}}]$ and n is the number of different probes for one locus). This value represents the deviation from normal (blood), where high values are weighted most. Only chromosomes with more than four informative markers are included. The $\hat{p}$ values tend to segregate into three clusters that correspond to the three genotypes AA, AB, and BB. Values close to 0 indicate BB, values close to 1 indicate AA, and intermediate values indicate AB. The absolute value of the difference between blood and tumor values, therefore, gives a measure of the shift from balanced heterozygosity toward allelic imbalance. Because $\hat{p}$ values are calculated by use of the ratio between the intensity of the A and of the B allele probes, it is not possible to distinguish between allelic loss and gain.

A

		Microsatellites					
Stage	Patient	D6S470 8.5MB	D6S1653 13.6MB	D6S288 15.0MB	D6S422 20.23MB	D6S1686 21.88MB	D6S1660 28.4MB
T2-4	341	AD	-	RH	RH	RH	n
	362	-	AD	-	-	n	n
	377	AD	RH	-	AD	RH	RH
	378	RH	n	RH	RH	RH	RH
	427	AD	AD	n	-	RH	RH
	564	n	AD	AD	AD	AD	AD
	697	RH	AD	-	AD	AD	n
	733	AD	-	-	AD	-	AD
	770	RH	-	-	AD	n	RH
Ta	3	RH	RH	-	RH	-	-
	32	n	n	-	RH	-	-
	38	RH	n	-	RH	-	-
	58	RH	n	-	RH	-	-
	60	n	n	-	RH	-	-
	73	RH	RH	-	RH	-	-
	92	RH	RH	-	RH	-	-
	115	RH	RH	-	RH	-	-
	154	RH	n	-	n	-	-
	252	n	RH	-	RH	-	-
	269	n	RH	-	RH	-	-
	272	RH	n	-	n	-	-
	327	RH	n	-	RH	-	-

B

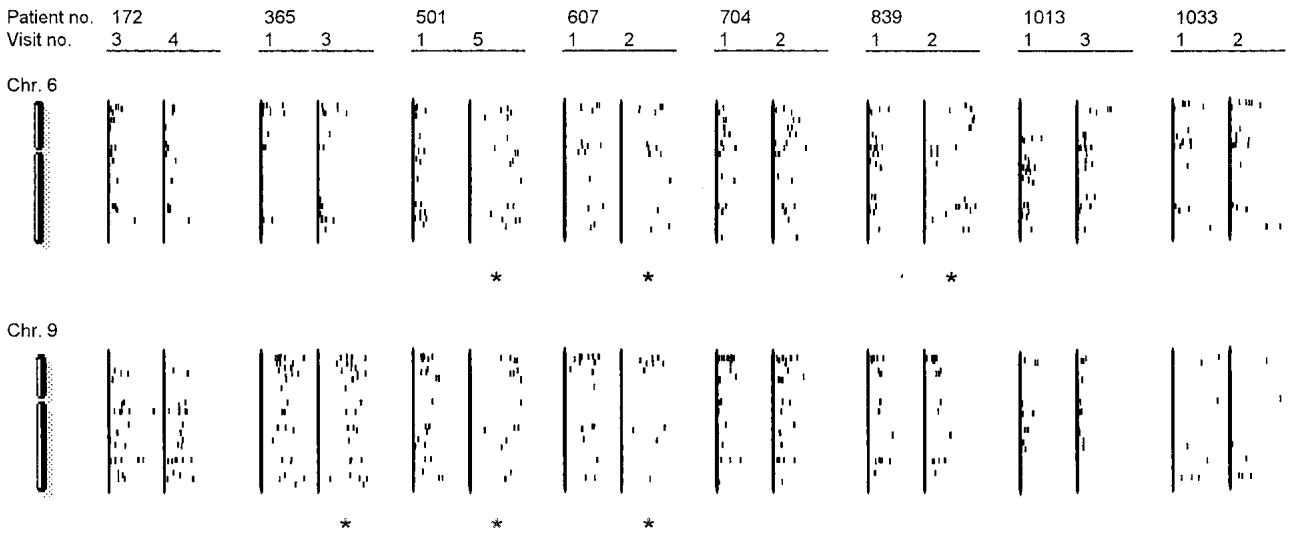

Fig. 2. A) Microsatellite analyses at the p end of chromosome 6. Nine muscle-invasive (T2–4) and 13 noninvasive (Ta) tumors were examined. AD = allelic deletion; RH = retention of heterozygosity; n = noninformative (homozygous or microsatellite-instable). The microsatellite and distance from the p end of chromosome 6 in megabases is shown at the **top. B)** Values of relative imbalance ($|\hat{p}_{\text{tumor}} - \hat{p}_{\text{blood}}|$) for chromosomes 6 and 9 are shown for the T1 tumor (**left**) and the T2–4 tumor (**right**). The *y*-axis (**black bar**) represents the full chromosome with the p end at the top. All informative markers are shown as **dots,** and the **distance from the black bar** indicates allelic imbalance. Only patients with two visits are included. Data from tumors that show indications of total chromosome loss are marked *.

For genomic mapping of the markers, we used the positions from the SNP database at the National Center for Biotechnology Information (NCBI, Bethesda, MD), build 92, contig build 21 (http://www.ncbi.nlm.nih.gov/SNP/). Only SNP markers with no more than one hit were used, except where differences in position did not influence the order of the SNP markers used in this study. This strategy allowed mapping of a total of 1075 SNP markers.

Microsatellite Analyses

DNA isolated for SNP analysis was used for microsatellite analysis. Primers were selected near the SNP loci from the home page of the NCBI. PCR amplification, electrophoresis, and scoring were carried out as described earlier *(2).* Allelic deletion was defined as the loss of a statistically significant part of one allele in tumor DNA compared with corresponding normal DNA. Tu-

mors with microsatellite instability were considered to be noninformative *(2)*.

Sequencing

Exons 5, 6, 7, and 8 of p53 were sequenced. Fragments representing each exon and its adjacent intron–exon boundaries were generated by PCR. Primers were as follows: p53 exon 5 and 6 sense = 5′-TCTTTGCTGCCGTGTTCCAGTTG-3′ and antisense = 5′-CTTAACCCCTCCTCCCAGAGACCC-3′; p53 exon 7 sense = 5′-CAGGTCTCCCCAAGGCGCACTG-3′ and antisense = 5′-CAGGGGTCAGCGGCAAGCAGAG-3′; and p53 exon 8 sense = 5′-GTAGGACCTGATTTCCTTACTGCCTCTTGC-3′ and antisense = 5′-ATAACTGCACCCTTGGTCTCCACCGC-3′. DNA was sequenced by using the DNA chain-terminating method, the BigDye Terminator Kit (Perkin-Elmer, Foster City, CA), and the ABI 377® sequencer (Applied Biosystems, Foster City, CA). For data analysis, we used the Sequence Navigator® program (Applied Biosystems). The sequences were compared with sequences obtained from GenBank.

Statistical Analyses

We used Fisher's exact test for analyzing 2 × 2 tables and the Mann–Whitney *U* test for nonparametric comparison of two datasets. For the Mann–Whitney *U* test, we used the Statistical Package for Social Sciences (Chicago, IL). All statistical analyses were two-sided. The two-sided value in Fisher's exact test was found by multiplying the one-sided value by 2.

RESULTS

All genotype calls presented below were generated by the software from Affymetrix, Inc. These data were then compared with data calculated by the use of an alternative $\hat{p}$ method, described above.

SNP Array Analysis

We used SNP arrays to scan the genome for allelic imbalances in patients with stage T1 bladder cancer that had progressed to higher stages. The SNP array, containing 1494 biallelic polymorphisms randomly distributed in the genome, was hybridized with the pooled products of 21 multiplex PCRs. The mean number of successfully identified calls was statistically significantly lower in tumor samples (1191 calls) than in blood samples (1226 calls) ($P = .009$, Mann–Whitney *U* test). In total, the median number of heterozygous loci was 343 (range = 309 to 370) of 1204 correctly identified loci.

Detection of Allelic Imbalances Generated by Affymetrix Calls

In the eight T1 tumors examined, allelic imbalance was found in a median of 8.5 loci (range = 2 to 57) in 348 informative loci (median; range = 327 to 368 informative loci). In the 11 T2–4 tumors examined, allelic imbalance was found in a median of 28 loci (range = 2 to 85) in 329 informative loci (median; range = 309 to 370).

Eight patients had one T1 and then a T2–4 tumor that we could examine. In these T2–4 tumors, we found a total of 281 loci with allelic imbalance. At these 281 loci, 123 (44%) were heterozygous in the previous T1 tumor, 59 (21%) were noninformative (AB_A, AB_B, or no signal), and 99 (35%) had allelic imbalance in the previous T1 tumor. In the T1 tumors from these eight patients, we found allelic imbalance at a total of 146 loci. In the subsequent T2–4 tumors in these patients, 21 (14%) of these loci were heterozygous, 26 (18%) were noninformative, and 99 (68%) had allelic imbalance. In six of the eight patients, zero to two allelic imbalances were present in the T1 tumor but not in the T2–4 tumor. In two patients, more than two allelic imbalances were present in the T1 tumor but not in the T2–4 tumor (four such loci in patient 172 and 11 in patient 839). Thus, these data indicate the mutual clonal origin of the T1 and T2–4 tumors in individual patients.

Allelic Imbalance Frequency, Tumor Stage, and TP53 Mutations

In general, we could subdivide the tumors into those with 32 or fewer imbalances and those with 33 or more imbalances, as well as into superficial and muscle-invasive tumors. The number 32 was chosen because the allelic imbalances cluster into a high-frequency group and a low-frequency group (mean = 32.3 allelic imbalances). It was remarkable that the group of stage T1 tumors contained tumors with both low and high frequencies of allelic imbalance, as did the stage T2–4 tumors. The level of allelic imbalance was generally higher in T2–4 tumors than in T1 tumors but not statistically significantly so. We then sequenced the TP53 gene and found four TP53 gene mutations in T1 tumors and four in T2–4 tumors (Table 1). All tumors with homozygous TP53 gene mutations (loss of one allele and mutation at the other) showed allelic imbalance of 17p. Tumors with high allelic imbalance showed homozygous TP53 mutations more often than tumors with low allelic imbalance ($P = .005$; two-sided Fisher's exact test), consistent with our recent findings of both chromosomal stable and unstable muscle-

Table 1. Total No. of allelic imbalances and status at the TP53 locus in each tumor*

				TP53 Status	
Patient No.	Visit No.	Stage	AI, total No.	SNP	Mutation
501	1	T1	2	NC	
172	3	T1	5	NC	
1013	1	T1	6	NC	GG→TT 919 (He)†
365	1	T1	8	NC	
607	1	T1	9	NC	
704	1	T1	11	NC	C→T 742 (He)‡
1033	1	T1	48	AI	G→C 396 (Ho)§
839	1	T1	57	AI	Del C 482 (Ho)‖
172	4	T2–4	2	NC	
341	1	T2–4	6	NC	
444	1	T2–4	8	NC	
365	3	T2–4	14	NC	
704	2	T2–4	15	NC	C→T 742 (He)‡
225	5	T2–4	18	NC	
1013	3	T2–4	26	NC	GC→TT 919 (He)†
607	2	T2–4	37	NC	
501	5	T2–4	47	AI/NC	
1033	2	T2–4	55	AI	G→C 396 (Ho)§
839	2	T2–4	85	AI	Del C 482 (Ho)‖

*Ho = homozygous mutation; He = heterozygous mutation; AI = allelic imbalance; NC = no change; SNP = single nucleotide polymorphism.

†GG→TT mutation at nucleotide 919 (alanine-307→serine, splice mutation).

‡C→T mutation at nucleotide 742 (arginine-248→tryptophan).

§G→C mutation at nucleotide 396 (lysine-132→asparagine).

‖Deletion of C at nucleotide 482 (stop codon at position 169).

invasive tumors *(2)*. We found no association between numbers of allelic imbalances and sex, age, numbers of tumors, time interval between tumors, outcome of disease, coexistence of carcinoma *in situ*, or smoking habits. It should be noted, however, that the sample was too small for firm conclusions to be reached.

Common Regions of Allelic Imbalance Defined by SNP Arrays

Allelic imbalance occurred heterogeneously in all chromosomes, ranging from two different loci with allelic imbalance on chromosome 1 (3%, two of 69 informative loci) to 26 loci with allelic imbalance on chromosome 6 (41%, 26 of 63 informative loci). To define chromosomes with common allelic imbalance in bladder cancer, we searched for chromosomes with a total of at least 25 allelic imbalances in at least four patients and found that chromosomes 6, 8, 9, 11, and 17 fulfilled these criteria (Table 2). Examining the allelic imbalances that occurred during disease progression from superficial stage T1 tumors to muscle-invasive stage T2–4 tumors revealed no new commonly affected chromosomes (Table 2).

The chromosomes with frequent alterations sometimes harbored hot spots for allelic imbalance, which may contain candidate tumor suppressor genes. Chromosome 6 had allelic imbalance at 11.1–22.3, 40.0–40.8, and 158.4–165.2 megabases (MB) from the p end and had a total loss or gain in two positions (patient 501, visit 5; patient 607, visit 2) (Fig. 1, B). Chromosome 8 had hot spots at 20.2–28.2, 80.3–92.8, and 118.6–127.5 MB, and one T2–4 tumor (patient 1033, visit 2) had lost or gained an entire chromosome 8. Chromosome 9 had hot spots at 40.0–48.0, 70.5–82.1, and 102.1–106.6 MB (Fig. 1, B), and two tumors (patient 501, visit 5; patient 607, visit 2) had lost or gained an entire chromosome 9 (Fig. 1, B). Chromosome 11 had an allelic imbalance hot spot at 11.1 MB, and chromosome 17 had an allelic imbalance hot spot at 0.9–3.8 MB (*see also* www.MDL.DK/sdata.html).

Because the p end of chromosome 6 was often affected and thus constituted a new area of interest in bladder cancer, six microsatellite loci in this region were examined. A new set of nine muscle-invasive tumors was examined, and allelic deletions were observed in at least one microsatellite in eight of these nine tumors. The three microsatellites that were most frequently deleted were examined in a new set of 13 Ta tumors. None of these tumors showed any allelic deletions (Fig. 2, A). This difference in allelic imbalance between Ta and T2–4 was highly statistically significant ($P<.001$; two-sided Fisher's exact test).

Association of SNP Findings and Microsatellite-Based Detection of Deletions

To verify that the SNP array-based detection of allelic imbalance was comparable to a known method, we analyzed microsatellites in selected areas of chromosomes 6, 8, 9, and 11. We

Table 2. Allelic imbalances and chromosomal location of the single nucleotide polymorphisms*

	No. of allelic imbalances†											Stage	
	Pt. 172, Vt. 3,4	Pt. 365, Vt. 1,3	Pt. 501, Vt. 1,5	Pt. 607, Vt. 1,2	Pt. 704, Vt. 1,2	Pt. 839, Vt. 1,2	Pt. 1013, Vt. 1,3	Pt. 1033, Vt. 1,2	Pt. 225, Vt. 5	Pt. 341, Vt. 1	Pt. 444, Vt. 1	T1‡	T2–4§
Chr 1	—,—	1,1	—,—	—,—	—,—	1,1	—,—	—,—	—	—	1	2/2	3/3
Chr 2	—,—	—,—	—,—	—,1	—,—	3,2	1,3	—,—	3	—	2	4/2	11/5
Chr 3	—,—	—,—	—,1	—,1	—,—	1,1	—,—	4,3	—	—	—	5/2	6/4
Chr 4	—,—	—,—	—,—	—,—	—,—	2,4	—,—	3,—	—	—	—	5/2	4/1
Chr 5	—,—	—,—	—,—	—,—	—,1	—,5	—,—	—,1	—	—	—	0	7/3
Chr 6	1,1	—,—	—,11	2,7	—,1	3,7	—,—	2,1	—	—	—	8/4	28/6
Chr 7	—,—	—,—	—,—	—,—	1,2	—,10	—,—	—,—	1	—	1	1/1	14/4
Chr 8	1,—	—,—	—,—	—,—	—,1	2,4	—,—	3,12	2	—	1	6/3	20/5
Chr 9	—,1	3,7	—,8	1,9	—,—	2,1	—,—	2,2	1	—	1	8/4	30/8
Chr 10	—,—	—,—	—,—	—,—	—,—	3,—	—,1	—,1	—	—	—	3/1	2/2
Chr 11	1,—	2,3	—,5	—,—	—,—	8,9	—,2	—,—	—	—	—	11/3	19/4
Chr 12	—,—	1,—	—,—	—,—	—,—	—,—	—,—	1,2	—	—	1	2/2	3/2
Chr 13	1,—	—,—	—,—	—,—	1,—	3,4	—,3	—,—	1	—	—	5/3	8/3
Chr 14	—,—	—,—	—,—	—,3	—,—	—,—	—,—	9,9	—	—	—	9/1	12/2
Chr 15	1,—	—,—	—,—	—,1	—,—	—,7	—,—	—,—	—	—	—	1/1	8/2
Chr 16	—,—	—,1	—,—	—,—	—,—	2,—	1,1	—,—	—	—	—	3/2	2/2
Chr 17	—,—	—,—	—,4	1,1	—,—	6,7	1,1	2,4	—	—	—	10/4	17/5
Chr 18	—,—	—,—	—,—	—,—	4,3	3,3	—,—	—,—	1	1	—	7/2	8/4
Chr 19	—,—	—,—	—,2	—,—	—,—	—,—	—,—	—,1	—	—	—	0	3/2
Chr 20	—,—	—,—	—,—	—,—	—,—	3,—	—,—	—,—	—	—	—	3/1	0
Chr 21	—,—	—,—	—,—	—,—	—,—	—,—	—,4	—,—	—	—	—	0	4/1
Chr 22	—,—	—,—	1,—	—,—	1,—	—,—	—,—	—,—	—	—	—	2/2	0
Chr X	—,—	—,—	—,—	—,—	—,—	1,—	—,—	1,1	—	1	—	2/2	2/2
Chr ?	—,—	1,2	1,16	5,14	4,7	14,20	3,11	21,18	9	4	1		
Total‖	5,2	8,14	2,47	9,37	11,15	57,85	6,26	48,55	18	6	8		

*For most patients, two tumors sampled from two different visits were examined. Pt. = patient; Vt. = visit; Chr = chromosome.

†Number of allelic imbalances detected in the single chromosome in the single patient. — = no allelic imbalances detected.

‡Total number of allelic imbalances in T1 tumors/number of T1 tumors with allelic imbalance.

§Total number of alleles lost in T2–4 tumors/number of T2–4 tumors with allelic imbalance.

‖Total number of allelic deletions in the single tumor.

found that the two methods agreed at 22 of 25 loci. The differences may be a result of either errors in mapping or the differential sensitivities of the two methods.

Detection of Allelic Imbalances as Generated From $\hat{p}$ Values

To quantitate allelic imbalance with respect to chromosomal location, we calculated the difference in $\hat{p}$ values between tumor and blood. The values for each chromosome were combined and plotted (Fig. 2, B). These plots offer an overview of the data for each chromosome and can easily be used to identify hot spots of imbalance.

DISCUSSION

The SNP array method is fast and reproducible. This method can be introduced in a more clinical setting because throughput time is shorter than that for microsatellite-based analyses and flexibility with respect to the genomic location of SNPs is equivalent. In the future, it should be possible to fabricate high-density SNP microarrays for predefined chromosomal locations, which could make noninformative areas informative.

We attempted to quantify the allelic imbalance by using $\hat{p}$ values obtained directly from the HuSNP™ LOH Utility version 1.0 software supplied by Affymetrix, Inc. The $\hat{p}$ value reflects the relative allele intensity, expressed as a value between 0 and 1. Using the $\hat{p}$ values instead of the genotype calls from a computer program allowed us to obtain information from more markers because many tumor sample calls did not pass the Affymetrix software quality tests. Although the nature of these tests is not publicly available, we believe that these failures were caused by the contamination of tumor DNA with a small amount of normal DNA from, for example, tumor-infiltrating lymphocytes, leading to a reduced and not completely absent signal from the allele lost in the tumor cells. The reduced but still detectable signal makes the call uncertain; thus, it is rejected. For assessing allelic imbalance, however, we empirically find that useful information is lost. A graphic plot of the many $\hat{p}$ values makes it easier to identify lost areas in tumors that may hold important tumor suppressor genes.

Our data are in line with previous studies of bladder cancer *(8,9)* that detected imbalance at chromosomes 8, 9, 11, 13, and 17 by use of comparative genomic hybridization. It is presently unclear why these chromosomes are altered, but chromosomes 1, 2, and 5 are rarely altered. Although various mechanisms leading to genomic imbalance, such as mutations in the anaphase-promoting complex *(12)*, have been examined, the actual mechanisms are still unknown. The RB gene on chromosome 13 is often lost in bladder cancer, but we did not detect systematic changes in this chromosome.

Allelic imbalances were more common in T2–4 tumors than in T1 tumors. However, both tumor types can be divided into two groups by the number of allelic imbalances detected; one group has higher numbers of allelic imbalances, and the other group has lower numbers. This observation is in accordance with our recent results *(2)* in which frequent allelic losses were detected with microsatellite probes close to the genes MDM2, TP53, CDKN1A (p21), CDKN2A (p16), RB1, E2F, and MYCL in approximately 50% of stage T2–4 tumors, but the rest of the tumors were surprisingly stable. In this study, we sequenced the TP53 gene and found that the difference between the high and low allelic imbalance groups could be the mutational inactivation of one allele and the loss of the other allele. TP53 is commonly inactivated in bladder cancer *(13)*, and these data clearly demonstrate that this inactivation may be closely associated with genomic imbalance because p53 is a quality-control gatekeeper that directs cells with allelic imbalances into apoptosis *(14)*. A similar association between TP53 mutations and a high frequency of loss of heterozygosity has been observed in lung cancers (deletions on 3p, 5q, 9p, 11p, and chromosome 17) and in liver cancers (deletions of 9p21–p23 and 16q21–q23) *(15,16)*. We observed four areas commonly affected at the p end of chromosome 6. These areas were more common in T2–4 tumors than in Ta tumors, indicating that allelic imbalance in 6p is a late event in bladder cancer. Chromosome 6 is frequently altered in ovarian cancer, and such alterations are associated with aggressive serous-type ovarian carcinomas *(17)*. No oncogenes, however, have been detected on chromosome 6 *(18)*. Multiple genes with a potential impact on carcinogenesis, including genes for endothelin-1, AP-2α, E2F3, multiple different histones, kinases, desmoplakin, and neuritin, are situated between 0 and 30 MB from the p end of chromosome 6 and may be candidate tumor suppressors.

SNP arrays have compared well with simple sequence length polymorphism *(5)*, comparative genomic hybridization *(5)*, and restriction fragment length polymorphism *(4)*. However, it must be stressed that relatively pure tumor tissue must be used for the multiplex PCR because contamination with 20% or more normal tissue statistically significantly affects the precision of the assay *(5)*. Therefore, we cannot exclude the possibility that more altered loci would have been detected if completely pure tumor cells, which are difficult to obtain from clinical samples, had been used. Results of standard microsatellite and restriction fragment length polymorphism analyses, which can tolerate more contaminating normal tissue, parallel our findings.

In conclusion, we have demonstrated that SNP arrays can detect allelic imbalance in bladder tumors, we have confirmed known areas of chromosomal losses, and we have identified new areas of allelic imbalance on chromosome 6. Two groups can be identified in both stage T1 and stage T2 tumors, one with smaller numbers of allelic imbalances and the other with larger numbers of allelic imbalances. It is interesting that both TP53 alleles were inactivated in tumors with a large number of imbalances ($P = .005$). Thus, SNP arrays are feasible for high-throughput, genome-wide screening for allelic imbalances, and arrays with higher SNP density should provide even more detailed information on chromosomal events.

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NOTES

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