

Genome-Wide Genetic Characterization of Bladder Cancer: A Comparison of High-Density Single-Nucleotide Polymorphism Arrays and PCR-based Microsatellite Analysis^{1,2}

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ABSTRACT

Most human cancers are characterized by genomic instability, the accumulation of multiple genetic alterations, and allelic imbalance throughout the genome. Loss of heterozygosity (LOH) is a common form of allelic imbalance, and the detection of LOH has been used to identify genomic regions that harbor tumor suppressor genes and to characterize different tumor types, pathological stages and progression. Global patterns of LOH can be discerned by allelotyping of tumors with polymorphic genetic markers. Microsatellites are reliable genetic markers for studying LOH, but typically only a modest number of microsatellites are tested in LOH studies because the genotyping procedure can be laborious. Here we describe the use of a new alternative approach to comprehensive allelotyping in which samples are genotyped for nearly 1500 single-nucleotide polymorphism (SNP) loci distributed across all human autosomal arms. We examined the pattern of allelic imbalance in human transitional cell carcinomas of the urinary bladder including 36 primary tumors and 1 recurrent tumor with matched normal DNAs. The call rate for all SNPs was $78.5 \pm 1.87\%$ overall samples. Overall, the median number of allelic imbalance was 47.5, ranging from 20 to 118. The mean number of allelic imbalances was 36.58, 51.30, and 67.78 for pT_a, pT₁, and \geq pT₂, respectively, and also increased by grade. The SNP microarray analysis result was validated by comparison with microsatellite allelotyping analysis of 118 markers in the same tumors. Overall, the two methods produced consistent loss patterns at informative loci. The SNP assay discovered previously undiscovered allelic imbalances at chromosomal arms 12q, 16p, 1p, and 2q. The detection of LOH and other chromosomal changes using large numbers of SNP markers should enable rapid and accurate identification of allelic imbalance patterns that will facilitate the mapping and identification of important cancer genes. Moreover, SNP analysis raises the possibility of individual tumor genome-wide allelotyping with potential prognostic and diagnostic applications.

INTRODUCTION

There are convincing data to support the hypothesis that a large number of genetic events play a role in the etiology and progression of human cancer. In addition to oncogene activation, the inactivation of tumor suppressor genes has been shown to play an important role in tumorigenesis (1). The silencing of tumor suppressor genes often involves two genetic events: the loss or recombination of large chro-

somal DNA regions containing one parental allele and a smaller mutational event (e.g., point mutation, localized deletion, promoter hypermethylation) inactivating the second allele (2).

Global patterns of LOH⁴ can be analyzed through allelotyping of tumors with polymorphic genetic markers from each chromosomal arm (3). Two allele RFLPs and Southern analysis gave way to simple-sequence-length polymorphisms such as PCR-based microsatellites, and both proved to be reliable genetic markers for studying LOH (4). However, only a modest number of polymorphic markers have been used in LOH studies because genotyping of many loci requires extensive time and labor. Furthermore, high-density genotyping is needed to tease out small deletions useful for the localization of a cancer gene and rare events that may define tumor behavior. Thus, high-throughput methods, such as CGH arrays⁵ (5) and SNP arrays⁶ (6, 7), have been introduced recently for genome-wide screening for chromosomal imbalance. However, CGH has limits of definition for small losses. Moreover, CGH can estimate the number of alleles but cannot distinguish between paternal and maternal from recombinational events (5, 8). SNPs can detect recombination events and may occur at more than three million sites in the human genome (approximately once in every 100–300 bases; Ref. 9), making 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 adenocarcinoma and in small cell lung carcinomas with high reproducibility and resolution (6, 7). This technique is potentially rapid, adaptable to clinical laboratory setting, and permits the analysis of a large volume of clinical samples (global genome analysis in one reaction).

Bladder tumors are predominantly TCCs but display significant variation in clinical behavior, propensity to recur, progression, and prognosis, which likely reflect genetic heterogeneity. Like most adult solid tumors, bladder cancers show a wide range of chromosomal numbers associated with a large number of structural and numerical chromosomal changes, suggesting diversity in the biology of these cancer cells (10). To understand the molecular mechanisms of this disease, it will be necessary to better define LOH patterns and ultimately identify the genes underlying these chromosomal abnormalities. Toward this end, we undertook a genome-wide allelotyping of TCCs of the bladder based on the Affymetrix HuSNP chip and validated our results by direct comparison with microsatellite analysis of the same tumors.

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² Supplementary data for this article are available at *Cancer Research Online* (<http://cancerres.aacrjournals.org>).

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⁴ The abbreviations used are: LOH, loss of heterozygosity; SNP, single nucleotide polymorphism; TCC, transitional cell carcinoma; FAL, fractional allelic loss; CGH, comparative genomic hybridization.

⁵ Internet address: java/Propub/genetics/ng0999_41.fulltext; java/Propub/genetics/ng0999_41.abstract.

⁶ Internet address: taf/dynapage.taf?file=/ncb/biotech/v18/n9/full/nbt0900_1001.html; taf/dynapage.taf?file=/ncb/biotech/v18/n9/abs/nbt0900_1001.html.

MATERIALS AND METHODS

Sample Collection. Primary tumor and peripheral blood samples were collected from 36 patients undergoing surgical resection of bladder cancer. A recurrent tumor from case number 32 was also collected. Lymphocytes were collected from blood and were used as the source of normal DNA. Tumor samples were promptly frozen at -80°C after initial gross pathological examination and microscopic dissection. DNA was isolated from tumor tissue or lymphocyte pellets by standard SDS/proteinase K digestion followed by phenol and chloroform extraction and ethanol precipitation (11).

Microsatellite DNA Markers and PCR-LOH Analysis. To perform a genome-wide allelotyping study, we used 118 microsatellite markers spanning all of the 39 nonacrocentric autosomal arms. Chromosomal localization of each marker was estimated by combining data from the Genethon genetic map and from the Genome Database (GDB)-integrated genetic and physical maps. One primer of each marker pair was end-labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Amersham, Arlington Heights, IL) using T4-polynucleotide kinase (Life Technologies, Inc., Gaithersburg, MD). Genomic DNA (50 ng) was subjected to 35 PCR cycles at a denaturing temperature of 95°C for 30 s, followed by various annealing temperatures ranging from 54°C to 58°C for 1 min, an extension step at 70°C for 1 min, and a final single extension step at 70°C for 5 min. PCR products were then separated in a denaturing 7% polyacrylamide-urea-formamide gel. Autoradiography was performed overnight at -80°C . LOH was scored in informative cases if a significant reduction ($>50\%$) in the ratio of the signal from the tumor allele was observed in comparison with the corresponding normal alleles in the adjacent lane. Analysis of all samples was carried out in a blinded fashion, without knowledge of pathological grade, stage, and clinical status.

SNP Chip Assay. Matched tumor and normal DNA samples were analyzed by using the HuSNP chip assay (Affymetrix, Inc., Santa Clara, CA) per the manufacturer's protocol⁷ and as described previously (7).

Data Analysis. GeneChip data analysis begins with assigning an experiment name to a probe array by creating an exp file. Scanning a probe array creates a data file or image file. From this data file, the software automatically generates a cell file by demarcating individual cells. A "probe cell" is the area on the surface of the array containing a unique oligonucleotide sequence. The pixel intensities within each probe cell are averaged, producing a cell file. Typical images are available at the Affymetrix website.⁸

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 a SNP was represented by four or five complementary 20-nucleotide 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 mismatch probes helped us to factor cross-hybridization out of the data analyses. The pattern recognition component of the software relies on the relative allele signal determined for each SNP and is described in the HuSNP Mapping Assay Technical Note, available from Affymetrix, Inc. (product 700318). This analysis can provide six possible calls: *AA*, *BB*, *AB*, *AB_A* (*i.e.*, *AB* or *AA*), and *AB_B* (*i.e.*, *AB* or *BB*). We considered no signal and *AB_A*, and *AB_B* calls to be noninformative. For all of the calculated results in this report, we used the calls generated from the software of Affymetrix, Inc. Allelic imbalance can be assessed when the individual SNP is polymorphic in the germ line (blood DNA), defined as informative and *AA* or *BB* in corresponding DNA from the tumor, indicating the loss of one allele or the amplification of the other allele.

Statistical Methods. The major statistical end point in this study was the correlation of SNP imbalance with LOH by microsatellite analysis over 39 chromosomal arms. Cross-tabulations were analyzed using χ^2 or Fisher's exact tests when appropriate. Stage (pT_a , pT_1 , $\geq pT_2$) and grade (G1, G2, and G3) were recorded when reported. Mean FAL was compared for sequence tandem repeats and SNPs across stage and grade status groups using simple linear regression models. All of the statistical computations were performed using the SAS system (12), and the *P*s reported are two sided.

RESULTS

Alteration Frequencies Determined by SNP Chips. Thirty-six primary bladder tumor and matched normal lymphocyte DNAs were genotyped using the HuSNP chip (Table 1). The software from Affymetrix, Inc., generated all of the genotype calls presented here (examples are presented in Fig. 1). The HuSNP chip performance (percentage passed) was 78.5 ± 1.87 over all of the samples yielding ~ 1172 SNPs scored per sample (Table 2). The rate did not differ significantly between normal and tumor samples. In total, the median number of heterozygous loci was 341 (range, 267–404) with an average coverage of one SNP per 8.7 cM. Frequent allelic imbalances ($\geq 50\%$) were observed at SNP loci on chromosomal arms 9p, 9q, 4q, 12q, 11p, 13q, 8q, 1p, 1q, 5q, 8p, 10q, 11q, 16p, 2q, 3p, 14q, 17p, 17q, and 18q (Fig. 2). Allelic imbalances in a frequency of 31–50% were found in chromosomal arms 3q, 4p, 6p, 10p, and 21q (Fig. 2). Many other chromosomes harbored imbalances at a frequency of $\leq 30\%$ (*e.g.*, chromosomal arms 6q, 7p, 7q, 15q, 18p; Fig. 2).

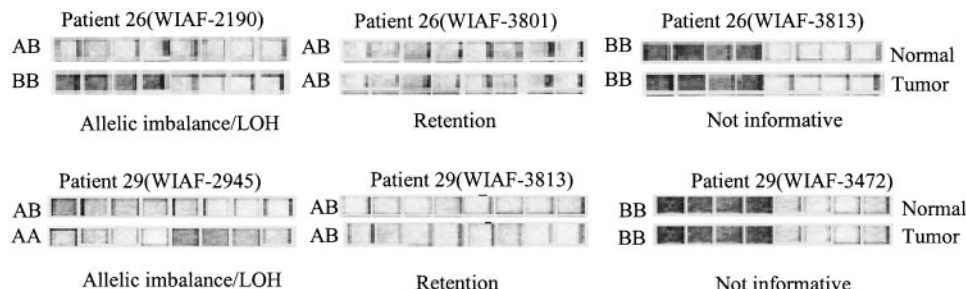
Alteration Frequencies Determined by Microsatellite PCR. Thirty of the 36 normal-and-tumor paired DNAs were allelotyped with 118 microsatellite markers distributed over 39 chromosomal arms. Overall, a relatively high percentage (more than 40%) of LOH was found for chromosomal arms, in order of frequency as follows: 9q [23 (76.67%) of 30], 9p (70%), 8p (50%), 20q(43%), 8q [13 (43.33%) of 30], 1q [12 (40%) of 30], 21q [12 (40%) of 30], and 5q [12 (40%) of 30; Fig. 2]. The frequency of allelic losses at 9p and 9q were nearly equally distributed throughout all of the tumor stages; the occurrence of allelic loss at 1p, 1q, 4p, 5q, 8p, 10q, 11q, 13q, 16p, 16q, 18p, 18q,

Table 1 Histopathological classification of bladder cancer samples

Sample no.	Cell type	Grade	Stage	Morphology
1	TCC	2	pT_a	Papillary
2	TCC	2	pT_a	Papillary
3	TCC	1	pT_a	Papillary
4	TCC	1	pT_a	Papillary
5	TCC	1	pT_a	Papillary
6	TCC	1	pT_a	Papillary
7	TCC	1	pT_a	Papillary
8	TCC	1	pT_a	Papillary
9	TCC	2	pT_a	Papillary
10	TCC	2	pT_a	Papillary
11	TCC	1	pT_1	Papillary
12	TCC	2	pT_1	Papillary
13	TCC	2	pT_1	Papillary
14	TCC	1	pT_1	Papillary
15	TCC	2	pT_1	Papillary
16	TCC	2	pT_1	Papillary
17	TCC	3	pT_1	Papillary/solid
18	TCC	2	pT_1	Papillary
19	TCC	2	pT_1	Papillary
20	TCC/glandular	2	pT_1	Papillary
21	TCC	2	pT_2	Papillary/solid
22	TCC	3	pT_2	Papillary/solid
23	TCC	2	pT_2	Papillary/solid
24	TCC	3	pT_3	Papillary/solid
25	TCC	3	pT_2	Papillary/solid
26	TCC	2	pT_2	Papillary/solid
27	TCC	3	pT_2	Papillary/solid
28	TCC	2	pT_2	Papillary/solid
29	TCC	3	pT_3	Papillary/solid
30	TCC	3	pT_2	Papillary/solid
31	TCC	3	pT_1	Papillary
32	TCC	3	pT_2	Papillary
32b (recur) ^a	TCC	3	pT_2	Papillary
33	TCC	1	pT_a	Papillary
34	TCC	3	pT_2	Papillary/flat
35	TCC	3	pT_2	Papillary
36	TCC	2	pT_a	Papillary

^a Sample no. 32b is recurrent tumor.⁷ Internet address: http://www.affymetrix.com/Download/manuals/husnp_manual.pdf.⁸ Internet address: http://www.affymetrix.com/support/technical/datasheets/husnp_data-sheet.pdf.

Fig. 1. Representative images of fluorescence intensities for SNP array hybridization to normal and tumor DNA samples. For each sample pair (sample numbers 26 and 29), a call of LOH, retention, or uninformative was made. *WIAF-2190*, *WIAF-3801*, *WIAF-2945*, and *WIAF-3472* are SNP markers on chromosomal regions 9q34.13, 9q33.3, 9q21.13, and 9p22.3, respectively.



20q, 21q, and 22q correlated with higher stages (examples are presented in Fig. 3).

Comparison of Microsatellite Analysis with the HuSNP Chip Assay. To verify that the SNP array-based detection of allelic imbalance was comparable with a known and established method, we compared the two genomic approaches. The range of consistency between the two methods in different loci varied from 50 to 100% (Table 3). Concordance (comparing at least one informative locus by SNP assay within 10 cM in both directions from the informative microsatellite marker) of $\geq 90\%$ were observed for *DIS228*, *D2S136*, *D2S126*, *D3S1268*, *D5S417*, *D5S108*, *D6S261*, *D12S95*, *D13S270*, *D14S288*, *D15S117*, *D15S116*, *D16S423*, *D19S246*, *D20S119*, and *D22S282* loci. Because of a limitation in the accuracy of physical mapping data for both SNPs and microsatellites (and because most chromosomal deletions are large and contiguous), we compared the two methods

Table 2. HuSNP chip performance (percentage passed)

Sample no.	All loci	Normal	Tumor
1	77.2	77.9	76.4
2	69.8	71.8	67.7
3	70.6	69.9	71.3
4	64.2	62.2	66.1
5	79.8	80.3	79.3
6	77.3	79.0	75.6
7	75.2	76.3	74.0
8	68.5	70.4	66.6
9	74.8	73.8	75.7
10	69.5	69.3	69.7
11	74.1	74.6	73.6
12	74.1	75.7	72.4
13	74.7	74.6	74.7
14	75.9	77.4	74.4
15	61.2	59.5	62.9
16	73.3	75.6	71.0
17	73.1	74.8	71.4
18	61.2	64.1	58.3
19	67.5	65.8	69.1
20	69	70	68
21	69	71	68
22	68	70	65
23	69	67	80
24	71	74	68
25	74	72	75
26	64	66	62
27	71	70	73
28	67.8	69.4	66.1
29	68	70	67
30	69	69	65
31	78	78	73
32	79	80	79
32 ^b	80	80	80
33	76	76	75
34	75	80	71
35	82	84	76
36	80	80	79
Average	78.5	78.8	78.2
SD	1.87	1.27	2.47

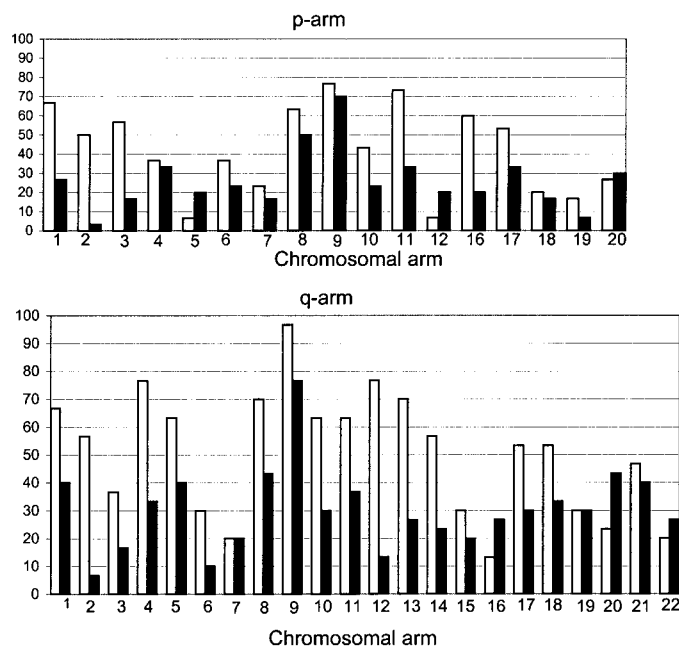


Fig. 2. Overall allelic imbalance/LOH fraction on p and q arms of the designated chromosome. The level of allelic imbalance/LOH corresponds well between SNPs (white) and microsatellites (black).

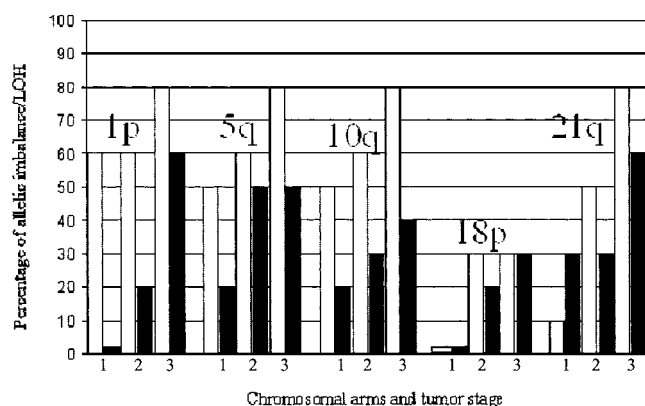


Fig. 3. Percentage of allelic imbalance/LOH on representative chromosomal arms. The level of allelic imbalance/LOH correlated with higher stages of tumor. 1, pT₀ tumor stage; 2, pT₁ tumor stage; 3, \geq pT₃ tumor stages. White bars, SNP analysis; black bars, microsatellite analysis. On chromosomal arm 1p, there were no losses in pT₀ tumors by microsatellite analysis, but the allelic imbalance detected by SNP assay was 60%. On chromosomal arm 18p, no allelic imbalance/LOH was detected in pT₀ tumors by either method.

by considering whole chromosomal arms. This comparison is shown in Table 4. In the supplementary data,² specific genome-wide information for each locus shows a generally high consistency across all of the chromosomal arms. Images of SNP markers and microsatellite markers

Table 3 Genome-wide consistency between microsatellite and SNP chip for LOH analysis by individual loci^a

Markers	Chr ^b	Match	Percentage (%)
D1S228	1p	10/11	90.9
D1S209	1p	1/1	100
D1S219	1p	14/16	87.5
D1S158	1q	19/23	82.6
AT3	1q	11/16	68.75
D2S162	2p	11/15	73.33
D2S147	2p	3/3	100
D2S136	2p	13/13	100
D2S111	2q	3/3	100
D2S143	2q	7/7	100
D2S126	2q	10/11	90.9
D3S1270	3p	9/9	100
D3S1597	3p	1/1	100
D3S1293	3p	13/15	86.66
D3S1292	3q	13/16	81.25
D3S1268	3q	9/10	90
D4S1582	4p	4/4	100
D4S404	4p	3/3	100
D4S174	4p	14/20	70
D4S1581	4p	3/4	75
D5S417	5p	9/10	90
D5S432	5p	6/7	85.71
D5S108	5p	9/10	90
D5S411	5p	9/12	75
D5S253	5q	13/15	86.66
D5S421	5q	13/22	59.09
CSFIR	5q	17/22	85
D5S504	5q	11/14	78.57
D6S260	6p	12/14	85.71
D6S265	6p	16/19	84.21
D6S261	6q	21/23	91.3
D6S292	6q	16/17	94.11
D7S481	7p	2/4	50
D7S507	7p	6/6	100
D7S488	7p	4/6	66.66
D7S495	7q	19/22	86.36
D7S486	7q	6/7	85.71
D8S1715	8p	1/2	50
LPL	8p	1/2	50
D8S261	8p	15/18	83.33
D8S257	8q	16/20	80
D8S275	8q	1/1	100
D8S273	8q	5/6	83.33
D9S144	9p	8/13	61.53
D9S1748	9p	20/25	80
D9S162	9p	5/5	100
D9S1752	9p	Not found	
D9S200	9p	10/16	62
D9S171	9p	5/5	100
D9S176	9q	15/18	83.33
D9S15	9q	12/19	63.15
GSN	9q	5/8	62.5
D9S12	9q	9/15	60
D10S226	10p	8/8	100
D10S249	10p	10/10	100
D10185	10q	13/13	100
D10S221	10q	13/15	86.66
D11S922	11p	15/19	78.94
D11S929	11p	8/11	72.22
D11S907	11p	11/17	64.7
D11S934	11q	18/24	75
D12S629	12p	10/12	83.33
D12S100	12p	1/1	100
D12S82	12q	4/5	80
D12S95	12q	16/17	94.11
D13S284	13q	3/5	60
D13S270	13q	16/17	94.11
D13S170	13q	1/1	100
D13S272	13q	4/4	100
D14S288	14q	23/25	92
D14S51	14q	1/2	50
D15S117	15q	8/11	72.72
D15S116	15q	21/23	91.3
D16S423	16p	10/11	90.9
D16S418	16p	5/7	71.42
D16S289	16q	12/14	85.71
D16S413	16q	12/16	75
CHRN1	17p	2/2	100
D17S1353	17p	18/23	78.26

Table 3 Continued^a

Markers	Chr ^b	Match	Percentage (%)
D17S952	17p	1/1	100
D17S804	17p	2/2	100
D17S250	17q	4/6	66.66
D17S579	17q	13/19	68.42
D18S59	18p	6/7	85.71
D18S52	18p	2/2	100
D18S67	18q	1/1	100
DCC	18q	19/23	82.6
D18S61	18q	1/2	50
D19S247	19p	5/5	100
D19S177	19p	10/12	83.33
D19S412	19q	4/5	80
D19S246	19q	20/21	95.23
D20S57	20p	6/9	66.66
D20S66	20p	4/5	80
D20S119	20q	11/12	91.66
D21S1257	21q	9/14	64.28
D21S263	21q	7/8	87.5
D21S259	21q	7/8	87.5
D21S11	21q	8/9	88.88
D21S1890	21q	1/1	100
D22S282	22q	15/16	93.75
ILRB1	22q	3/3	100

^a The consistency of allelic imbalance/LOH between the HuSNP assay and microsatellite analysis. See "Materials and Methods" for a description of how consistency was calculated.

^b Chr, chromosome arm.

from the same region of chromosomal arms from different patients are shown in Fig. 4.

The HuSNP chip assay detected a high frequency of allelic imbalance ($\geq 50\%$) in the 36 primary bladder cancers on chromosomal arms previously described (13–16). In addition, the assay detected a high incidence of allelic imbalance at chromosomal arms 12q, 16p, 1p, and 2q that was not previously reported. The pattern of allelic imbalance by SNP chips analysis is remarkably similar to that shown by microsatellite analysis, yet it clearly provides more information because of the presence of additional markers in regions sparsely populated by the microsatellites that we tested in both techniques. LOH of 9p and 9q were the most common events in bladder cancers, and the average number of losses (FAL) increased with more advanced pathological stage and grade by both of the techniques (Fig. 5). In one recurrent tumor from case number 32, allelic imbalance at 39 SNP loci were identical to those seen on the index tumor. Additionally, 84 new SNP losses (mostly on chromosomes 2, 8, and 9) were identified in the recurrent tumor.

DISCUSSION

The present study describes a high-resolution, genome-wide allelotyping of 36 primary human bladder cancers using the HuSNP chip. We validated our SNP assay data with "gold standard" microsatellite analysis using a subset of 30 normal and tumor DNA pairs in both assays. Observed heterozygosity (median, 341 SNPs) matched the expected distribution of heterozygosity as defined using biallelic SNP markers (9) and in previously reported HuSNP data (7). Previous allelotyping analyses of bladder cancer by our group (14) and others (16) were restricted to particular chromosomal regions or arms, or else used a relatively low density of markers. Very few reports have presented allelotyping data on multiple sites in the same tumor using two different methods. Our results represent, to date, the highest resolution of bladder cancer allelotyping with genome-wide coverage and allow for more comprehensive analysis. The HuSNP chip assay is high throughput and more automated than PCR-based microsatellite analysis at detecting LOH, but neither technique is currently infallible in identifying LOH. For fine mapping studies, an initial HuSNP chip

Table 4. The concordance of SNP and microsatellite analysis by chromosomal arms^a

Chr. arm	Match	Percentage (%)
1p	22/29	75.86
1q	24/28	85.71
2p	29/29	100
2q	24/28	85.71
3p	23/24	95.83
3q	23/26	88.46
4p	26/29	89.65
4q	20/26	76.92
5p	25/28	89.28
5q	26/29	89.65
6p	24/29	82.75
6q	24/27	88.88
7p	23/27	85.18
7q	24/27	88.88
8p	25/28	89.28
8q	27/27	100
9p	21/28	75
9q	28/29	96.55
10p	17/21	80.95
10q	30/30	100
11p	18/25	72
11q	24/28	85.71
12p	23/25	92
12q	22/24	91.66
13p	ND	ND
13q	21/25	84
14p	ND	ND
14q	20/29	68.96
15p	ND	ND
15q	22/25	88
16p	21/24	87.5
16q	25/28	89.28
17p	25/30	83.33
17q	24/27	88.88
18p	21/23	91.3
18q	27/29	93.1
19p	26/28	92.85
19q	25/27	92.59
20p	19/25	76
20q	15/17	88.23
21p	ND	ND
21q	25/29	86.2
22p	ND	ND
22q	24/27	88.88

^a The criteria for concordance was set up based on the informative markers on individual chromosomal arms. See "Materials and Methods" for details.

^b Chr., chromosomal; ND, not determined.

assay may need to be followed by additional allelotyping using dense polymorphic markers (SNPs or microsatellites) tested by individual assays.

Currently, the standard method for detecting LOH is PCR amplification of a specific locus, followed by size separation of the allele products on a denaturing polyacrylamide gel, followed by autoradiography. By this method, most studies have been limited to testing just a few chromosomal arms. Moreover, labor-intensive gel-based microsatellite assays are difficult to automate and are not readily scalable (17). They also entail additional labor in having to individually radioactive- or fluorescence-label many individual markers. This approach is also expensive and not readily available to most clinical laboratories. Scanning any portion of a chromosomal arm or region may miss smaller deletions especially valuable for the localization of a cancer gene (*e.g.*, *p16* on 9p). It remains to be seen whether SNP analysis can detect homozygous deletions, but preliminary evidence suggests that homozygous deletions can be resolved in microdissected tumor specimens as shown for microsatellites (18). Finally, scoring for LOH can be very subjective with microsatellite analysis and generally requires great expertise.

As expected, the degree of accordance between the two methods

was reasonably high. The results obtained by HuSNP assay were generally reproducible by microsatellite analysis when we compared locus to locus (Table 3). Moreover, when we compared the two methods by chromosomal arms (Table 4), the concordance of the two methods was very robust. The highest degree of discrepancy was observed in chromosomal arm 14q (32.04%). Chromosomal arm 14q (19) is a region of frequent cytogenetic alterations in bladder cancer but does not always reflect interstitial deletions or LOH. Thus, the nature of chromosomal alterations may also be responsible for the discrepancy in other chromosomal arms such as 11p, 9p, and 4q, which are also frequently altered in bladder cancer but have produced mixed results by different assays (20–22). Therefore, in cases with high discordance, the results need to be confirmed using additional techniques such as FISH or real-time PCR. Real-time PCR has the advantage that it can be performed using small numbers of tumor cells, and the need for normal reference DNA can be circumvented. In addition, this method will give exact information about the copy number of a given gene (23).

In several cases, a discrepancy appeared with the detection of LOH by microsatellite analysis but no detectable chromosomal imbalance by the HuSNP chip assay. This is probably caused by a no-signal genotype call either in tumor or in normal DNA or in both. This problem can be solved by increasing the number of SNPs for the specific loci and by developing a more sensitive method for the generation of calls. When we take into account the cutoff values for LOH detection by microsatellite analysis and the HuSNP assay threshold for the definition of loss of genetic material in our study, we conclude that microsatellite analysis may be somewhat more sensitive for the detection of genetic loss at a particular locus. However, re-evaluation of the respective SNP calls by using *Ps* (6) did not suggest that this will hamper the automation of the assay and may simply raise questions for generated calls by the quality control software. Most of the differences in our comparison study are probably attributable to: (a) limitation of mapping data for both microsatellite and SNPs; (b) differences in resolution of microsatellites and SNPs; (c) amplification efficiency and differential sensitivity of the two methods; (d) technical limitations such as a no-signal genotype call by the Affymetrix software; and (e) the presence of bad SNPs in the array.

The HuSNP chip assay provides several distinct advantages over microsatellite analysis: (a) the assay is accurate, automated, and readily adaptable to the clinical setting and high-density mapping. SNPs can be amplified by multiplex PCR (24) in contrast with microsatellite markers that generally require individual amplification reactions or at best only a limited multiplex assay; (b) analysis of the genetic alterations with the HuSNP assay saves considerable time over microsatellite analysis; (c) the assay involves multiplex amplification and other methods that can be completed in one day. All of the 36 samples were amplified and analyzed by SNP analysis by a single investigator in 6 weeks. Conventional microsatellite analysis of these samples consumed the time of more than one person/year; (d) the SNP array method is also a molecular technique that allows the detection of chromosomal imbalances in tumor DNA prepared from fresh or archival material. Archival pathology specimens are a valuable resource for the genetic analysis of tumors but are limited in the quantity and quality of the extractable DNA. Formalin, the most commonly used fixative for pathology tissue specimens, has been shown to reduce the size of PCR segments (often to <200 bp) that may be amplified from the samples (25, 26). Tissues frozen in OCT media for frozen section diagnosis suffers less of a direct insult to DNA quality but are still subject to handling and storage exposure that may result in DNA fragmentation. Ideally, a genomic analysis technique for pathology specimens would maximize the data obtained from nanogram quantities of low-molecular-weight DNA. The HuSNP array has yielded genotype results that were reliable and

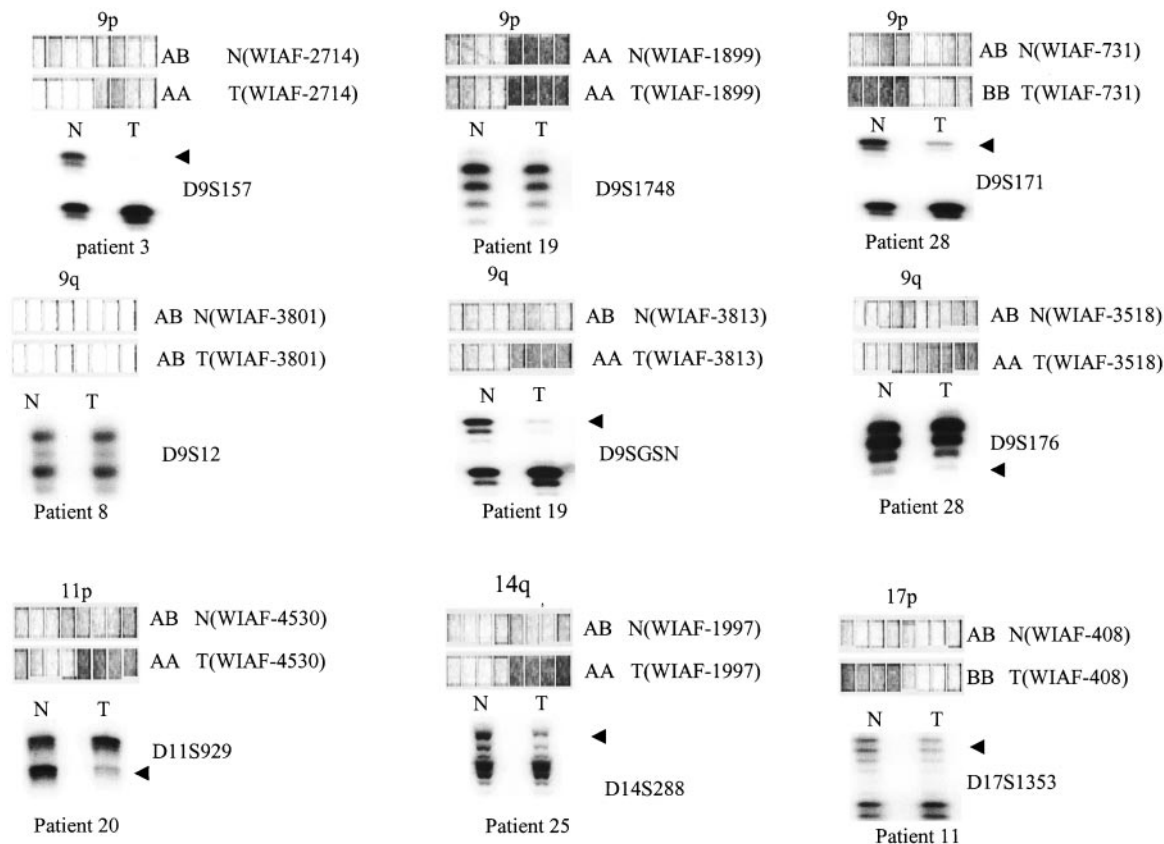
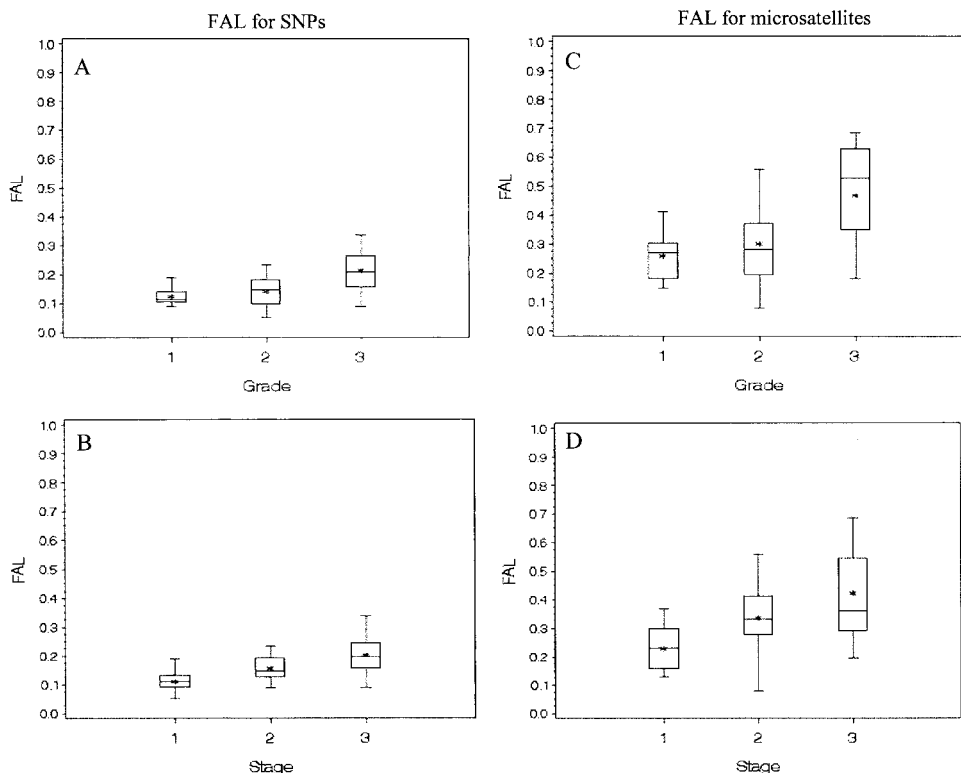


Fig. 4. Representative images of HuSNP and microsatellite allelic loss data for chromosomal arms 9p, 9q, 11p, 14q, and 17p. For each normal (N) and tumor (T) pair: *top panel*, the SNP results; *bottom panel*, the microsatellite results. For the SNP data, allelic imbalance was called based on the automated software-generated data and heterozygosity (AB) in the normal lymphocyte DNA. For the microsatellite data, LOH was scored in informative cases if a significant reduction (<50%) in the ratio of the signal from the tumor allele was observed in comparison with the corresponding normal allele in the adjacent lane. *Arrowheads*, presence of allelic loss.

Fig. 5. FAL index for all of the TCC grades (A) and stages (B) by the SNP analysis. *Bars*, the mean for each group of subjects (mean FALs are 0.12, 0.14, and 0.22 for grade 1, grade 2, and grade 3, respectively; mean FALs for stages are 0.11, 0.16, and 0.20 for pT_a, pT₁, and ≥ pT₂, respectively). The loss of all informative markers occurred significantly ($P = 0.04$) more often in stage 3 than in stage 2. Statistical comparison of stage 3 versus stage 1 was significant ($P = 0.0002$), whereas comparison of stage 1 versus stage 2 identified a trend ($P = 0.08$). The P s for grade 3 versus grade 2, grade 3 versus grade 1, and grade 2 versus grade 1 are 0.0007, 0.0007, and 0.62, respectively. *C*, and *D*, the FAL index for microsatellite analysis by grade and stage. (Stage 1 versus stage 2, $P = 0.09$; stage 1 versus stage 3, $P = 0.003$; stage 2 versus stage 3, $P = 0.16$; grade 1 versus grade 2, $P = 0.49$; grade 1 versus grade 3, $P = 0.005$; grade 2 versus grade 3, $P = 0.01$).



concordant for both fixed and frozen tumor (27); and (e) a minimal quantity (120 ng) of sample DNA is needed for each SNP assay. If the frequency of SNP heterozygosity is 50% lower than microsatellites, to analyze 750 microsatellite loci (one-half of the 1500 SNP loci) requires 15 μ g of DNA, which in some cases can be impossible to obtain from small clinical or paraffin-embedded samples.

There are also some limitations to the SNP assay: (a) a lower average heterozygosity of SNPs (0.33) compared with sequence tandem repeat. However, the identification and mapping of additional SNP markers is rapidly advancing; this will be helpful to have more informative loci in the region of interest; (b) the present version of the HuSNP array contains some regions of the genome in which the SNPs are clustered, in contrast to other sites in which SNPs are underrepresented. Efforts are under way to customize a second-generation HuSNP array that will contain 10,000 SNPs and will provide more information on genome localization.

The most frequent allelic deletion/allelic imbalances were at 9p and 9q. This is in agreement with other recent reports (28, 29). Molecular mapping studies have shown several candidate tumor suppressor genes on chromosome 9 (30–32), and it is probable that loss of chromosome 9 is an early event of bladder tumor formation. The other most frequently affected genetic alterations from our samples were on chromosome 1, 8, 16, 14, and 21, as described previously by other approaches (33, 34). Both FALs and specific losses were clearly associated with stage and grade progression. Our present data confirm and extend the previous findings of genetic alterations in bladder cancer on chromosomes 3, 4, 10, 13, and 17. Some chromosomal arms like 5p, 7q, 12p, 16q, 18p, 19p, 20p, 20q, and 22q were rarely altered in our set of samples.

In summary, our findings revealed high concordance between the HuSNP array and conventional LOH analysis by microsatellites. We were able to perform a genome-wide comparison with microsatellites where previously reported data (6, 7, 35) confirmed only a small panel of microsatellites on a limited number of chromosomal arms. Moreover, we used the latest SNP mapping information from Affymetrix, which has been compared with the whole genome sequence and is greatly improved over prior reports. We thus confirmed that allelic losses at multiple sites of the genome are frequent in bladder cancer, and we identified new areas of allelic imbalance. The data from this study validate the use of HuSNP arrays for the genotyping of human cancers and emphasize the potential of such high-throughput approaches for use in the clinical setting.

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