Progressive increase of genetic alteration in urinary bladder cancer by combined allelotyping analysis and comparative genomic hybridization

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Received September 11, 2008; Accepted November 10, 2008

DOI: 10.3892/ijo_00000222

Abstract. Bladder cancer is the ninth most common cancer in the world. Urothelial carcinoma (formerly known as transitional cell carcinoma) comprises the majority of bladder cancers. In order to decipher the genetic alteration leading to the carcinogenesis of urothelial cancer, we performed genome-wide allelotyping analysis using 384 microsatellite markers spanning 22 autosomes together with comparative genomic hybridization (CGH) in 21 urothelial cancer. High frequency of allelic imbalance was observed in chromosome arm 1q (61.9%), 3p (61.9%), 4q (66.67%), 8p (57.14%), 9p (76.2%) and 9q (66.67%). Allelic imbalance with frequency above average was also observed in chromosome arm 2q, 10p, 10q, 11p, 11q, 12q, 13q, 15q, 17p and 19q. The allelic imbalance of each case and fractional allelic loss for each chromosome was associated with higher tumor grade and stage ($P<0.05$). We have also delineated several minimal deletion regions on chromosome 3p, 4q, 8p, 9p, 9q, 11p, 13q, 16q and 17p. By CGH analysis, common chromosomal alterations included gain of 1p, 1q, 12q, 16p, 17q and 19p as well as loss of 4q and 9p in most of the cases. Our findings may provide valuable information to locate putative oncogenes and tumor suppressor genes in the carcinogenesis of bladder cancer in this locality.

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Key words: allelotyping, comparative genomic hybridization, bladder cancer

Introduction

Bladder cancer is the sixth most common cancer in the world (1). More than 200,000 new cases are diagnosed annually leading to >100,000 deaths per year worldwide (2). Urothelial cancer (formerly known as transitional cell carcinoma, TCC), which comprises the majority of bladder cancer displays multiple metachronous and synchronous multifocal occurence. Upon initial presentation, >80% of urothelial cancer are low-grade (grade 1 or 2), superficial tumors (≤pT1) (3). The 5-year survival rates for patients with such non-invasive tumors have a favorable prognosis in >80% of the cases (4). However, most of these patients are known to suffer local recurrence after endoscopic treatment (5). On the other hand, 20% of the tumors are of high-grade (grade 3) and stage (≥pT2) with a much less favorable prognosis and often progress rapidly. Therefore, understanding the molecular mechanism of bladder cancer would provide more information on the natural history of this disease and enable development of more effective treatment modalities.

Development of cancer is associated with multiple and accumulated genetic alterations in oncogenes and tumor suppressor genes (6). Previous cytogenetic studies found that loss of 3p, 11p, 13q, monosomy of chromosome 9 and trisomy of chromosome 7 existed in bladder cancer (7-10). LOH (loss of heterozygosity) study suggesting the loss of a tumor suppressor gene (TSG), has been reported for many chromosome arms in bladder cancer including 3p, 8p, 9p, 9q, 13q and 17p (11-17). Previously, CGH (comparative genomic hybridization) study also found that high incidence of loss and gain of chromosome arms 1q, 2q, 3p, 4q, 9p&q, 13q and 17q in bladder cancer (18-20).

To elucidate the critical genetic events leading to the carcinogenesis of bladder cancer, we performed combined allelotyping analysis and comparative genomic hybridization (CGH) on 21 cases of bladder TCC with different grade and stage. The frequency of chromosomal deletion and the extent of deleted regions on all autosomal arms were determined.

Minimal deletion regions (MDR), which identified novel tumor suppressor loci were also mapped in this study.

Materials and methods

Tissues samples. Twenty-one frozen samples of bladder tumor tissue from transurethral resection specimens were obtained at the Prince of Wales Hospital. There were 5 grade 1 cases, 11 grade 2 cases and 5 grade 3 cases. The male to female ratio was 2.5:1 and their ages ranged from 48 to 94 years with the median of 70 years. Detailed clinicopathological parameters are listed in Table I.

DNA extraction. DNA were extracted by phenol/chloroform method. H&E-stained sections from each tumor sample were examined by an experienced pathologist (K.F. To) to confirm their histological diagnosis and assess the tumor content. If tumor content (number of tumor cells/total number of cells) was <80%, tumor content was enriched by micro-dissection using a fine needle under a dissection microscope, as previously described (21). For normal control, peripheral blood of the corresponding patients were used.

Genome-wide allelotyping. Genomic-wide allelotyping was performed in 21 cases of urothelial cancer samples. ABI PRISM linkage Mapping Set MD-10 (Applied Biosystems, Foster City, CA) containing 382 fluorescent-labeled microsatellite markers that spanned 22 autosomes was used. The average interval of loci is ~10 cM. The markers were grouped into 27 panels and each panel contained 10-20 primer pairs. Multiplex PCR examining 2 loci was performed in a 7.5 μl reaction volume containing 60 ng of DNA, 2.5 pmoles of each primer, 1X PCR buffer II, 0.2 mM dNTP, 2.5 mM $MgCl_2$ and 0.6 unit of AmpliTaq Gold DNA polymerase (Applied Bioystems). One hundred and ninety-two PCR reaction were performed at a time in an ABI PRISM 877 integrated thermal cycler (Applied Biosystems) at 95˚C for 15 min, follwed by 10 cycles of 94˚C for 15 sec, 55˚C for 15 sec and 72˚C for 30 sec and another 22 cycles of 89˚C for 15 sec, 55˚C for 15 sec and 72˚C for 30 sec. PCR products were pooled and separated by electrophoresis in 4% polyacrylamide gel with 6 M urea on an ABI PRISM 377 automated DNA sequencer. Paired up normal and tumor samples were loaded into consecutive lane which contained overlapping alleles of the same panel. Three fluorescent-labeled dyes, FAM, HEX and NED, were displayed on the ABI PRISM 377 as blue, green and yellow color, respectively. The data collected were analyzed using GeneScan analysis software v3.1 (Applied Biosystems). Fig. 4 illustrates the principle and procedures of allelotyping analysis.

Assessment of allelic imbalance. For each informative locus of the urothelial cancer samples and its corresponding normal control, allelic imbalance (AI) was calculated. It was defined by calculating the allelic ratio (AR) of normal DNA (N1/N2) to the ratio of tumor DNA (T1/T2), where AR was the ratio of peak height of the smaller allele (allele 1) to that of the larger allele (allele 2). Allelic imbalance was considered when the AI value was <0.5 or >1.5. In this experiment, both allelic loss and gain were referred as allelic imbalances.

Table I. Clinicopathological parameters of 21 bladder UC cases.

Case	Gender	Age	Grade	MI[a]	Staging
5	M	94	1	N	Ta
24	F	70	1	N	Ta
27	M	64	1	N	Ta
43	M	53	1	N	Ta
50	M	48	1	N	Ta
55	M	87	2	N	Ta
56	M	61	2	N	Ta
57	M	71	2	N	Ta
3	M	70	2	N	T1
6	F	84	2	N	T1
33	M	84	2	N	T1
38	F	72	2	N	T1
45	M	59	2	N	T1
22	F	74	2	Y	T2,N0
47	M	65	2	Y	T3a,N0
34	M	68	2	Y	T3b,N0
8	F	73	3	N	T1
19	M	70	3	N	T1
26	M	63	3	N	T1
52	F	76	3	N	T1
23	M	82	3	Y	T2,N0

[a]MI, muscle invasiveness; Y, yes and N, No.

Comparative genomic hybridization (CGH) and digital image analysis. CGH was performed as previously described (22). In brief, tumor DNA and normal reference DNA were labelled with biotin-16-dUTP and digoxigenin (dig)-11-dUTP (Boehringer-Mannheim, Mannheim, Germany), respectively, by nick translation. Biotin-labelled tumor DNA and gender mismatched dig-labelled normal reference DNA (800 ng each) were precipitated together with 40 μg of Cot-1 DNA (BRL, Gaithersburg, MD). The mixed probe was then hybridized to slides with metaphase cells prepared from the blood of a healthy male donor. Biotin-labelled DNA was detected by avidin-conjugated fluorescein isothiocyanate (FITC) (Vector, Burlingame, CA) and dig-labelled DNA was detected by mouse monoclonal anti-dig antibody (Sigma, St. Louis, MO) and rabbit anti-mouse IgG-conjugated tetramethylrhodamine isothiocyanate (TRITC) (Sigma). Digital images of TRITC, FITC and DAPI fluorescence were captured separately by 3 band pass filters (TRITC, FITC and DAPI) set in a cooled CCD camera connected to a Zeiss (Jena, Germany) fluorescence microscope and calculated with a digital imaging system (ISIS3; Metasystems, Sandhausen, Germany).

Statistical analysis. Comparison of fractional allelic loss (FAL) or AI to clinicopathological parameters were assessed by Kruskal-Wallis Test or Mann-Whitney test whichever appropriate. All statistical analysis was performed by SPSS software (version 10.0; SPSS, Inc). A two-sided P-value of <0.05 was considered statistically significant.

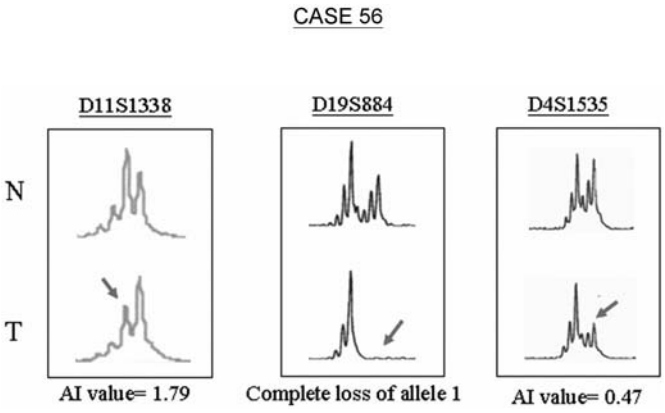

Figure 1. Representative results of microsatellite analysis in D11S1338, D19S884 and D4S1535 of patient #56. PCR reaction for each microsatellite markers was performed in bladder cancer tissue (T) and corresponding peripheral blood (N) which was acted as control. For informative markers, it should result in the amplification of both paternal and maternal alleles in peripheral blood sample. Allelic imbalance (AI) resulted in differential loss of either of the alleles (D11S1338 and D4S1535) or complete loss of one allele (D19S884) can be visualized by peak height or calculated by AI values as indicted (please refer to Materials and methods). Arrows indicate the loss of each allele.

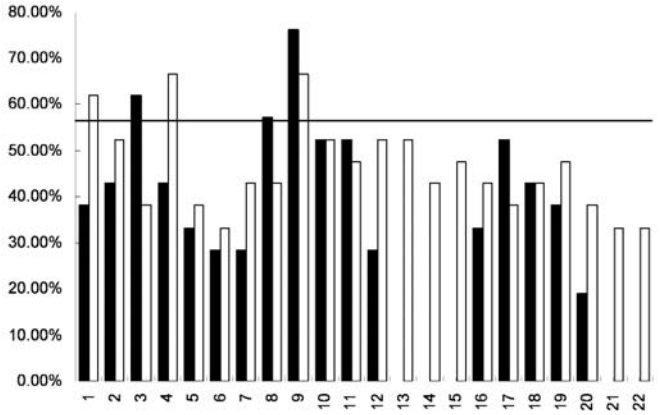

Figure 2. Summarized results of allelic imbalances at each chromosomal arm in 21 cases of urothelial cancer. Black and white bars denote short (p) and long (q) arms, respectively. The solid line represents the cut-off baseline and is defined as the mean percentage of allelic imbalance plus one standard deviation (56.74%).

Results

Progressive increase in allelic imbalance (AI) on 21 bladder cancer patients. We have investigated the allelic status of 21 urothelial cancer samples using a panel of 382 microsatellite markers mapped to 22 autosomes. An average of 257 (67.2%) informative loci was detected in the samples. Representative results of allelic imbalance at selected chromosomes and microsatellite loci are illustrated in Fig. 1. Allelic imbalances (AI) of each cases and each chromosomal arm were calculated. The AI% of the cases ranged from 1.52 to 69.64%. Frequencies of AI for individual chromosomal arms ranged from 19.05% (20p) to 76.19% (9p) (Fig. 2). The mean percentage of AI was 44.69±12.05%. In this experiment, AI >56.74% (mean percentage + 1SD) was considered to be significant. Significant allelic imbalances above the baseline (56.74%) were identified on chromosomal arms 1q (61.90%), 3p (61.90%), 4q (66.67%), 8p (57.14%), 9p (76.19%) and 9q (66.67%) (Fig. 2). In addition, chromosomal arms with frequencies of allelic imbalances higher than the mean percentage (44.69%) were also identified on 2q (52.38%), 10p (52.38%), 10q (52.38%), 11p (52.38%), 11q (47.62%), 12q (52.38%), 13q (52.38%), 15q (47.62%), 17p (52.38%), and 19q (47.62%).

Minimal deletion region (MDR) was identified in several chromosome arms. In this experiment, several MDRs on the chromosomal arms that were frequently deleted in urothelial cancer especially in high-grade tumors were identified. These regions were located on chromosomes, 3p, 8p, 9p, 9q and 16q. Fig. 3 summarized the nine MDRs that were defined by the tumors containing deletion on these chromosome arms. Frequent deletion (>50%) was found in 4q35.1 to 4qter, 8p21.1 to 8p22, 9p13.2 to 9p21.3 and 9q34 to 9qter. Moreover, deletion of 16q22.3 to 16q23.1 and 17p13.1 to 17p13.2 was significantly associated with staging and grading respectively (Kruskal-Wallis, P=0.008).

Increased allelic imbalance was correlated with advanced stage tumor. The fractional allelic loss (FAL) for each tumor was also determined. In this experiment, FAL ranged from 0.08 in case 50 to 0.97 in case 34 (Fig. 4). FAL of each case was significantly correlated with higher grade (Kruskal-Wallis, P=0.014); stage (Kruskal-Wallis, P=0.007); and muscle invasiveness (Mann-Whitney, P=0.001) of the tumor. On the other hand, allelic imbalances of chromosomal arm 2p, 2q, 3p, 3q, 5q, 8q, 14q, 16p, 16q, and 17p and 18p were significantly associated with higher grade or stage of the tumor (Kruskal-Wallis, P<0.05) (Table II).

CGH identified chromosome gains and losses in urothelial cancer. Although the allelic imbalance was presented in several chromosomal arms, some of observations may be due to chromosomal gain. To gain insight into this issue, we performed comparative genomic hybridization (CGH) on 20 urothelial cancer samples (one sample did not have enough DNA) and 2 bladder cancer cell lines (J82 and UM-UC-3). Common chromosomal alteration included gain of 1p, 1q, 12q, 16p, 17q and 19p as well as loss of 4q and 9p in most of the cases (Fig. 5). In this study, gain of chromosomes were detected more frequently than losses. Among them, gain of chromosome 1p (75%) was frequently observed. This result is in agreement with the previous studies of Chinese bladder cancer patients on different geographic locations (23,24). By combining data from allelic imbalance and CGH, chromosomal region that were previously classified as deletion may be due to gene amplification (Fig. 6). However, the genetic alteration identified by CGH has lower frequency than the changes identified by microsatellite markers. It may be due to the fact that CGH has a much lower resolution than our microsatellite analysis.

Discussion

In the present study, a total of 382 loci at 22 autosomes of 21 bladder cancer samples were examined. This genome-wide allelotyping study has generated an accurate and clear-cut profile of the chromosomal abnormalities of bladder cancer.

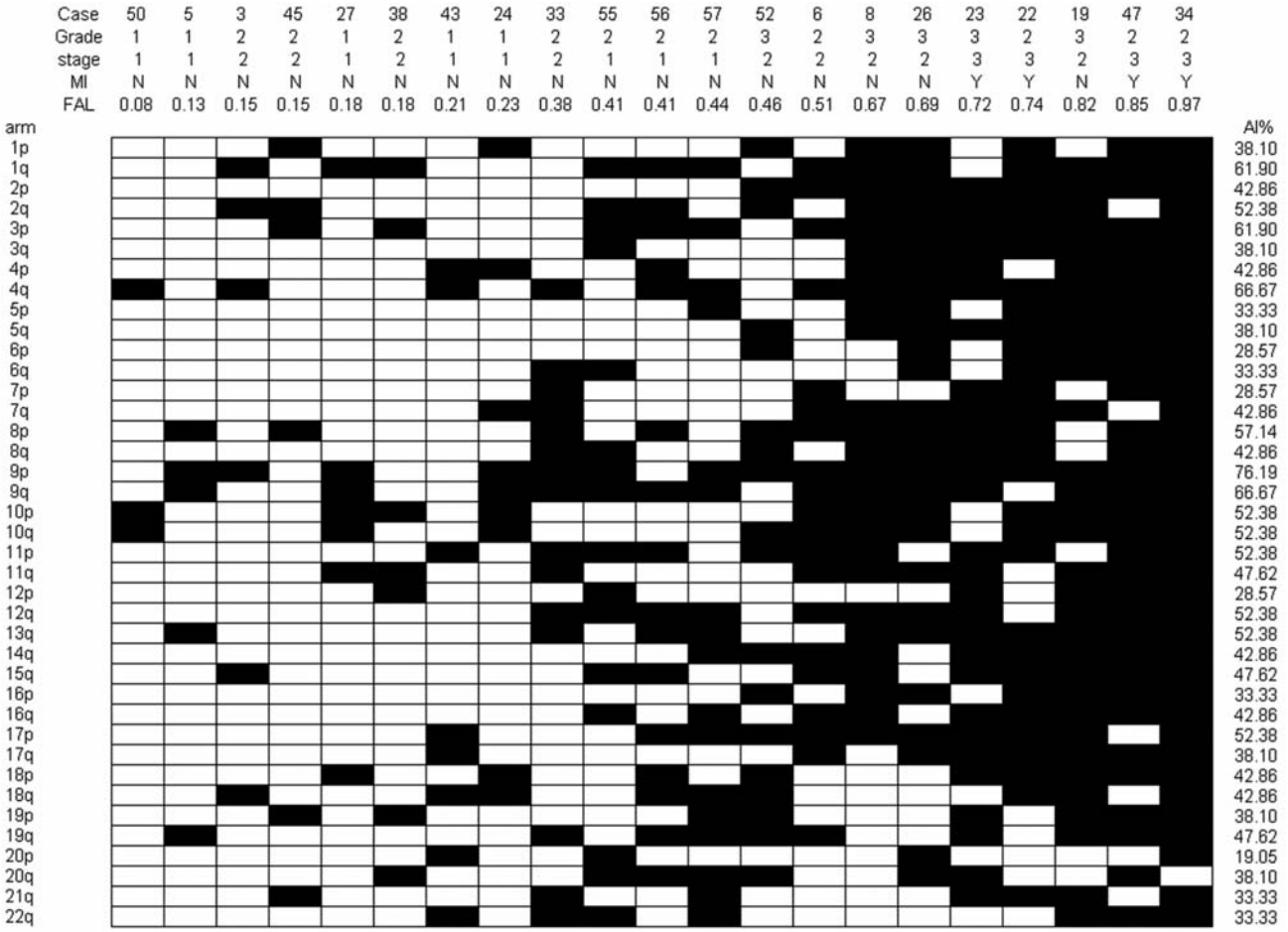

Figure 3. Allelotyping analysis of 21 cases of urothelial cancer. Top panel, case number, grade and stage of the cases. The fractional allelic loss (FAL) for each case and percentage of allelic imbalance (AI%) for each chromosome arm are shown. (■, AI; □, retention of heterozygosity).

Chromosomal region	markers in between	Approx. size	cases defined with MDR: case	50	5	3	45	27	38	43	24	33	55	56	57	52	6	8	26	23	22	19	47	34	MDR frequency	*P* (grade)	*P* (stage)
			grade	1	1	2	2	1	2	1	1	2	2	1	1	2	2	2	2	3	3	2	3	3			
			stage	1	1	2	2	1	2	1	1	2	1	1	1	2	2	2	2	3	3	2	3	3			
3p14.2 to 3p24.2	D3S1266 and D3S1285	43.2cM																							23.8%	0.325	0.37
4q35.1 to 4qter	D4S1539 and D4S426	40.6cM																							52.4%	0.176	0.055
8p21.1 to 8p22	D8S550 and D8S505	41.0cM																							52.4%	0.176	0.055
9p13.2 to 9p21.3	D9S157 and D9S1817	23.2cM																							57.1%	0.97	0.563
9q34 to 9qTer	D9S290 and D9S158	22.1cM																							57.1%	0.97	0.563
11p15.3 to 11p15.5	D11S4046 and D11S902	18.2cM																							47.6%	0.267	0.474
13q11 to centromere	D13S175 and D13S217	6.8cM																							47.6%	0.176	0.074
16q22.3 to 16q23.1	D16S503 and D16S516	21.6cM																							33.3%	0.354	**0.008**
17p13.1 to 17p13.2	D17S831 and D17S799	27cM																							47.6%	**0.008**	0.232

Figure 4. Summary of 9 minimal deletion regions (MDR) discovered on 21 cases of urothelial cancer. The chromosome region, size and corresponding microsatellite markers for each MDR are indicted. The P-value (Kruskal-Wallis test) for grade and stage for each MDR is also shown (■, AI; □, retention of heterozygosity).

In this study, frequent allelic imbalances were found in 1q, 2q, 3p, 4q, 8p, 9p and q, 10p and q, 11p and q, 12q, 13q, 15q, 17p, and 19q. Moreover, the data demonstrated an increase in chromosomal aberrations upon higher grade and stage of tumors which was in keeping with previous studies that increased genomic instability was observed in tumor development and progression (12,25).

Bladder cancer development and progression is thought to result from an accumulation of multiple genetic events, which provide a selective growth advantage for the cancer cells (12). Some genetic changes have been associated with superficial tumors, whereas others are commonly associated with invasion, suggesting that certain genetic abnormalities may be responsible for initiation, while others are responsible for progression (12). Identifying the genetic pathways involved in bladder cancer development would help us to better understand the natural history of this disease and develop more effective treatment modalities.

In allelotyping analysis, the differential peak heights between alleles can be due to gain or loss of one allele, leading to allelic imbalance. In order to verify the allelic status of polymorphic status, comparative genomic hybridization

Table II. Association between allelic imbalance (AI) on chromosomal arm and tumor grade/stage.

		Tumor grade				Tumor stage			
Chromosome arm	AI[a]	1	2	3	P-value[b]	Ta	T1	≥T2	P-value
2p	+	5[c]	7	0	0.006	8[c]	4	0	0.003
	-	0	4	5		0	5	4	
2q	+	5	5	0	0.008	6	3	1	0.152
	-	0	6	5		2	6	3	
3p	+	5	2	1	0.006	5	3	0	0.114
	-	0	9	4		3	6	4	
3q	+	5	7	1	0.039	7	6	0	0.015
	-	0	4	4		1	3	4	
5q	+	5	8	0	0.004	8	5	0	0.004
	-	0	3	5		0	4	4	
8q	+	5	6	1	0.043	7	5	0	0.019
	-	0	5	4		1	4	4	
14q	+	5	6	1	0.043	7	5	0	0.019
	-	0	5	4		1	4	4	
16p	+	5	8	1	0.027	8	5	1	0.026
	-	0	3	4		0	4	3	
16q	+	5	5	2	0.094	6	6	0	0.041
	-	0	6	3		2	3	4	
17p	+	4	6	0	0.038	5	4	1	0.015
	-	1	5	5		3	5	3	
18p	+	3	7	2	0.681	5	7	0	0.036
	-	2	4	3		3	2	4	

[a]+, Retention of heterozygosity; -, LOH or AI. Only chromosome arms with $p<0.05$ are shown. [b]Calculated from Kruskal-Wallis test. [c]Number of cases.

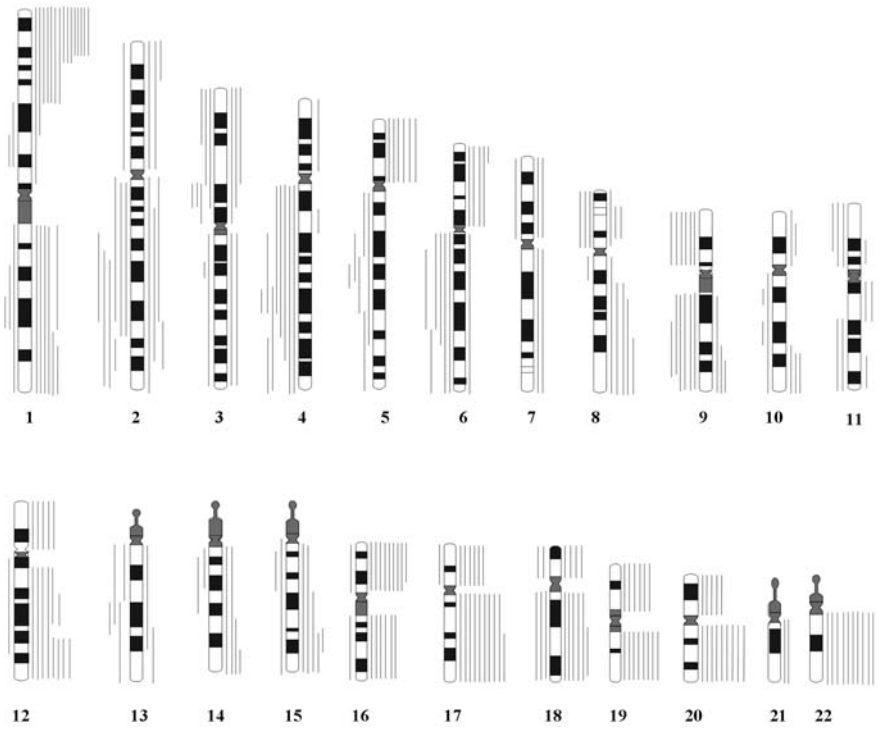

Figure 5. A summary ideogram of gains (right) and losses (left) of chromosomal regions seen by CGH in 20 cases of urothelial cancer.

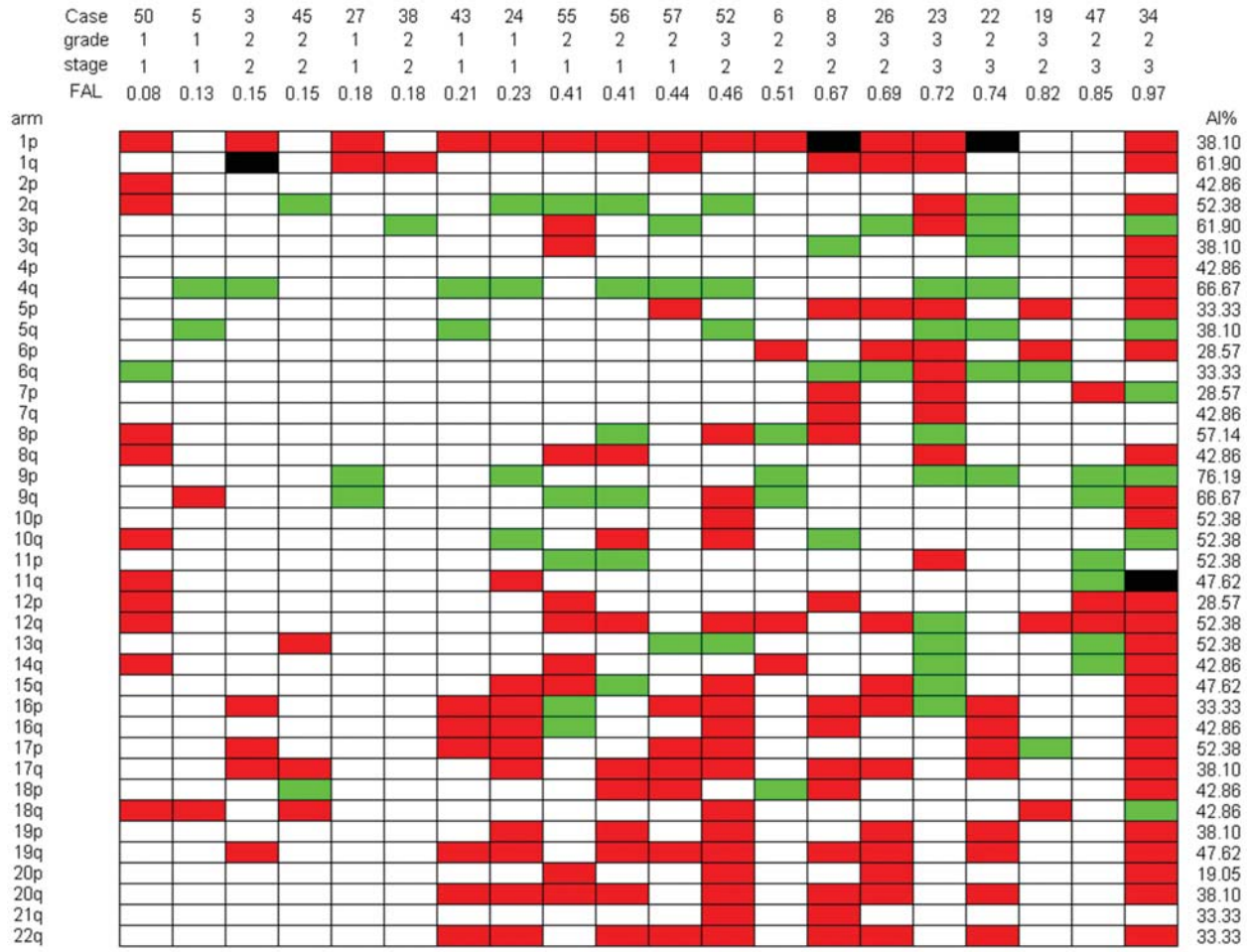

Figure 6. Summary of allelotyping and CGH analysis on 20 cases of urothelial cancer. The result from CGH on each chromosomal arm is indicated by color box; green, gains; red, losses; black, both, gains and losses. The FAL and AI% of each case and chromosomal arm as determined by allelotyping analysis are indicated.

(CGH) has to be performed. CGH results from our present study, and those of others, found that frequent chromosome losses are on 3p, 4p, 6q, 9p, 9q, 8p, 11p, 13q, 17p, and 18q while chromosomes gains are on 1q, 3q, 5p, 6p, 7p, 8q, 10q, 11q, 13q, 17q, 20q (12,19,20,25-30). As a result, the high frequency of allelic imbalance of chromosome 1q, 10q, and 11q, detected in this study was probably due to chromosomal gain.

In this study, we have identified that several MDR regions on chromosome arms were frequently deleted in bladder cancer suggesting that important tumor suppressor genes are located in this region. LOH of chromosome 3p was found in 61.9% in all our cases. Furthermore, we delineated a 43.2cM MDR region on 3p14.2 to 3p24.2 flanked by D3S1266 and D3S1285. This result confirmed previous studies that chromosome 3p is frequently deleted in bladder cancer (11-13,26,28). Previously, we have also demonstrated that genetic and epigenetic mechanisms cooperate together to down-regulate *RASSF1A*, one of the important tumor suppressor genes residing on this chromosomal region (31). Taken together, deletion of 3p may be a common event in bladder cancer.

Loss of chromosome 8 was also found in 57% of all our cases. Among those cases, a 41.0cM MDR on 8p22 was found. Frequent LOH of this region has been described previously in bladder cancer (14,32,33). Homozygous deletion has also been identified in prostate cancer and medulloblastoma (34-36) suggesting that multiple TSGs are located in this region. *Fez1/Lzts1* (leucine zipper, putative tumor suppressor 1) is a potential tumor suppressor gene located at this region (37). Expression of FEZ1/LZTS1 was reduced or absent in lung, gastric and bladder cancer (38-40). Introduction of FEZ1/LZTS1 into *Fez1/Lzts1*-negative bladder, prostate and breast cancer cells results in suppression of tumorigenicity and reduced cell growth *in vitro* and *in vivo* (37,39,41). FEZ1/LZTS1 was found to be associated with microtubule components and interacts with $p34^{cdc2}$ at late S-G_2/M stage *in vivo* (41). Functional experiment also revealed that FEZ1/LZTS1 inhibited cancer cell growth by stabilizing active $p34^{cdc2}$ during mitosis and that alterations of Fez1 led to early exit from mitosis (41). Taken together, *FEZ1/LZTS1* may be a potential TSG in this chromosomal region and participates in the carcinogenesis of bladder cancer.

MDR has also been found in chromosome arm 9p and q. In bladder cancer, previous studies found that loss of chromosome 9 was the most frequent deletion region suggesting that multiple TSG residing in this chromosome (12). On chromosome 9p, an MDR was found between 9p13.2 and 9p21.3 where the negative cell cycle regulators p14, p15 and p16 reside. Homozygous deletion of these

regions was common in bladder cancer and cell lines (42-46). On chromosome 9q, an MDR was found on 9p34 to 9pter. This region was the second most frequently deleted region in chromosome 9 (47-50). Recently, *DAP kinase* (death-associated protein kinase) and *DBCCR1* (deleted in bladder cancer gene 1) which is located at this region was found to be hyper-methylated in bladder cancer (51-53) thus suggesting that these 2 genes may be the candidate TSGs located in this region. MDR has been mapped on 16q22.3 to 16q23.1, however, no known TSGs were mapped within this region. TSGs such as *E-cadherin* which involves cell-cell interaction is located adjacent to this region. Loss of *E-cadherin* was correlated with grade, stage and poor survival in bladder cancer (54,55).

Although the resolution (~10 Mb) in this study is much lower than the studies performed by array CGH (25,29) or high density SNP microarray (56,57), our current study based on microsatellite markers have identified several genetic alterations in bladder cancer of Hong Kong Chinese population, which may have unique carcinogenesis pathway from other geographical locations. For example, a study found that arsenic exposure resulted in different genetic abnormalities in bladder cancer patients of Taiwan (23). Thus, our results provide valuable information to better understand carcinogenesis and biology of bladder cancer in this locality.

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