Bladder Cancer Stage and Outcome by Array-Based Comparative Genomic Hybridization

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Abstract

Purpose: Bladder carcinogenesis is believed to follow alternative pathways of disease progression driven by an accumulation of genetic alterations. The purpose of this study was to evaluate associations between measures of genomic instability and bladder cancer clinical phenotype.

Experimental Design: Genome-wide copy number profiles were obtained for 98 bladder tumors of diverse stages (29 pT_a, 14 pT_1, 55 pT_{2-4}) and grades (21 low-grade and 8 high-grade superficial tumors) by array-based comparative genomic hybridization (CGH). Each array contained 2,464 bacterial artificial chromosome and P1 clones, providing an average resolution of 1.5 Mb across the genome. A total of 54 muscle-invasive cases had follow-up information available. Overall outcome analysis was done for patients with muscle-invasive tumors having "good" (alive >2 years) versus "bad" (dead in <2 years) prognosis.

Results: Array CGH analysis showed significant increases in copy number alterations and genomic instability with increasing stage and with outcome. The fraction of genome altered (FGA) was significantly different between tumors of different stages (pT_a versus pT_1, $P = 0.0003$; pT_a versus pT_{2-4}, $P = 0.02$; and pT_1 versus pT_{2-4}, $P = 0.03$). Individual clones that differed significantly between different tumor stages were identified after adjustment for multiple comparisons (false discovery rate < 0.05). For muscle-invasive tumors, the FGA was associated with patient outcome (bad versus good prognosis patients, $P = 0.002$) and was identified as the only independent predictor of overall outcome based on a multivariate Cox proportional hazards method. Unsupervised hierarchical clustering separated "good" and "bad" prognosis muscle-invasive tumors into clusters that showed significant association with FGA and survival (Kaplan-Meier, $P = 0.019$). Supervised tumor classification (prediction analysis for microarrays) had a 71% classification success rate based on 102 unique clones.

Conclusions: Array-based CGH identified quantitative and qualitative differences in DNA copy number alterations at high resolution according to tumor stage and grade. Fraction genome altered was associated with worse outcome in muscle-invasive tumors, independent of other clinicopathologic parameters. Measures of genomic instability add independent power to outcome prediction of bladder tumors.

Bladder cancer comprises of highly heterogeneous tumors. Approximately 70% of tumors are superficial (stages pT_a, pT_1, pT_{is}), whereas the remaining are muscle infiltrating (stages pT_2-pT_4) at the time of initial presentation. Muscle invasive tumors are thought to arise *de novo* or within an area of carcinoma *in situ*, as well as occasionally developing from previously superficial tumors. Patients with superficial and invasive tumors have remarkably different 5-year survival rates (1) reflecting their biological differences.

Like other solid tumors, it has been suggested that this morphologic heterogeneity originates from underlying genetics leading to diverse pathways of tumor development and progression. There are a number of studies indicating that there is considerable variability in the degree to which bladder tumors are altered at the chromosomal level and the spectrum of alterations can vary depending on the grade of differentiation and the tumor stage.

Cytogenetic and loss of heterozygosity studies of bladder carcinomas and cell lines have revealed a number of recurrent genetic aberrations including amplifications or gains on 8q22-24, 11q13, 12q14-15, 17q21, and losses on chromosomes 9, 8p22-23, 13q, and 17p (2, 3). Some of these aberrations have been associated with the pathologic stage and/or outcome of

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Note: Supplementary data for this article can be found at http://cc.ucsf.edu/people/waldman/bladder/blaveri.cgh.

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bladder cancer. The advent of array-based comparative genomic hybridization (CGH) has allowed the high-throughput mapping of DNA copy number alterations in bladder cancer (4, 5) and in other tumors (6, 7). The patterns of copy number alterations identified by array CGH have been reported to aid in differentiating tumors into more biologically and clinically relevant subtypes and the higher resolution has led to precise mapping of the boundaries of amplified and deleted regions indicating candidate genes relevant to cellular control pathways.

Array CGH allows a unique view of the genomic instability that a tumor has undergone before diagnosis. Both the amount of genomic copy number alteration and the specific loci involved are defined in one analysis. Different representations of instability, such as whole chromosome gain or loss, partial or complete chromosome arm changes, high-level amplification, and homozygous deletions, are easily quantified. The type, degree, and locations of these changes may have prognostic and therapeutic implications.

In this study, we report array-based CGH analysis in a set of 98 transitional cell carcinomas of the bladder of different stage, grade, and outcome. A high frequency of DNA copy number alterations was detected involving regions previously documented in bladder cancer. Most importantly both the overall frequency of alterations and the frequency of individual clones were significantly different between tumors with different clinicopathologic characteristics. The fraction of genome altered (FGA) was significantly associated with overall outcome in patients with muscle-invasive disease.

Materials and Methods

Samples and DNA preparation. Freshly frozen tissue was collected from 98 patients with bladder cancer from the University of California San Francisco Medical center from 1991 to 2003. Patients provided signed informed consent for participation in this study that was approved by the University of California San Francisco institutional review board. For each case, all blocks from the surgical specimen were reviewed to determine pathologic grade, stage, and histologic tumor type. Tumors were staged according to the American Joint Committee on Cancer (8) and graded according to the WHO and International Society for Urological Pathology classification system (9).

Before DNA extraction, an initial H&E-stained frozen section was reviewed to assess tumor quality and content. Normal and necrotic tissue were excluded by trimming of the frozen block. A tumor sample was considered suitable for study if the proportion of tumor cells was higher than 70%. DNA was extracted from tissue using standard proteinase K digestion followed by phenol/chloroform extraction (protocol available at http://cc.ucsf.edu/people/waldman/Protocols). Normal genomic DNA was obtained from Promega (Madison, WI) and was used for reference.

Array comparative genomic hybridization. Human array 2.0 chromium surface arrays were provided by the University of California San Francisco Cancer Center Array Core. Each array consisted of 2,464 bacterial artificial chromosome and P1 clones printed in triplicate, providing representation of the entire genome at a 1.5 Mb resolution (10).

A total of 500 ng of each sample DNA was random prime labeled using the BioPrime kit (Invitrogen, Carlsbad, CA; protocol available at http://cc.ucsf.edu/people/waldman/Protocols). Tumor DNA was labeled with FluoroLink Cy3-conjugated dUTP and normal genomic DNA was labeled with FluoroLink Cy5-conjugated dUTP (Amersham Pharmacia, Piscataway, NJ). Excess primers and nucleotides were removed using Sephadex G50 columns (Amersham Pharmacia). Labeled tumor and reference DNA were combined with 100 μg Cot1 DNA (Invitrogen) and the mixture was precipitated using 3 mol/L sodium acetate. Precipitated sample was dissolved in a solution containing 600 μg yeast tRNA, 9% SDS, 50% formamide, 10% dextran sulfate, and 2× SSC. The DNA probe was then denatured at 73°C for 15 minutes, cooled at 37°C for 30 minutes, and applied to the array. The slide was finally placed at 37°C for 48 hours in a sealed hybridization chamber containing 50% formamide/2× SSC for humidity. After hybridization, each slide was washed with 50% formamide and 2× SSC twice at 45°C for 12 minutes, followed by two washes in phosphate buffer with 0.1% NP40 (pH 8.0). Slides were mounted in 90% glycerol in phosphate buffer containing 4′,6-diamidino-2-phenylindole at a concentration of 0.3 μg/mL and coverslipped.

A number of normal male DNA versus normal female DNA hybridizations were done to identify outliers and polymorphic clones, which were excluded from further analysis.

Image and data processing. Three, 16-bit fluorescence single-color intensity images (4′,6-diamidino-2-phenylindole, Cy3, and Cy5) were collected from each array using a charge coupled device camera (Sensys, Photometric, equipped with a Kodak KAF 1400 chip) coupled to a 1× optical system, as previously described (11). The Spot 2.0 software program (available at http://www.jainlab.org/downloads.html) was used for image analysis (12). Intensities were determined for each spot by subtracting local background from foreground intensities. The following spots were excluded for further analysis: (*a*) spots that were <15 pixels in size, (*b*) spots with a correlation of <0.9 of the two fluorescent dyes or ones that were in the bottom 10th percentile, (*c*) spots that had a ratio of (reference intensity) / (sum of reference and test intensity) <0.1, and (*d*) spots with a test plus reference intensity of <200 or ones that were in the bottom 20th percentile. For each clone, the average ratio of test over reference intensity was calculated from the three replicate clones on the array. Clones whose ratio was derived from only one of three replicates and clones whose replicate ratios had a $\log_2$ SD > 0.33 were discarded. The human DNA sequence draft at http://genome.ucsc.edu (July 2003 freeze) was used to map clones. The $\log_2$ ratios for each case were median centered to zero. Thresholds for determining chromosome gain or loss were defined separately for each individual sample using a discrete time hidden Markov model (13) as implemented in the aCGH package of BioConductor open source software (http://www.bioconductor.org), correcting for varying signal-to-noise in each sample. A clone was declared aberrant if its absolute value exceeded the tumor-specific threshold computed as 2.5 times the estimate of the SD of the experimental noise for a given profile. A clone was considered amplified if it was part of a region (no larger than 10 Mb) whose absolute $\log_2$ ratio exceeded 0.9 and also exceeded the $\log_2$ ratio of its immediately flanking regions by at least 0.5. The algorithm also identified regions (also narrower than 10 Mb) whose $\log_2$ ratios exceeded immediately flanking segments by 0.9. The latter rule permitted the detection of amplifications originating out of regions present at decreased copy number. Clones with $\log_2$ ratios <−0.75 were considered high-level losses. These levels were chosen empirically due to the expected presence of normal cells in the specimens and tumor heterogeneity and are concordant with the thresholds used in the previously published array CGH articles (14).

The frequency of gains and losses for a given clone in a group of interest was calculated as the proportion of samples in which a clone was gained or lost in that group. As our CGH arrays do not cover the entire genome, each clone was assigned a genomic distance equal to the sum of half of the distance between its center and that of its two adjacent clones to quantitate the fraction of the genome altered.

Statistical analysis. Clones with significantly different copy number between different tumor groups were identified using a *t* statistic with pooled variance. Adjustment for multiple comparisons was made using false discovery rate.

Kruskal-Wallis nonparametric test was used to assess significance of the different measures of genomic instability (FGA, number of whole

chromosome changes, number of copy number transitions within a chromosome, total number of chromosomes containing transitions, amplifications, and deletions) between tumors of different stage, grade, and outcome.

Survival probabilities were estimated using Kaplan-Meier or Cox proportional hazards analysis in *R*. Variables were defined in either continuous or dichotomous fashion.

Prediction analysis for microarrays (15) was used to identify clones that could best predict each bladder tumor class. Prediction analysis for microarrays uses a modified version of the nearest centroids classification method, which "shrinks" the centroids by means of soft thresholding. Ten-fold cross-validation was used to choose a threshold, which minimized classification errors and selected a list of predictive genes. Missing values were estimated across the data set, using a 10 nearest-neighbor impute engine.

One-way unsupervised hierarchical clustering was done for tumors using Ward's method of linkage and Euclidean distance as a metric.

Results

Clinicopathologic characteristics of the samples. Ninety-eight primary bladder tumors of transitional histology were studied. These included 29 stage pT_a (21 low grade and 8 high grade), 14 pT_1, 15 pT_2, 25 pT_3, and 15 pT_4 tumors (all pT_1-pT_4 tumors were high grade; Table 1). The median age for all patients was 69 years. Transurethral resection was used to sample 27 of the 29 stage pT_a tumors and 11 of the 14 pT_1 tumors, whereas cystectomy was done for 51 of the 55 stage pT_2 or higher tumors. Outcome information was available for 54 patients with muscle-invasive disease (stage pT_2 or higher). Fifteen of these patients survived with a median follow-up of 42.8 months, whereas 39 patients died with a median survival of 8.8 months. A listing of clinicopathologic information can be found in Supplementary Data (Clinical.xls).

Stage- and grade-specific genomic profiles. Array-based CGH identifies copy number gains and losses on a clone-by-clone basis. Such copy number gains and losses were defined using tumor-specific thresholds for each sample (median threshold for all samples ± 0.25). A representative profile of array CGH of a primary bladder tumor is shown in Fig. 1. A file containing all primary data $\log_2$ ratios can be found in Supplementary Data (log2ratios.xls).

Table 1. Clinicopathologic characteristics

	n (%)
Stage	
pT_a	29 (29.6)
pT_1	14 (14.3)
pT_2	15 (15.3)
pT_3	25 (25.5)
pT_4	15 (15.3)
Gender	
Male	70 (71.4)
Female	28 (28.6)
Lymph node status	
Negative	27 (49)
Positive	16 (29)
Unknown	12 (22)
Clinical outcome*	
Alive (≥24 mo)	10 (23)
Dead (<24 mo)	34 (77)

*Muscle invasive tumors only.

Specific loci harboring recurrent alterations across tumors were seen. There was a high frequency of aberrations involving whole chromosomes, whole chromosome arms, and consecutive clones on chromosome arms. The frequency of copy number changes showed characteristic patterns according to stage (Fig. 2) and grade (Supplementary Data: GradeFrequency). Stage pT_a tumors had a 12% average frequency of alterations. The most frequent alteration in stage pT_a tumors was loss of clones across the entire chromosome 9 occurring with an average frequency of 47% for 9p and 46% for 9q. The clone containing *CDKN2A/p16* (CTB-65D18) had the highest frequency of loss in pT_a tumors at 65%. Stage pT_1 tumors had a higher average frequency of alterations than pT_a tumors (27%). The highest average frequency losses in this tumor set were on 11p (54%), whereas the highest average frequency gains were on 8q (55%). These tumors had a similar frequency of loss of clones on chromosome 9p (51%) and 9q (43%) as pT_a tumors. Finally, muscle-invasive tumors had an average frequency of alterations of 18%. Although these tumors retained the same spectrum of aberrations as pT_1 tumors, they generally showed lower frequencies. Clones with the highest frequency of loss in these tumors were on 8p (29%), whereas clones with the highest frequency gains were on 20q (26%). A list of the most frequently recurrent aberrations with their corresponding frequencies by chromosome arm can be seen in Table 2.

A direct comparison of tumors of different stage and grade was made to identify individual clones significantly altered between the groups. Robustness of the findings was assured by performing adjustment for multiple comparisons (false discovery rate ≤ 0.05). A list of the differentially altered regions between the different stages can be seen in Supplementary Data (stage.comparison.regions), whereas a comprehensive list of all these clones is in Supplementary Data (pT_a.vs.pT_1.signclones, pT_a.vs.pT_{24}.signclones, and pT_1.vs.pT_{24}.signclones).

Genomic instability. Differences in the extent of the genome affected were identified among the tumors of different stage and grade. The extent of the genome affected was defined as the FGA (as represented by the clones on our array). The median FGA for all the tumors studied was 17%. Stage pT_a tumors had the lowest median FGA at 9%, whereas pT_1 tumors had the highest median FGA at 27% (Kruskal-Wallis, $P = 0.0003$). Interestingly, muscle-invasive tumors had a median FGA of 16%, which was lower than that of pT_1 tumors (Kruskal-Wallis, $P = 0.03$; Fig. 3). When pT_a tumors were separated into low and high grade, low-grade tumors had a much lower FGA (median FGA = 8%) than that of high-grade tumors (median FGA = 20%; Kruskal-Wallis, $P = 0.097$; Supplementary Data: GradeFGA). A plot of the FGA distribution according to stage and grade can be seen in Supplementary Data (FGArange). Interestingly, a total number of seven pT_a and six muscle-invasive tumors with the lowest FGA (<3%) had no whole chromosome or chromosome arm changes, but showed only gains and losses of individual clones scattered over the genome. These clones do not seem to be due to polymorphisms or outliers based on the information from our normal-normal control hybridizations.

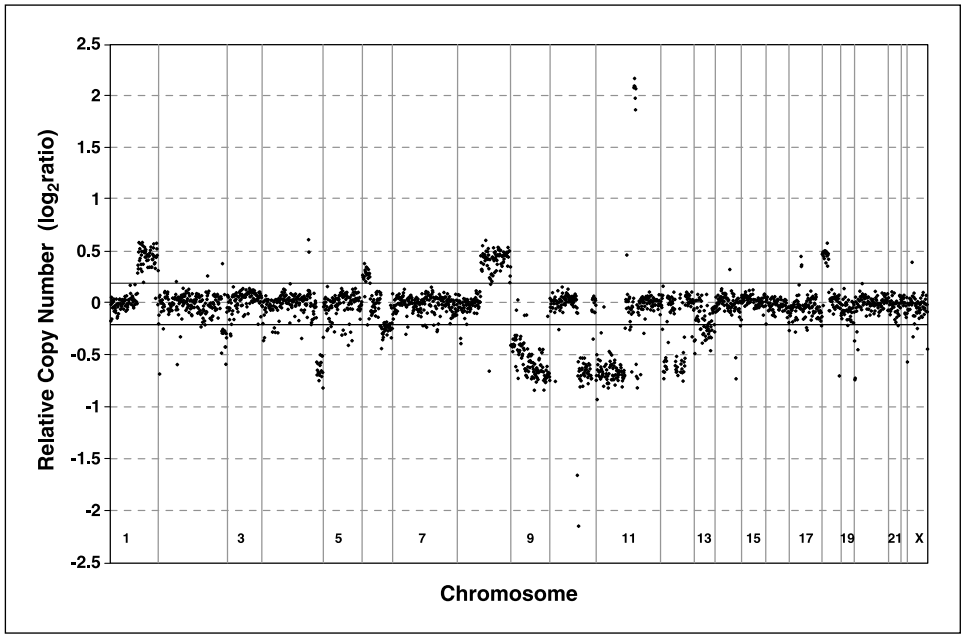

Fig. 1. Whole-genome array CGH profile from a primary bladder cancer (stage pT_a, low grade). Copy number changes relative to normal, sex-matched DNA are shown for each clone. Horizontal solid lines, tumor-specific $\log_2$ ratio threshold of 0.2 that was used in this case to determine clones gained (+0.2) or lost (−0.2). Clones are ordered from chromosomes 1 to 22 and within each chromosome on the basis of their map position (http://genome.ucsc.edu/version July 2003). Two of the most frequent changes in bladder cancer are seen, high-level gains (amplifications) in the region of cyclin D1 (11q13), and loss of clones across the entire chromosome 9.

FGA is a measure of genomic instability, representing the overall burden of gains and losses in each tumor. Other measures representing more specific mechanisms of chromosome alterations can also be defined, including (*a*) the number of whole chromosome changes, (*b*) the number of copy number transitions within a chromosome (representing unbalanced translocations or deletions), and the total number of chromosomes containing such transitions, (*c*) amplifications, and (*d*) deletions.

For all tumors, the number of copy number alterations involving whole chromosomes (median = 1) was significantly less frequent than alterations affecting entire chromosome arms or portions of arms (median = 8; Kruskal-Wallis, $P < 0.0001$).

Superficial tumors showed significant differences in all of the measures of genomic instability assessed according to both stage and grade. Stage pT_1 tumors had a greater number of whole chromosome changes ($P = 0.012$), number of transitions ($P = 0.044$), number of chromosomes containing transitions ($P = 0.004$), and number of amplifications ($P = 0.035$) than stage pT_a tumors. The number of high-level losses did not differ significantly between these two stages. When pT_a tumors were compared according to their grade, higher-grade tumors showed significantly increased number of transitions ($P = 0.019$) and number of chromosomes containing transitions ($P = 0.012$) compared with low-grade tumors. However, the number of whole chromosome changes ($P = 0.584$), number of amplifications ($P = 0.535$), and number of high level losses ($P = 0.203$) did not reach significance, probably due to the small number of tumors in the high-grade pT_a tumor group ($n = 8$).

Stage pT_a tumors showed a significantly lower level of all the measures of chromosomal instability assessed when compared with muscle-invasive tumors, including the number of whole chromosome changes ($P = 0.015$), number of transitions ($P = 0.006$), number of chromosomes containing transitions (0.0002), and number of amplifications ($P = 0.003$). Interestingly, there were significantly more high-level losses in pT_a tumors than in muscle-invasive tumors ($P = 0.02$). When pT_1 tumors were compared with muscle-invasive tumors, pT_1 tumors showed overall a greater level of all the measures of genomic instability calculated but they did not

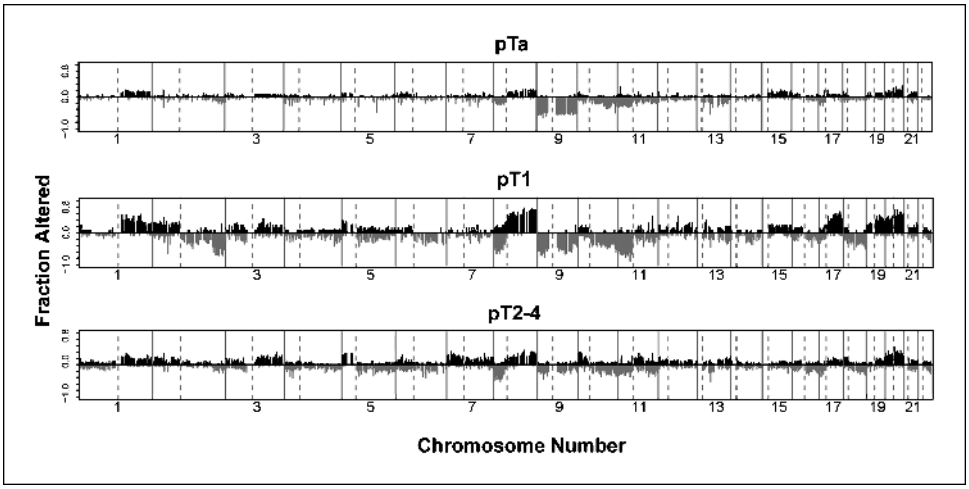

Fig. 2. Array CGH according to tumor stage. Copy number frequency plots for 29 pT_a (21 low grade and 8 high grade), 14 pT_1, and 55 muscle-invasive (pT_{2-4}) tumors. Gains are shown above and losses below the 0 horizontal. Vertical dashed lines, centromeres.

Table 2. Most frequent genomic alterations by chromosome arm

Stage		Chromosome arm	Frequency (%)		Chromosome arm	Frequency (%)
pT_a	Gain	20q	17	Loss	9p	47
					9q	46
					11p	24
					10q	20
					13q	17
					8p	16
					17p	15
pT_1	Gain	8q	55	Loss	11p	54
		20q	47		9p	51
		19q	36		9q	43
		1q	34		8p	38
		20p	33		18q	32
		17q	32		2q	32
		19p	27		10q	29
		5p	25		6q	23
		2p	22		18p	21
		3q	18		17p	18
					13q	17
					5q	16
					14q	15
					16q	15
pT_{2-4}	Gain	5p	27	Loss	8p	29
		20q	26		9p	21
		8q	23		11p	18
		10p	21		17p	17
		20p	21		18q	17
		7p	20		10q	16
		3q	18		5q	15
		1q	17		6q	15
					16q	15

NOTE: The frequencies of gains and losses on each chromosome arm were averaged according to stage. Only average frequencies of ≥15% are shown.

reach statistical significance. The only exception was the number of high-level losses, which was higher in pT_1 than in muscle-invasive tumors ($P = 0.05$).

Regions of high-level amplification. High-level amplifications were identified in 45 of the 98 patients (6 pT_a, 8 pT_1, and 31 pT_{2-4}) and affected 233 clones. Twenty-nine of these clones showed amplification in three or more cases. A list of these amplified clones with their chromosomal location is shown in Table 3.

The six most frequently amplified clones on 6p22.3 span a region of ~2 Mb between RP11-43B4 and RP11-273J1. These clones were present in significantly higher frequencies in the muscle-invasive tumors ($P = 0.002$). Genes in this region include *E2F3* and *CDKAL1*. This area is a recurrent area of amplification in bladder cancer and four of the six clones that were found most frequently amplified in this study were the identical clones identified in our previous bladder CGH study on a different tumor set (4). The chromosome 6p22.3 CGH profile of tumors with at least three clones amplified in the critical region of amplification is available in Supplementary Data (Amplifications).

Eight of the clones on chromosome 8q22-24 were amplified in at least four cases. Two of these eight clones were also reported as amplified in our previous array CGH study. The most frequently amplified clones on the 8q region were RP11-128P9 (133,448,746-133,448,897 kb) and RP11-45B19 (135,479,232-135,641,838 kb), including the *KCNQ3* and *ZNF406* genes, respectively.

The 12q15 region of amplification contained three clones, which were amplified in at least four cases. All but one of the cases that contained amplifications of these three clones were superficial tumors. The most frequently amplified clones were CTB-136O14 (67,488,237-67,674,625) and RP11-15L3 (68,217,525-68,403,913 kb) and contained the *FRS2*, *CCT2*, *VMD2L3*, and *MDM2* genes.

Deletions. High-level losses were identified in 59 of the 98 patients (19 pT_a, 10 pT_1, 30 pT_{2-4}) affecting 349 clones, of which 59 clones showed deletions in three or more cases. A list of the deleted clones is shown in Table 4.

As expected, the clones with the highest frequency of high-level losses were located on chromosomes 9p24-13 (5,873,409-35,799,611 kb) and 9q21-34 (68,683,031-1,333,886,959 kb).

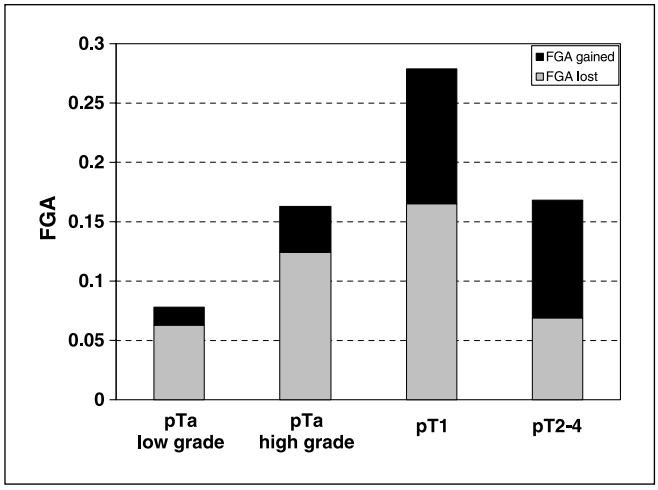

Fig. 3. FGA by tumor stage. *Black column*, FGA gained; *gray column*, FGA lost. The FGA for each tumor was quantitated by assigning for each clone a distance equal to the sum of one half of the distance between its own center and that of its neighboring clones. Stage pT_a tumors had the lowest median FGA (9%), whereas pT_1 tumors had the highest (27%). Muscle-invasive tumors had a median FGA of 16%.

Clone CTB-65D18 containing *CDKN2A* was the most commonly deleted clone on 9p (20 of 98 tumors), whereas clone RP11-9M16 containing *AKNA* and *DFNB31* was the most common deleted clone on 9q (10 of 98 tumors). Quantitative real-time PCR was used as an independent method to confirm the *CDKN2A* homozygous deletion. Taqman analysis showed single-copy loss of multiple chromosome 9 regions, with further loss of the *CDKN2A* sequence in a subset of cell lines and tumors, agreeing with our CGH analyses.[5]

Other deleted clones were located on 2q14-37 (118,783,032 kb), 8p23 (2,057,936 kb), 10q22-24 (78,595,996-99,294,901 kb), and 11p15-12 (2,852,537-40,886,858 kb).

Outcome. A total of 54 muscle-invasive cases had follow-up information available (median survival 11 months; Supplementary Data: Clinical.xls). For analysis of associations with outcome, subsets of contrasting patients were selected with good prognosis (alive >2 years, $n = 10$) and bad prognosis (dead in <2 years, $n = 34$). There were no significant differences in the distribution of stage or nodal status between the two prognosis groups.

The FGA was significantly lower in the muscle-invasive patients with good prognosis (median FGA = 3.5%) than patients with bad prognosis (median FGA = 22.4%; $P = 0.002$). Other measures of genomic instability were also significantly lower in the patients with good versus bad prognosis, including whole chromosome changes ($P = 0.01$), number of transitions ($P = 0.05$), number of chromosomes with transitions ($P = 0.016$), and the number of high-level losses ($P = 0.015$). However, the difference in the number of amplifications did not reach significance ($P = 0.09$).

Univariate Cox proportional hazards analysis showed significant association between overall survival and FGA as a continuous ($P = 0.013$) or dichotomized variable ($P = 0.0049$) in muscle-invasive patients, with low FGA associated with longer overall survival. For dichotomized analysis, a threshold of FGA (10%) was chosen as the optimum balance between sensitivity (88%) and specificity (70%) as determined by receiver operator characteristic analysis. FGA was not associated with any other clinicopathologic variables tested. Univariate analysis showed association between overall survival and the presence of amplified clones ($P = 0.033$), deleted clones ($P = 0.052$), chromosomes with transitions ($P = 0.055$), and whole chromosome changes ($P = 0.07$). Extent of invasion (stage pT_2, pT_3, or pT_4), nodal status, sex, and age were not significantly associated with outcome in these muscle-invasive patients.

Multivariate analysis using a Cox proportional hazards model was done to identify statistically independent factors in overall survival. When variables considered above (in the univariate model) were chosen in a stepwise reduction, FGA was the only independent predictor of overall survival when analyzed as a continuous or dichotomous variable.

Cox proportional hazards analysis was also used to identify individual clones that were associated with prognosis. This analysis identified 137 individual clones, the majority of which were gained on chromosomes 2p25-11 (8,710,225-88,494,982 kb), 5p13-15 (3,055,081-31,667,201 kb), 8q22 (99,202,014-101,125,862 kb), and lost on 5q11-34 (54,222,747-160,719,699 kb), 8p23-21 (2,080,313-19,617,041 kb), 8p12 (28,889,000-31,264,731 kb), 10q21-26 (64,541,582-128,795,405 kb), and 17q21-25 (30,082,000-80,815,074 kb).

The prediction analysis for microarrays method of supervised classification was used for outcome prediction in these muscle-invasive patients with good versus bad prognosis. Using cross-validation to reduce bias due to overfitting, a classification success rate of 71% was obtained based on 102 unique clones (55 of these clones were also identified in the Cox proportional hazards analysis described above). The majority of prediction analysis for microarrays predictive clones were located on chromosomes 8 (23 clones on 8p and 18 clones on 8q) and 5 (7 clones on 5p and 17 clones on 5q). The combination of clones on chromosomes 5q and 8p did not add significantly to prediction of overall outcome.

Unsupervised classification of muscle-invasive patients grouped the tumors into three clusters (Fig. 4). Interestingly, the tumors in these three clusters had different median FGA (Kruskal-Wallis rank sum, $P < 0.0001$). The tumors in the left cluster had FGA = 3.8%, in the middle cluster FGA = 13.5%, and the patients in the right cluster had FGA = 35.6%. Kaplan-Meier survival analysis showed significant association with outcome when done based on these tumor clusters ($P = 0.019$; Fig. 4). Patients in the left cluster were associated with the best survival and also had the lowest FGA value.

Discussion

Bladder cancer consists of a heterogeneous group of tumors that differ in their types and range of genetic alterations (16, 17). As it has been reported for other solid tumors, one hallmark of the pathway of tumor development and progression is an increase in genome complexity. In this study, array CGH was used to dissect the spectrum of alterations in bladder cancer and to identify recurrent aberrations that may contain cancer-related genes. Different types of genomic instability were

[5] In preparation.

also assessed to better understand the underlying mechanisms of genomic instability during tumor development. Such measurements are helpful in further stratifying tumors into more homogeneous and clinically relevant subgroups and can be used to develop markers to better predict clinical outcome.

This study has confirmed and extended at high resolution previously reported loci showing alterations in bladder cancer. Many of the same regions were previously reported by other investigators using loss of heterozygosity (18, 19), chromosomal CGH (2, 3, 20–26), and/or array-based CGH (4, 5). Apart from detecting regions of recurrent aberrations, individual clones that were significantly different between tumors of different stage and grade were identified. Overall, there was an increase in the levels of chromosomal alterations for both increasing grade and stage and strong similarities were found in the levels of chromosomal aberrations between pT_1 and muscle-invasive tumors similar to previous reports (21). Loss of clones across the entire chromosome 9 was the most frequent alteration in stage pT_a tumors, and in some tumors it was the only alteration identified. The frequency of loss for 9p versus 9q was balanced in tumors from all pathologic stages.

Other recurrent alterations present at high frequency across different stages were the loss of chromosome 8p accompanied by a gain of 8q. This characteristic transition pattern of chromosome 8 has also been reported in other types of tumors, including colon, prostate, and breast. In all these tumors, extensive areas of distal 8p are lost. Interestingly, five CGH studies (including ours) using different tumor types (bladder, colon, prostate, and fallopian tube) identified the same clone (bacterial artificial chromosome RP11-121F7) as homozygously lost on 8p23.2. This clone encompasses the *CSMD1* gene (CUB and Sushi multiple domains 1), the function of which is currently unknown. The identification of a functionally significant gene in this region has the potential as a diagnostic or therapeutic target with wide application to solid tumors. Muscle-invasive tumors showed roughly the same spectrum of changes as pT_1 tumors. However, gains on chromosome 7p and 7q were seen at significantly higher frequencies in the muscle-invasive tumors. Chromosome 7 aneusomy has been previously shown by fluorescence *in situ* hybridization to be significantly different between pT_1 and pT_{2-3} tumors (27) and has been associated with tumor aggressiveness (28).

In this study, significant differences in the FGA were identified among the tumors of different stage, grade, and patient outcome. Stage pT_a tumors had the lowest levels of alteration, and stage pT_1 showed a much higher overall FGA.

Table 3. Amplifications in the 98 bladder tumors

Clone	Chromosome band	Start position*	Genes	No. tumors†
RP11-193J5	1q24	163,240,622		4
RMC01P52	1p34	39,779,782	*MYCL1*	3
RP11-43B4	6p22	20,117,736	*ID4*	3
RP11-159C8	6p22	20,442,777	*E2F3*	5
CTD-2018P8	6p22	20,622,576	*CDKAL1*	4
RP11-3D15	6p22	21,715,615	*SOX4*	5
RP11-273J1	6p22	22,030,457	*LOC401237*	4
RP11-10G10	8q22	101,112,122	*SPAG1, RNF19*	3
RP11-145G10	8q24	128,499,737		3
RP11-85M7	10p14	6,649,014	*SFMBT2*	3
RP11-33J8	10p14	7,199,612		3
RP11-35I11	10p14	8,922,795		3
RP1-88B16	11q13	69,133,863	*CCND1prox*	9
CTD-2192B11	11q13	69,133,897	*CCND1*	8
RP1-4E16	11q13	69,134,005	*CCND1*	8
RP1-128I8	11q13	69,134,005	*CCND1*	7
RP1-162F2	11q13	69,134,063	*CCND1end*	6
RP1-17L4	11q13	69,134,124	*FGF4*	7
CTB-36F16	11q13	69,134,163	*FGF3*	8
CTC-437H15	11q13	69,134,772	*EMS1, CTN1*	5
RP11-120P20	11q13	70,129,383	*SHANK2*	4
GS-7N12	11q13-14	74,760,360	*PAK1*	5
CTD-2222B22	11q23	116,613,723	*PCSK7*	3
CTB-136O14	12q14	67,488,237	*MDM2*	6
RP11-15L3	12q15	68,217,525	*FRS2, CCT2 LRRC10,VMD2L3*	6
CTB-82N15	12q14	68,366,100		4
RP11-183A20	13q33	106,617,308	*TNFSF13B*	3
RP11-140E1	19q13	43,372,950	*DPF1, PPP1R14A, SPINT2, KCNK6*	3
RP11-118P21	19q13	43,861,826	*ACTN4*	3

*Based on University of California Santa Cruz human DNA sequence draft of July 2003 freeze.
†Clones with amplifications in at least three tumors are shown.

Table 4. Deletions in the 98 bladder tumors

Clone	Chromosome band	Start position*	Genes	Number of tumors†
RP11-98C1	2q14	118,783,032	*FLJ39081, INSIG2*	3
RP11-504L12	2q35	217,012,575	*PECR*	4
RP11-247E23	2q36	223,880,100		4
RP11-188B21	2q37	233,910,314	*TNRC15, KCNJ13*	4
RP11-21K1	2q37	235,977,579		3
RP11-116M19	2q37	236,916,706	*CENTG2*	7
CTB-172I13	2 q tel	Telomere		4
RP11-79N22	4p15	18,622,376		3
RP11-194B9	4p15	28,103,518		3
RP11-6L19	4q32	167,513,183	*TLL1*	3
RP11-252I13	5q21	107,005,165	*EFNA5*	3
RP11-81C5	5q22-23	115,231,364	*APG12L, AP3S1*	3
RP11-58F7	7q36	156,989,618	*PTPRN2*	3
RP11-117P11	8p23	2,057,936	*MYOM2*	5
RP11-82K8	8p23	2,080,313	*MYOM2*	3
RP11-246G24	8p23	2,355,758		3
RP11-112G9	8p23	10,038,625	*MSRA*	3
RP11-254E10	8p23	11,164,156		3
RP11-165O14	9p24	5,873,409	*MLANA, KIAA2026, RANBP6*	3
CTD-2006L14	9p24	5,892,906	*MLANA, KIAA2026, RANBP6*	3
RP11-264O11	9p23	10,578,156		9
RP11-109M15	9p22	16,141,129		8
CTB-65D18	9p21	21,958,037	*CDKN2A*	20
RP11-33O15	9p21	22,823,087		5
RP11-17J8	9p13	34,511,041	*UNQ470*	4
RP11-165H19	9p13	34,589,621	*C9orf23, DCTN3, ARID3C, OPRS1, GALT, IL11RA, CCL27, CCL19, CCL21*	3
RP11-61G7	9p13	35,799,611	*SPAG8, HINT2*	3
RP11-19O14	9q21	68,683,031	*TRPM3*	3
RP11-8L13	9q21	16,996,818		3
RP11-14J9	9q21	73,221,555		4

FGA seemed to stabilize in the muscle-invasive cases, with a slight decrease relative to pT_1 tumors. Low-grade superficial tumors had a significantly lower FGA than did high-grade tumors. Interestingly, some of the low-grade pT_a tumors and even some of the muscle-invasive tumors had a very low FGA (<3%). These tumors had no gross chromosomal alterations whatsoever by array CGH, but only gains or losses of a few individual clones. The alterations of these individual clones may represent true genomic changes, although hybridization noise or even polymorphisms affecting specific clones in individual patients might also be suspected. This observation of very low FGA in certain tumors suggests that some bladder tumors evolve by mechanisms that result in little chromosomal change, whereas others undergo marked chromosomal rearrangements. This variability may be due to differences in tumor initiation, processes of genomic instability, or the individual genotypic background of the tumor. It is possible, for example, that the tumors with few chromosomal changes arise in epithelial stem cells already having an active telomerase so that telomere crisis with associated extensive genomic rearrangement is avoided. Similarly, tumor initiation and progression associated with defects in mismatch repair might lead to tumors with few chromosomal abnormalities, although this is reported to be rare in bladder cancer. Overall, in our tumor set, copy number alterations affecting chromosome arms or portions of the arms (transitions) were more frequent than whole chromosome changes. This was most prominent in low-grade pT_a tumors where 5 of 21 (24%) of the tumors had transitions and no whole chromosome changes. These observations are consistent with a model in which telomeric dysfunctions and breakage-fusion-bridge events occur at an earlier stage in tumor development than errors in whole chromosome segregation. It is interesting to note that most of the tumors analyzed in this study exhibited both numerical aberrations and highly complex structural aberrations, increasing from pT_a to pT_1 and muscle-invasive tumors.

One surprising finding in this study was the significantly higher FGA in stage pT_1 tumors compared with muscle-invasive tumors and the absence of significant differences between these stages in the other measures of genomic instability assessed. Perhaps, over time, tumors acquire a more "stable" genome, already optimized for growth, invasion, and dissemination, making it less probable that additional lesions will confer further advantage. Direct evaluation of the rate of genomic

Table 4. Deletions in the 98 bladder tumors (Cont'd)

Clone	Chromosome band	Start position*	Genes	Number of tumors†
RP11-57N18	9q21	73,439,444	*LOC138932*	4
RP11-8D10	9q21	80,348,013		3
RP11-9I18	9q22	88,624,086	*LOC340515*	5
RP11-128O12	9q22	91,549,881	*C9orf10OS*	7
RP11-54O15	9q22	93,006,894	*C9orf3*	5
RP11-5K11	9q31	98,443,107	*TEX10, MGC17337*	3
RP11-4O1	9q31-32	110,119,611	*SUSD1*	3
RP11-9M16	9q32	112,531,818	*AKNA, DFNB31*	10
RP11-229N14	9q33	115,037,234	*ASTN2*	5
GS1-135I17	9q34-tel	133,886,959	*VAV2*	3
RP11-17O5	10q22.2	78,595,996	*KCNMA1*	4
CTB-46B12	10q23	89,287,772	*PTEN*	3
RP11-129G17	10q23	89,676,027	*FLJ11218*	3
RP11-8D20	10q24	97,762,569	*DNTT*	4
RP11-19K9	10q24	99,294,901	*CRTAC1*	3
RP4-693L23	11p15	2,852,537	*CDKN1C*	4
RP11-47D7	11p15	11,610,583	*AF116621*	3
RP11-62G18	11p15	19,596,327	*NAV2*	4
RP11-11A11	11p15	19,912,236	*NAV2*	6
RP11-72A10	11p12	36,741,042		3
RP11-34F8	11p12	40,886,858		4
CTD-2012D15	11q22	104,333,213		4
RP11-36M5	11q23	113,531,008	*ZNF145, NNMT*	4
RP11-8K10	11q23	119,094,962	*PVRL1*	3
RP11-15J15	11q24	125,423,862	*CDON*	3
RP11-20M1	11q24	125,859,929	*KIRREL3*	3
RP11-52B21	13q14	45,018,239	*CHDC1*	4
RP11-4B17	18q23	72,858,573	*MBP*	3
RP11-7H17	18q23	75,214,350	*ATP9B, NFATC1*	3

*Based on University of California Santa Cruz human DNA sequence draft of July 2003 freeze.
†Clones with deletions in at least three tumors are shown.

instability during tumor evolution might best be measured using matched pairs of samples from the same patient at different stages of progression (e.g., pT_1 versus pT_3). The decrease in genomic alterations in muscle-invasive tumors might also have been due to increased normal cell contamination compared with superficial tumors, with a larger component of stroma and inflammation within the tumor. However, tumor blocks were grossly dissected to assure that at least 70% of tumor cells was present. Previous cell line mixing studies have shown that this degree of admixture does not affect detection of copy number alterations. Further, comparison of different dissection procedures (gross block dissection versus fine microdissection yielding 95% tumor cells) showed that the frequency of alterations did not significantly increase with purity >70%.[6]

Higher-stage tumors might be expected to show increased tumor heterogeneity and aneuploidy. Array CGH detects DNA alterations present in common within the tumor cell population, and so it is not able to account for tumor heterogeneity. Similarly, the complexity of DNA copy number alterations present in an aneuploid tumor may lead to decreased detection of such alterations due to the difficulty during array CGH data analysis of defining copy number gains and losses in these tumors. Different algorithms used for analysis of array CGH data may also affect the detection of genomic alterations in an aneuploid background.

There is wide agreement that recurrent genomic aberrations may highlight chromosomal regions that are important for tumor development. The importance of recurrent aberrations involving gene dosage is particularly clear. In many cases, these aberrations contain known oncogenes and tumor suppressor genes whose expression levels are altered by genomic changes. The large size of most aberrations detected by chromosomal CGH or cytogenetics makes it difficult to narrow down candidate genes. However, using array CGH, the regions of high-level aberrations (amplifications and homozygous deletions) can be studied at much greater resolution and their boundaries can be identified with greater precision. However, identifying the relevant cancer-related genes is still difficult because most aberrations are large, containing multiple genes. For example, the region of recurrent amplification on

[6] Unpublished data.

chromosome 11q13 containing *CCND1* also contains growth factors *FGF19*, *FGF4*, and *FGF3*, and actin-binding oncogene *EMS1*. It is possible that the concurrent impaired expression of all these genes is necessary for a particular tumor phenotype and may drive the appearance of high-level recurrent rearrangements in this region. In these cases, array CGH can help refine the number of candidate genes that would need to be further characterized.

Clinical outcome for patients with muscle-invasive bladder cancer is only partially predicted by tumor stage. Grade is not a useful prognostic marker in this tumor group because they are all high grade. Although specific genetic changes have been described, which are associated with higher disease stage (at 1p, 3p, 13q, 17p, 20p), they did not predict outcome independent of standard clinicopathologic parameters (19, 22). A large number of additional molecular markers have also been nominated as predictors of outcome in muscle-invasive bladder cancer. However, conclusions from these studies have been inconsistent, and few if any of the markers is able to discriminate good versus poor prognosis groups sufficiently to have clinical utility (29). In this study, FGA was significantly higher in patients with bad clinical outcome (died in <2 years) versus in patients with good outcome (alive in >2 years). This was independent of stage, nodal status, and age, although these other clinical variables (stage, age, sex, nodal status) were not themselves significantly associated with prognosis in this group of 54 patients. Multivariate Cox proportional hazards analysis identified FGA as the only independent prognosticator of overall outcome.

Unsupervised classification separated muscle-invasive patients into three groups. These groups were significantly associated with overall outcome. Further, the tumor cluster with the worst outcome had the highest FGA, providing additional evidence for the correlation of FGA with outcome in patients with muscle-invasive disease. However, for FGA to be applied in a clinical setting, a cutoff would be necessary. When, in this study, FGA was used as a dichotomous variable, a threshold of <10% was best for identifying patients with poor prognosis. This threshold was chosen after performing receiver operator characteristic analysis to determine the optimum balance between sensitivity and specificity of FGA in predicting patients with worse outcome. Further studies in independent data sets would be necessary to fine tune such a threshold and make it applicable in a wider clinical setting. In the future, this could be applied for choice of more aggressive therapies, and in some cases sparing therapy in patients with better outcome. The quantitative nature of FGA is also appealing for its development as a clinical marker. FGA can be used as a means to determine the "genomic grade" of a tumor in a similar way to the histologic stage or grade, and together with a panel of other molecular markers can be used to predict outcome of patients with muscle-invasive disease.

Supervised classification and Cox proportional hazards analysis applied to individual clones identified clones associated with outcome that were localized predominantly on chromosomes 8p, 8q, 5p, and 5q. Chromosome 8p deletions have been found frequently in several tumor types, associated with invasive tumor growth. Chromosome 5 aberrations have also been associated with increasing bladder cancer stage (30).

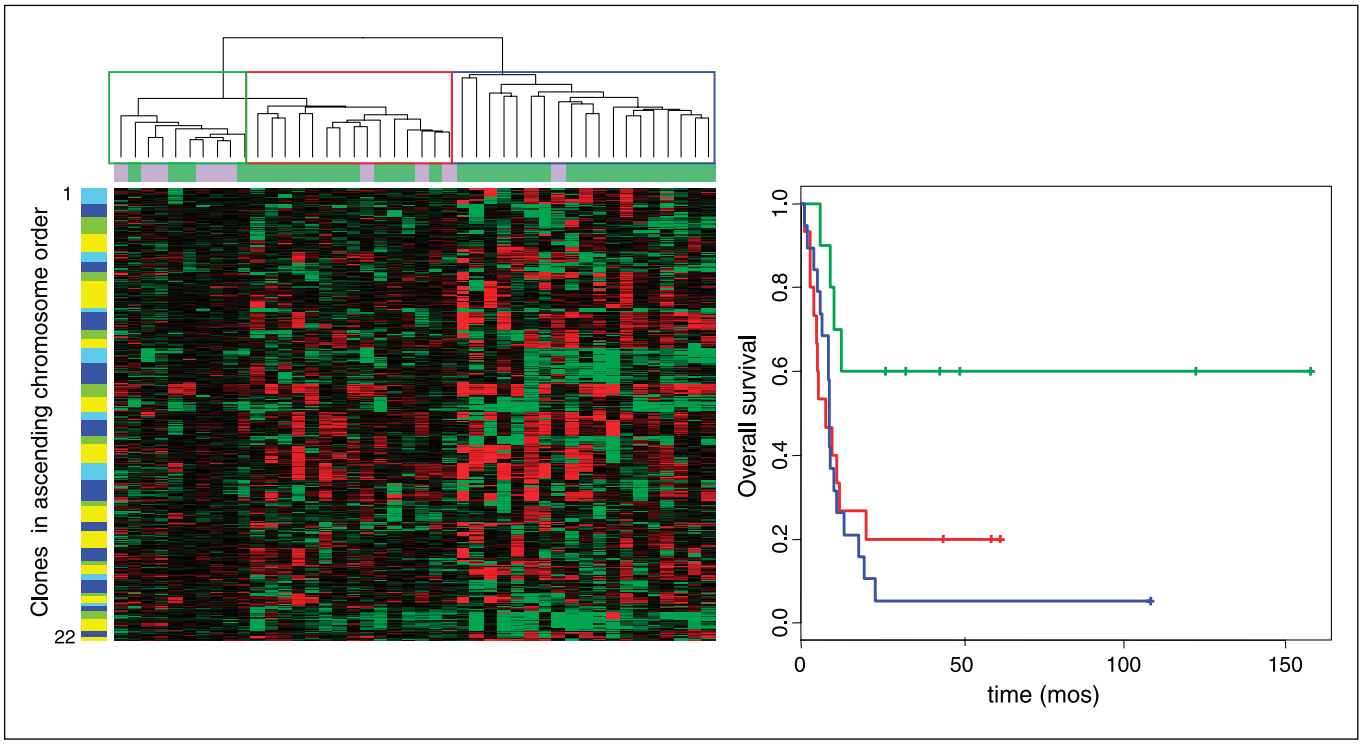

Fig. 4. Unsupervised hierarchical clustering of muscle-invasive bladder tumors (n = 44). Left, clustering of tumors shows three large clusters (top: green, red, blue). The horizontal band at top shows tumor columns with good prognosis (alive >2 years, n = 10) in purple, and bad prognosis (dead in ≤2 years, n = 34) in green. Rows represent individual clones on chromosomes in ascending order (light and dark blue for the p and q arms of odd chromosomes and green and yellow for the p and q arms of even chromosomes). Decreased copy numbers are in red and increased copy numbers are in green. Right, Kaplan-Meier survival analysis showing significant association with outcome (P = 0.019) when done based on tumor groups defined by unsupervised clustering (on the left): *green*, left cluster; *red*, middle cluster, and *blue*, right cluster.

In this study, alterations on either 8p or 5q were able to successfully predict overall outcome in patients with muscle-invasive disease. However, their combination did not have the same success.

This study shows at high resolution the range of specific genomic changes present in bladder cancer. Tumors also showed a wide range in the pattern of alterations, suggesting that alternative mechanisms of genomic instability may play a role in this tumor type. The FGA was independently associated with outcome in this tumor set, and may reflect a "genetic grade" for development more informed choices for therapeutic decision making. These studies should be validated, especially using independent tumor sets from multiple institutions.

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