Centromeric Copy Number of Chromosome 7 Is Strongly Correlated with Tumor Grade and Labeling Index in Human Bladder Cancer

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ABSTRACT

The relationship between interphase cytogenetics and tumor grade, stage, and proliferative activity was investigated in 27 transitional cell carcinomas of the urinary bladder. Using fluorescence in situ hybridization with chromosome-specific DNA probes, the copy number of pericentromeric sequences on chromosomes 7, 9, and 11 was detected within interphase nuclei in touch preparations from tumor biopsies. Monosomy of chromosome 9 was detected in 9 of 22 cases (41%), while tetrasyom for chromosomes 7 and 11 was detected in 10 of 26 (38%) and 6 of 23 (26%) cases, respectively. Copy number of chromosome 7 was the most highly correlated with increasing tumor grade \((r^2 = 0.616, P < 0.001)\), Spearman rank correlation \) or increasing pathological stage \((r^2 = 0.356, P < 0.002)\). Copy number for chromosome 9 did not correlate with either grade or stage \((P > 0.05)\). Tumor labeling index \(LI\) was determined either by autoradiographic or by immunohistochemical methods. Increasing \(LI\) by either method correlated with increasing copy number for all three chromosomes \((r^2 = 0.473, P < 0.002 \) for 7; \(r^2 = 0.384, P < 0.01 \) for 11; and \(r^2 = 0.316, P < 0.05 \) for 9). Since high tumor grade, stage, and \(LI\) are all indicative of more aggressive tumor behavior and worse prognosis, these findings suggest that polysomy, especially for chromosome 7, may be highly predictive for bladder tumor aggressiveness.

INTRODUCTION

Numerous specific genetic aberrations have been implicated in bladder cancer. Cytogenetic studies have defined numerical and structural changes involving chromosomes 1, 3, 5, 7, 9, 11, and 17 \((1-8)\). Flow cytometric analysis of DNA content has shown a correlation between DNA ploidy, S-phase fraction, and tumor grade \((9-14)\). Analyses of allelic loss rates for sites on 9q, 11p, and 17p have shown a high fraction of tumors with such losses \((15)\). In particular, it has been shown \((16)\) that losses of 9q occur independently of tumor grade, whereas 17p allelic loss is more specifically associated with higher grade tumors. While the prognostic significance of these genetic and cytogenetic changes has yet to be demonstrated in prospective studies, there is increasing evidence that the specific genes involved may modulate tumor proliferation \((17-20)\).

Tumor labeling index or S-phase fraction has been correlated with prognosis in bladder tumors \((10, 12)\). S-phase fraction can be measured directly by incorporation of \([3H]\)thymidine or BrdUrd into tumor DNA \(^4\) or by immunohistochemical staining of cell cycle-specific antigens such as Ki67 \((21)\) or PCNA \((22)\) expressed only in proliferating cells. The present study was undertaken in order to study the relationships in bladder cancer between genotypic and phenotypic abnormalities. Tumor grade, tumor stage, and tumor labeling index, as measured by BrdUrd incorporation or PCNA labeling, were used to characterize tumor phenotype, while tumor genotype was defined by cytogenetic abnormalities, as detected by FISH with DNA probes specific for chromosomes 7, 9, and 11.

MATERIALS AND METHODS

Clinical Material. All tumor samples were obtained fresh from the Medical Center and affiliated hospitals, University of California, San Francisco. All patient protocols were reviewed by the Institutional Review Board. Tumors were analyzed for pathological stage and grade \(T1\), papillary noninvasive; \(T2\), invading into lamina propria; \(T3\), deep invasion of muscularis; \(T4\), metastatic) and pathological grade \((23)\), without knowledge of interphase cytogenetic or labeling index results.

Fresh unfixed tumor samples were collected during cystoscopy and stored in Hanks' balanced salt solution prior to sample processing. Adequate biopsy material was always first collected for histopathological diagnosis. Touch preparations were generated by gently touching the urethelial surface of the biopsy to a dry microscope slide. This brief contact allowed an adequate number of single tumor cells to adhere to the slide surface. Five to 10 such touch preparations were made from each biopsy specimen. Slides were then air dried at room temperature overnight and stored under nitrogen at \(-20^\circ\)C. Whenever possible, 5-mm x 5-mm x 1-mm slices were also cut from cystectomy specimens, and touch preparations were made as described.

After touch preparations were made, tumor slices were incubated with BrdUrd \((100 \mu M\) BrdUrd, 10 \(\mu M\) fluorescein isothiocyanate, hyperbaric oxygen in RPMI) for 2 h at \(37^\circ\)C, to label cells undergoing DNA synthesis. Tissue was then fixed in 70% ethanol, embedded in paraffin, and sectioned for immunocytochemical staining using monoclonal antibodies to BrdUrd (IU4, 1:10,000; Caltag) and cyclin/PCNA (19A2, 1:200; Boehringer Mannheim). Standard indirect immunoperoxidase staining was used (Vectastain Elite ABC kit; Vector Laboratories). The proportion of tumor cells staining positively for BrdUrd and PCNA was determined from 2000 cell counts, by selecting 4-6 high power \((x40)\) fields showing the greatest concentration of stained nuclei.

Controls used for \(in situ\) hybridization were peripheral blood lymphocytes from healthy human volunteers. Lymphocytes in metaphase were obtained after short term culture by standard procedures. Control slides were stored under nitrogen at \(-20^\circ\)C until use.

Flow Cytometry. When adequate material was available, single-cell suspensions were generated by finely mincing tissue, and cells were prepared according to the method of Vindelov et al. \((24)\). Alternatively, a vigorous bladder wash specimen taken at the time of biopsy was used for flow analysis. The FACScan flow cytometer (Becton-Dickinson, Mountain View, California) was used to determine DNA ploidy, S-phase fraction, and labeling index \(LI\).

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San Jose, CA) was used for analysis, with doublet discrimination gating and CellFit (Becton-Dickinson) software. A clearly defined second peak comprising more than 20% of total cells was necessary to define aneuploidy.

Chromosome-specific Probes. The probes used were specific for pericentricomeric sequences on chromosome 7 (p15.3; H. Willard, Stanford University), chromosome 9 (pHUR9; B. Moyzis, Los Alamos National Laboratory), and chromosome 11 (pLC11A; H. Willard). Probes were labeled by nick translation using either biotin-11-dUTP (Bethesda Research Laboratories) or digoxigenin-11-dUTP (Boehringer Mannheim). Approximately 5-35% of thymidine residues were substituted during the reaction.

In Situ Hybridization. The hybridization was carried out as previously described (25), with modifications (26). Slides were first fixed for 5 min each in three changes of freshly prepared Carnoy's solution (3:1 methanol/acetic acid). Hybridizations containing 5-10 ng labeled probe, 2.5 μg carrier DNA, 55% formamide, and 10% dextran sulfate, in 2x SSC, were incubated overnight at 37°C. Slides were washed 3 times for 10 min each in hybridization wash buffer (2x SSC, 50% formamide for chromosome 7, 55% formamide for chromosomes 9 and 11) at 45°C and then 2 times in 2x SSC, first at 45°C and then at room temperature.

To minimize nonspecific staining, slides were then incubated for 5 min in ADB (5% Carnation milk power, 4x SSC, pH 7, 0.02% sodium azide, 0.1% Triton X-100). Slides on which cells had been hybridized to biotinylated probes were incubated for 25 min at room temperature in FITC-avidin (Vector Laboratories) diluted 1:200 in ADB. Slides on which cells had been hybridized to digoxigenin-labeled probes were incubated for 30 min in FITC-conjugated antidigoxigenin antibody (Boehringer Mannheim) in ADB. Slides were then rinsed 10 min each in 4x SSC, 4x SSC/0.1% Triton X-100, and 4x SSC. Slides were then rinsed in distilled water, air dried, and counterstained with either 0.25 μg/ml propidium iodide (Sigma) or 0.2 μg/ml 4',6-diamidino-2-phenylindole (Molecular Probes) in anti-fade solution (27). If necessary, biotinylated probe signals were amplified by preblocking in ADB for 5 min, incubating 30 min in 1:100 goat antiavidin/ADB, washing in 4x SSC, blocking again in ADB, and incubating 25 min in FITC-avidin diluted 1:200 in ADB.

Scoring of Interphase Nuclei. A touch preparation from each biopsy was stained with Giemsa and analyzed to determine the proportion of malignant cells present. Those cases with <50% tumor cells in the touch preparations (by conservative cytological estimate) were censored from the study. Additionally, six cases showed no tumor in hematoxylin- and eosin-stained sections of the biopsy and were excluded. When the majority of cells were tumor cells by cytological criteria, benign fibroblasts and inflammatory cells were readily recognized and were excluded from further cytogenetic analysis.

After hybridization, slides were scored for the number of hybridization signals in each nucleus, using an epifluorescence microscope equipped with a 63x NA 1.3 oil immersion objective. If possible, 200-500 nuclei were scored for each hybridization. Nuclei in which the nuclear boundary was broken or torn were considered damaged and were censored from analysis. Similarly, squashed, smeared, clumped, or overlapped nuclei were ignored. Each hybridization was accompanied by a control hybridization using normal lymphocytes. If the control displayed increased monosomy (>10%), parallel tumor preparations displaying weak signals were repeated, because the likelihood of missing signals during the counting procedure was high.

The copy number category (monosomic, disomic, trisomic, tetrasomic, pentasomic, and above) reflects the major (>50%) or dominant (20-50%) population present. If no aneuploidy (i.e., other than two copies/nucleus) subpopulation was present at >50% of the total, then the largest aneuploidy subpopulation having >20% of total cells was used. Thus, a tumor was considered disomic if no other subpopulations had >20% of total cells.

RESULTS

Clinical. Twenty-seven cases of transitional cell carcinoma of the bladder were analyzed. Table 1 shows tumor grade, stage, age, sex, labeling indices, and interphase cytogenetics for the three chromosomes tested. The worst pattern present for nuclear grade and pathological stage was recorded for each case. The mean age was 68 years, and 90% of the tumors were from men. Four of 27 patients were treated with various intravesical chemotherapeutic reagents prior to biopsy.

Interphase Cytogenetics. The copy number distributions of chromosomes 7, 9, and 11 in control interphase lymphocytes are shown in Fig. 1. The sensitivity of the reaction was apparent from the high proportion of nuclei having two signals. Because only 3.3, 4.2, and 5.0% of control nuclei had one target copy

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<th>PCNA LI (%)</th>
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* M, male; F, female.
B, bladder wash; T, disaggregated tissue.
Centromeric copy number of major or dominant population.
Previously treated with intravesical chemotherapy.
for chromosomes 7, 9, and 11, respectively, it was possible to define, with high confidence, true tumor monosomy when the proportion was above 20%. Conversely, the specificity of hybridization reactions was demonstrated in metaphase chromosomes, in which only two specific targets were present.

Photomicrographs of four different hybridizations are shown in Fig. 2. Case 20 (Fig. 2A) was easily characterized, showing three small well defined signals in each nucleus with the probe for chromosome 7. Case 25 (Fig. 2B) also is the result of hybridization with probe specific for chromosome 7. Most nuclei showed four signals, although in this case the signals were larger than in case 20 and slightly spread. Two smaller nuclei having only two signals were also present. Some of the signals were split into couplets, which were scored as single signals. Fig. 2C shows nuclei from case 11 after hybridization to chromosome 9 probe, showing one copy in each cell. Cytoplasmic shadows could be seen staining nonspecifically with propidium iodide. Nonspecific hybridization to nuclear DNA was also apparent as much smaller and fainter fluorescein signals. Monosomy for chromosome 11 in case 5 is shown in Fig. 2D. A minor population of nuclei having two signals was also present. In this photomicrograph, cytoplasmic fluorescence appears green.

A summary of the interphase FISH results for two representative tumors is shown in Fig. 3. In case 11, two patterns of copy number distribution were seen. Chromosome 9 was present predominantly as one copy/nucleus. Chromosomes 7 and 11 were detected predominantly as two copies/nucleus. This tumor was scored as monosomic for 9 and disomic for 7 and 11. Note that there were small populations of nuclei which had

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Fig. 2. Photomicrographs of four different tumors after fluorescence in situ hybridization. Probes were biotinylated and then stained with FITC-avidin (green). Nuclei were counterstained with propidium iodide (red) or 4′,6-diamidino-2-phenylindole hydrochloride (blue). Green cytoplasmic fluorescence is the result of autofluorescence and nonspecific staining. Multiple exposures were used to combine colors. Original magnification, ×63. A, case 20, showing trisomy 7. B, case 25, showing tetrasomy 7. C, case 11, showing monosomy 9. D, case 5, showing monosomy 11.
one copy of chromosomes 7 and 11, most likely due either to overlap causing apparent fusion of two signals into one or to weaker signals which were missed during scoring. These sub-populations, always <10%, were also present in control preparations. The small populations (~5%) of nuclei having three copies of chromosome 7 was less likely due to artifact, however. These trisomy 7 nuclei most likely represented true heterogeneity of copy number distribution, because nuclei with such increased copy number are not present in control hybridizations in the absence of larger populations with four or more signals.

Case 27 in Fig. 3 showed a more heterogeneous distribution of centromeric copy numbers. Large sub-populations (30-50%) of nuclei having either two or four copies of chromosomes 7 and 9 were present. Chromosome 11 was present predominantly at two copies/nucleus. This tumor was scored as tetrasomic for 7 and 9 and disomic for 11.

Table 1 shows the clinical, proliferative, and cytogenetic information for all of the cases analyzed, ordered with increasing pathological grade. Although sufficient tissue for flow cytometric analysis was available for only nine cases, a good correlation was seen between DNA index and total centromeric copy numbers. In case 9, there was a small (10% of total cells) peak seen by flow cytometry with a DNA index of 1.8. Interestingly, there was a similar minor population of nuclei analyzed by FISH with four copies of chromosome 7 (20%) and 11 (16%) and <4% tetrasomy for chromosome 9 (data not shown).

The copy number distributions, for the 27 tumors, for each of the three chromosomes tested are summarized as percentages in Table 2. Chromosome 7 was probed in 26 cases. Ten of these (38%) showed tetrasomy for chromosome 7, while eight cases (31%) showed disomy. Note from Table 1 that five cases (cases 4, 5, 14, 16, and 24) showed a positive imbalance of chromosome 7 (i.e., 9 and 11 both had fewer copies than did 7), but no tumors had a negative imbalance for chromosome 7.

Chromosome 9 was probed in 22 cases. Nine of the 22 cases (41%) were monosomic for chromosome 9. Five of these monosomic cases (cases 9–11, 18, and 22) and one disomic case (case 24) had a negative imbalance and two cases (cases 3 and 8) had a positive imbalance for chromosome 9.

Chromosome 11 was probed in 23 tumors. Eight cases (35%) were disomic and six (26%) tetrasomic. There was a positive imbalance in one disomic (case 2) and one tetrasomic (case 19) case and a negative imbalance in one disomic (case 27) and one trisomic (case 21) case.

### Table 2

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Fig. 4. Relationship between tumor grade and centromeric copy number for chromosomes 7, 9, and 11. □, grade 1; ■, grade 2; ○, grade 3; †, grade IV.
The copy numbers for chromosomes 7, 9, and 11 were generally correlated with each other. The correlation between chromosomes 7 and 11 was the strongest \( r^2 = 0.619, \ P = 0.0001 \), simple regression), followed by 7 and 9 \( r^2 = 0.209, \ P = 0.037 \) and 9 and 11 \( r^2 = 0.127, \ P = 0.123 \).

Tumor Grade and Interphase Cytogenetics. Tumor grade was strongly associated with chromosome copy number for chromosomes 7 and 11 (Spearman rank correlation, \( r^2 = 0.616, \ P < 0.001 \) and \( r^2 = 0.524, \ P < 0.001 \), respectively) and was not quite significant for chromosome 9 \( r^2 = 0.140, \ P < 0.10 \). As shown in Fig. 4, all grade I tumors were either monosomic or disomic for chromosome 7, while all grade III tumors were polysomic. Grade II tumors were more evenly distributed. These relationships were also apparent for chromosome 11 but less strongly so for chromosome 9.

Tumor Stage and Interphase Cytogenetics. Tumor stage was also correlated with chromosome copy number, although not as strongly as grade (Table 1 and Fig. 5). Stage Ta tumors were more likely to be monosomic or disomic, and stage T2-T3 tumors were more likely to be polysomic. The correlation between stage and chromosome 7 copy number (Spearman rank correlation, \( r^2 = 0.356, \ P < 0.002 \)) was stronger than that between stage and chromosome 11 copy number \( r^2 = 0.284, \ P < 0.01 \); there was no significant correlation between chromosome 9 copy number and tumor stage \( r^2 = 0.025, \ P > 0.25 \).

BrdUrd Labeling Index. Tumors were labeled in vitro with BrdUrd, to detect the fraction of cells in the S (synthesis)-phase of the cell cycle (the tumor labeling index). The LI showed a strong correlation with pathological grade (Spearman rank correlation, \( r^2 = 0.814, \ P < 0.001 \)) and stage \( r^2 = 0.537, \ P < 0.001 \). BrdUrd LI was compared to the chromosome copy number, as determined by FISH. Fig. 6 shows the strong correlation which existed for chromosomes 7 (Spearman rank correlation, \( r^2 = 0.473, \ P < 0.002 \)), 9 \( r^2 = 0.316, \ P < 0.05 \),
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Fig. 7. Relationship between PCNA labeling index and centromeric copy number for chromosomes 7, 9, and 11.

and 11 ($r^2 = 0.384, P < 0.01$). For chromosome 7, all monosomic and disomic tumors had a BrdUrd LI less than 5.1%, while only one tumor with greater copy number had a labeling index of <10% (this was a stage Ta tumor and had a LI of 4.8%). A similar relationship was present for chromosome 11 but was not present for chromosome 9.

**PCNA Labeling Index.** PCNA is an auxiliary protein to DNA polymerase δ (28). Like BrdUrd, it can be detected immunohistochemically and is a reflection of the fraction of cycling tumor cells. The PCNA LI showed a good correlation with pathological grade (Spearman rank correlation, $r^2 = 0.444, P < 0.005$) and stage ($r^2 = 0.444, P < 0.005$). PCNA LIs were compared to chromosome copy number as determined by FISH. Fig. 7 shows the distribution of PCNA LI with chromosome copy number for chromosomes 7 (Spearman rank correlation, $r^2 = 0.519, P < 0.001$), 9 ($r^2 = 0.384, P < 0.005$), and 11 ($r^2 = 0.569, P < 0.002$).

**DISCUSSION**

Numerical cytogenetic alterations in 27 carcinomas of the urinary bladder have been characterized in interphase cells, using fluorescence in situ hybridization with DNA probes specific for pericentromeric regions on chromosomes 7, 9, and 11. These tumors showed a high incidence of chromosomal aneuploidy; only one of the 27 tumors analyzed was disomic for all three chromosomes tested. There was a high correlation seen between the BrdUrd and PCNA labeling indices and tumor grade, tumor stage, and chromosomal pericentromeric copy number. This correlation was strongest for chromosome 7 copy number and weakest for chromosome 9 copy number.

Chromosomal copy number, as defined by FISH with pericentromeric probes, is only an estimate of the copy number of sequences carried on that chromosome. It should be emphasized that each nuclear signal which was counted represented the hybridization target only. Other regions on these chromosomes coding for other gene sequences may have been present at greater or fewer copy number. It should be noted that centromeric copy number determined by FISH does not increase with DNA replication. G2 cells show only two hybridization spots for each centromeric signal, because sister chromatids have not yet separated. This is evident from the virtual absence of nuclei with four signals in preparations of proliferating lymphocytes.

Analyses of primary bladder cancers using banded cytogenetics have shown numerous nonrandom aberrations, with increasing numbers of numerical and structural aberrations present in more advanced disease. Both monosomy 9 and trisomy 7 have been proposed as primary (initiating) changes in bladder cancer, since they are sometimes the sole cytogenetic lesion detected (4, 8). Structural alterations of chromosome 11 have also been noted, particularly deletions on 11p (1, 29). Although flow cytometric analysis of DNA content of tumor cells was only possible in a fraction of cases in this study, the strong correlation between FISH and flow analysis seen in other studies (30, 31) was substantiated. Flow cytometric analysis does not discriminate between chromosomes, however. Aneuosoemies, as detectable by FISH, which affect just one or a few chromosomes, are frequently missed in tumors with a diploid DNA index.

The correlation between chromosomal copy number and tumor proliferation or grade may be direct and specific or indirect and nonspecific. Results reported here may specify a direct connection between chromosome 7 copy number and tumor behavior. There may be genes on chromosome 7 (epidermal growth factor receptor, for example) which, when present in extra copy number, lead to increased tumor proliferation. Alternatively, the strong correlation between chromosome 7 copy number and tumor proliferation may be a general phenomenon, in which genetic instability (and chromosomal aneuploidy) leads to any of a number of specific gene mutations or deletions on other chromosomes, and this, in turn, may cause increased tumor proliferation. The tighter correlation between chromosome 7 copy number and tumor proliferation, as compared to chromosomes 11 and 9, might be due to an increased likelihood for random loss of 9 or 11, rather than an amplification of specific gene(s) on chromosome 7.

A high incidence of allelic loss in chromosomes 9, 11, and 17 (and low levels of loss in the other chromosomes tested) has been reported in bladder cancer (15, 16, 33). The pattern of allelic loss, when detected, is usually consistent with >90% of tumor cells having that loss (32), strongly suggesting that a...
genetic change associated with allelic loss will lead to an increased proliferative advantage for that tumor clone. Thus, finding a high S-phase fraction in tumors carrying specific genetic aberrations is not surprising.

A strong correlation was seen in this study between tumor grade and both BrdUrd and PCNA labeling indices. This association was also seen in a larger series of bladder tumors. The correlation between LI and grade is consistent with tumor grade being a descriptor of nuclear activity. Proliferating cells generally have larger, less condensed nuclei and more mitoses than nonproliferating cells, characteristics used to define higher tumor grade. Pathological stage is less clearly a marker of proliferation than of tumor invasiveness and is less strongly associated with either labeling index or chromosome copy number in our study.

In summary, the results of this study demonstrate a strong correlation in human bladder cancer between genotypic abnormalities and tumor phenotype. Changes in centromeric copy number reflect genotypic modification. An increase in copy number, particularly of chromosome 7, is associated with a less favorable phenotype, including high nuclear grade, high pathological stage, and high BrdUrd and PCNA labeling indices. Conversely, a disomic or monosomic genotype, particularly for chromosome 7, is associated with a more favorable phenotype. The causal mechanism for this relationship is unclear, although it may possibly involve expression or suppression of specific genes on chromosome 7. Prospective follow-up of these patients will establish the relationship between the observed genotype and phenotype and actual clinical outcome.

Note Added in Proof

Hopman et al. (Hopman, A. H. N., Moesker, O., Smeets, W. G. B., Pauwels, R. P. E., Voojjs, G. P., and Ramaekers, C. S. Numerical chromosome 1, 7, 9, and 11 aberrations in bladder cancer detected by in situ hybridization. Cancer Res., 51: 644–651, 1991) have recently reported that FISH analysis of 40 bladder tumors showed prevalence of numerical aberrations for chromosomes 7, 9, and 11 similar to those reported in this paper.

REFERENCES

Centromeric Copy Number of Chromosome 7 Is Strongly Correlated with Tumor Grade and Labeling Index in Human Bladder Cancer

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