Featured Article

Centrosome Hyperamplification Predicts Progression and Tumor Recurrence in Bladder Cancer

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ABSTRACT

Purpose: Recent studies have reported that centrosome hyperamplification (CH) is closely related to chromosomal instability in bladder cancer. In this study, we investigated whether CH could be used as a prognostic biomarker for patients with bladder cancer.

Experimental Design: CH was evaluated by immunohistochemistry in 50 bladder cancers (≤pT1: 43; ≥pT2: 7). In addition, numerical aberrations of chromosomes 7, 9, and 17 and gain of 20q13, on which the Aurora-A gene is located, were evaluated by fluorescence in situ hybridization, and DNA ploidy was assessed. Preliminary experiments on eight bladder cancer cell lines found that six had over 5% of CH cells associated with a gain of 20q13 and overexpression of Aurora-A; therefore, CH-positive cases (CH+) were defined as those having over 5% of cells with ≥3 centrosomes per cell.

Results: CH+, 20q13 gain, chromosomal instability, and DNA aneuploidy were detected in 30 (60%), 18 (36%), 22 (44%), and 19 (38%) patients, respectively. There were significant differences in tumor number, grade, recurrence, and progression between the CH+ and CH− groups. The later had significantly higher recurrence-free and progression-free survivals than the former (P = 0.0028 and P = 0.0070, respectively, log-rank test). Multivariate analysis revealed that CH+ was the strongest predictor for tumor recurrence in nonmuscle invasive (pTa and pT1) bladder cancer (hazard ratio, 1.882; 95% confidence interval, 1.161–3.325; P = 0.0094).

Conclusions: Detection of CH may provide crucial prognostic information about tumor recurrence in bladder cancer.

INTRODUCTION

Transitional cell carcinoma (TCC) of the bladder ranks as the second most common malignancy of the genitourinary tract, with more than 13,000 cases reported in Japan in 1998. Approximately three-quarters of all bladder cancers are low grade and noninvasive superficial tumors. Despite a favorable prognosis with respect to mortality, 70% of patients have tumor recurrence, and 15% of these develop disease progression (1). Identification of the genetic or epigenetic changes underlying tumor recurrence or disease progression could provide useful prognostic information in such patients. To date, assays for several biomarkers, including BTA (bladder tumor antigen) and NMP22 (nuclear matrix protein), and a multitarget fluorescence in situ hybridization (FISH) assay (2), have been reported to have superior diagnostic abilities over conventional urinary cytology in bladder cancer. However, these tests provide little evidence for predicting patient prognosis. P53 alterations, as detected by immunohistochemical staining, may be an important prognostic indicator for disease progression (3), although the phenotype does not always reflect genotypic change.

The centrosome is a major microtubule organizing center for the formation of the bipolar mitotic spindles and plays an important role in accurate chromosome segregation to daughter cells (4). Accumulating evidence has suggested that centrosome hyperamplification (CH; ref. 5), which leads to the formation of multipolar spindles and unequal segregation of chromosomes, is both common and a major factor for chromosomal instability (6) in several human malignancies (7, 8). Overexpression of STK15/BTAK/Aurora-A kinase, a centrosome-associated serine/threonine kinase, the gene of which is located on 20q13, has been reported to lead to aneuploidy (9), cell transformation, and CH (10). Recent studies have reported that CH is closely related not only to the tumor grade and DNA ploidy but also to chromosomal instability in bladder cancer (11, 12). In this study, we examined whether CH could be used as a prognostic biomarker for patients with bladder cancer. Additionally, the relationship between CH and 20q13 gain was investigated in bladder cancer. To the best of our knowledge, this is the first report to show the striking association of CH with patient prognosis.

MATERIALS AND METHODS

Cell Culture Specimens. Eight established human bladder cancer cell lines (KK47, RT-4, T24, EJ-1, 5637, J82, TCC-
SUP, and SCaBER) were used for preliminary experiments (Table 1). Cells (3 × 10^5) were seeded on 75 cm² culture flasks (Corning, Corning, New York), cultured in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO₂ and 95% air at 37°C, and then harvested in the exponential phase (days 4 to 5) for comparative genomic hybridization analysis. Chamber slides (4.0 cm²; Nunc, Naperville, IL) containing each cell line plated for comparative genomic hybridization and digital image analysis were applied to the established cancer cell lines as described previously (15). In this study, aliquots of DNA extracted from tumors and lymphocytes were labeled by nick-translation with Spectrum Green and Spectrum Red (Vysis, Downers Grove, IL), respectively. A digital image analysis system (QUIPS XL, Vysis) was used. Gain and loss of DNA sequence copy numbers were judged by Green/Red ratios of >1.2 and <0.8, respectively.

### Indirect Immunofluorescence
For immunostaining of centrosomes and Aurora-A protein, cells were fixed with 10% formalin/methanol for 20 minutes at 25°C, washed with PBS, and permeabilized with 0.5% Triton X in PBS for 5 minutes at 25°C. Cells were then incubated with a blocking solution (10% normal goat serum in PBS) for 1 hour and subjected to immunostaining.

For immunostaining of centrosomes, a mouse monoclonal anti-γ-tubulin antibody (Sigma) was applied as described previously (16). The antibody-antigen complexes were detected with an Alexa 488- or 568-conjugated goat antirabbit IgG antibody (Molecular Probes, Eugene, OR) for 1 hour at 37°C.

Immunostaining of Aurora-A protein was done on established cancer cell lines. Cells were probed with a rabbit polyclonal anti-Aurora-A antibody (kind gift from Dr. Hideyuki Saya, Kumamoto University, Kumamoto, Japan) for 1 hour at room temperature. Antibody-antigen complexes were detected with an Alexa 594-conjugated goat antirabbit IgG antibody (Molecular Probes) for 1 hour at 37°C.

The number of centrosomes in each cell was determined by observing >100 cells in each sample under an epifluorescence microscope (Olympus, Tokyo, Japan) at ×1,000 magnification. CH was defined as 3 or more centrosomes per cell (16).

Overexpression of Aurora-A protein was defined as when more than 20% of the >100 cells counted showed strong cytoplasmic expression (9).

### FISH
To determine the gain of 20q13 and the variant fractions for chromosomal instability, dual- and multicolor FISH techniques were applied to specific regions at 20q13 and chromosomes 7, 9, and 17, respectively. Commercially purchased 20q13 probes (LSI ZNF217 and 20q13.2 amplicon) labeled with Spectrum Orange (Vysis) were cohybridized with centromeric probes specific for chromosome 20 (D20Z1) labeled with fluorescein (Q Biogene, Amsterdam, Netherlands). Numerical aberrations of chromosomes 7, 9, and 17 were detected with CEP 7 (D7Z1), CEP 9, and CEP 17 (Vysis), respectively. The fixation, hybridization, and posthybridization procedures were done according to protocols recommended by the supplier. More than 100 nuclei were counted under an epifluorescence microscope equipped with triple bandpass filter sets (DAPI/Spectrum Green/Spectrum Orange) in combination with a single passband filter (Spectrum Aqua, Vysis, Downers Grove, IL).

Cases were classified as 20q13 gain when over 60% of cells had >5 signals per cell for 20q13 (9).

The fraction of cells, the chromosome number of which was different from the modal chromosome number, was calculated and defined as the variant fraction, an index of chromosomal instability. Chromosomal instability was tentatively defined as over 25% of the average variant fractions of
chromosomes 7, 9, and 17, with reference to the data of Len-gauer et al. (6).

**Measurement of Nuclear DNA with a Laser Scanning Cytometer.** The nuclear DNA content was measured with a laser scanning cytometer (LSC 101, Olympus, Tokyo, Japan) as described previously (17, 18). A histogram was generated, and the DNA ploidy was determined. The DNA index was calculated according to published principles (19). Diploid and aneu-ploid tumors were classified as DNA index $<=1.2$ and $>1.2$, respectively (20).

**Statistical Analysis.** Statistical analysis was done with the JMP 4.0 statistical software (SAS Institute, Cary, NC). The
probability of survival was calculated by the Kaplan-Meier method, and statistical differences were evaluated by the log-rank test. A contingency table with either χ² test or Fisher’s test was applied for the univariate analysis. The Cox proportional hazard model was applied for multivariate analysis with a step-down procedure until all of the factors remained significant. For all of the statistical tests, \( P < 0.05 \) was considered significant.

RESULTS

Preliminary Experiments

Table 1 summarizes the results of the preliminary experiments on 8 established bladder cancer cell lines. Two cell lines (KK47 and RT-4) had <5% of cells with CH, whereas the other 6 cell lines (T24, EJ-1, 5637, J-82, TCC-sup, and SCaBER) had percentages >5%. The two cell lines with <5% CH cells showed consistent features of negative 20q gain by comparative genomic hybridization, 20q13 gain by FISH, and Aurora-A protein overexpression, whereas all of the 6 cell lines with >5% CH cells showed positive 20q gain by comparative genomic hybridization, 20q13 gain by FISH, and Aurora-A protein overexpression. KK47 showed the normal number (one or two) of centrosomes with disomy (two copies) of chromosomes 7 and 9 (Fig. 1A), whereas TCC-sup showed CH associated with numerical aberrations of chromosomes 7 and 9 (Fig. 1B). On the basis of these preliminary experiments, positive CH cases (CH+) were defined as those in which over 5% of the cells had CH in tumor tissue specimens; representing a slight modification of the criteria of Pihan et al. (8).

Clinical Study

Overview. The patient characteristics of the clinical specimens and the results of the study are summarized in Table 2. In total, 50 bladder cancer patients (41 men and 9 women, average age 66.9 years, range 33 to 88) were studied. The histopathological grade was 1, 2, and 3 in 5, 31, and 14 cases, respectively. The pathological stage was Ta, T1, and T2 in 9, 34, and 7 cases, respectively. For adjuvant therapy, intravesical instillation therapy with Bacillus Calmette-Guérin was done in patient numbers 30, 31, 40, and 44 and adjuvant chemoradiation therapy in patient numbers 18, 19, 26, 29, 35, and 45 (Table 2).

Table 1. Centrosome Hyperamplification in Bladder Cancer

<table>
<thead>
<tr>
<th>Centrosome Hyperamplification</th>
<th>CH+</th>
<th>CH-</th>
</tr>
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<tbody>
<tr>
<td>CH+</td>
<td>20q13 gain</td>
<td>30 (60%)</td>
</tr>
<tr>
<td>CH-</td>
<td>20q13 gain</td>
<td>18 (36%)</td>
</tr>
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</table>

CH+ 20q13 gain, chromosomal instability and DNA aneuploidy were detected in 30 (60%), 18 (36%), 22 (44%), and 19 (38%) cases, respectively. The percentage of cells with CH was 15.5 ± 6.5% (mean ± SD) in CH+ patients but only 1.7 ± 1.2% in CH− patients (\( P < 0.0001 \), unpaired \( t \) test). The percentage of cells with more than 5 copies of 20q13 was 87.2 ± 10.4% (mean ± SD) in patients with 20q13 gain but only 4.8 ± 8.0% in patients without 20q13 gain (\( P < 0.0001 \), unpaired \( t \) test).

Fig. 1 Representative cases with and without centrosome hyperamplification and the relationship with numerical aberrations. Centrosomes were evaluated by immunohistochemistry with an anti-γ-tubulin antibody followed by FISH with centromeric probes in the same cells. Superimposed figures were then created with LuminaVision (Mitani Corporation, Hukui, Japan). A. KK47. Note the two signals for centrosomes (green) with disomy of chromosomes 7 (red) and 9 (blue). B. TCC-sup. Note the centrosome hyperamplification (green) with polysomy of chromosomes 7 (red) and 9 (blue).
Relationship between CH and Patient Prognosis

Tumor recurrence was observed in 23 of 43 (53.5%) non-muscle invasive bladder cancer (Ta and T1) patients with a mean follow-up of 43.3 months (range, 4 to 94 months). CH+/H11002 patients had a significantly higher recurrence-free survival than CH+/H11001 patients for nonmuscle invasive bladder cancer (P = 0.0028, log-rank test; Fig. 2A). Multivariate analysis revealed that CH+ was the strongest predictor for tumor recurrence in nonmuscle invasive bladder cancer (hazard ratio, 1.882; 95% confidence interval, 1.161–3.325; P = 0.0094; Table 4).

Disease progression was observed in 10 (Six distant and four local progression) of the total of 50 (20.0%) bladder cancer patients with a mean follow-up of 40.8 months (range, 1 to 94 months). All cases with disease progression had CH+/H11001, irrespective of the type of disease progression. CH+ patients had a significantly higher progression-free survival than CH+ patients (P = 0.0070, log-rank test; Fig. 2B), whereas CH+ was not an independent prognostic factor for disease progression in multivariate analysis (data not shown).

DISCUSSION

Malignant tumors display two types of genetic instabilities: microsatellite instability arising from defects of mismatch repair genes and chromosomal instability caused by abnormal chromosome segregation (6). Our data strongly supports the hypothesis that CH drives chromosomal instability during tumor development (7).

Accumulating evidence has suggested that overexpression of STK15/BTAK/Aurora-A kinase, the gene of which is located on 20q13, induces aneuploidy (9), chromosomal instability, and CH (10). Our data shown in Table 1 supports the close association of CH with Aurora-A overexpression. A gain of the copy number of 20q13 was significantly associated with the variant fractions of chromosomes 7, 9, and 17 (data not shown) as well as CH. All of the 18 cases (100%) with 20q13 gain had CH+/H11001, which indicated that 20q13 gain leads to CH in bladder cancer. Also, 60% of the CH+/H11001 cases had 20q13 gain, which suggests that more than half the CH cases were caused by amplification or gain of the STK15/BTAK/Aurora-A kinase gene. This finding may partially support data indicating that more than half Aurora-A protein overexpression is associated with amplification.

![Figure 2A](image1.png)  
**Fig. 2A** Relationship between centrosome hyperamplification and patient prognosis. A. Recurrence-free survival in patients with nonmuscle invasive bladder cancer stratified by the centrosome hyperamplification. The CH− group has a significantly higher recurrence-free survival than the CH+ group for nonmuscle invasive bladder cancer (P = 0.0028, log-rank test). B. Recurrence-free survival in patients with grade 2 nonmuscle invasive tumors stratified by the centrosome hyperamplification. The CH− group has a significantly higher recurrence-free survival than the CH+ group (P = 0.0129, log-rank test).

![Figure 2B](image2.png)
tion of the Aurora-A gene in bladder cancer (9), whereas its gene amplification is not correlated to its overexpression in pancreatic cancer (21). Presumably, overexpression of the mRNA for STK15/BTAK/Aurora-A kinase without gene amplification (10, 21), or another mechanism, may cause CH in the discrepant cases (5, 22).

The role of chromosome 9 monosomy has been established in the oncogenesis of superficial bladder cancer (23, 24). This specific alteration is likely to be independent of CH, because 9 of 20 (45%) cases with CH− had chromosome 9 monosomy (data not shown), and monosomy may occur because of the nondisjunction of daughter chromosomes (25). The 5 recurrent cases without CH may have had other pathways for developing the recurrence.

Several biomarkers for detecting cancer, or for predicting patient outcome, have been published in the literature (2, 3). However, the clinical significance of biomarkers has not been fully elucidated in bladder cancer. Multitarget FISH assay may be a promising detection tool for bladder cancer. Numerical aberrations of chromosomes 7, 9, and 17 have been shown to be correlated with tumor recurrence as well as the clinicopathological background in bladder cancer patients (23, 24). Immunohistochemical detection of mutant p53 has been established as a technique for predicting patient outcome (3); although a drawback to this assay is a potential subjective bias in the interpretation of the results. In contrast, CH is determined by counting the number of signals and may thus show relative objectivity. On the basis of these observations, the CH detection assay may be a novel and powerful molecular tool for predicting patient outcome in bladder cancer. Regarding the definition of CH+, the tentative cutoff value of 5% may be justified by the clinical data that the mean value + 3 SD was 5.3% in the CH− group.

One may be skeptical in that the close association of CH with tumor recurrence could have been biased by other factors, because the CH+ group was linked to several clinicopathological parameters reflecting tumor recurrence. However, CH could also predict the tumor recurrence in patients who had grade 2 nonmuscle invasive tumors, which have been reported to have a highly variable prognosis in bladder cancer. Additionally, CH was detected in precursor lesions to several human cancers including *in situ* carcinomas of the uterine cervix, prostate, and the female breast (7, 8). CH and overexpression of Aurora-A were reported as an early event in rat mammary carcinogenesis (26).

In this study, more cases had CH+ and lacked chromosomal instability (30%) than those with chromosomal instability that lacked CH (4.5%), indicating that CH preceded chromosomal instability (8). These findings suggest that CH is likely to be an early event in oncogenesis that leads to chromosomal instability and eventually confers higher malignant behaviors on tumors, including tumor recurrence or disease progression.

Chemoradiation therapy was done in 6 patients whose CH statuses were CH+ (*n* = 4) and CH− (*n* = 2). The 4 patients with CH+ developed tumor recurrence (4) and disease progression (3), whereas the 2 patients with CH− had no recurrence or disease progression. This preliminary data suggests that CH might be used to determine adjuvant chemotherapy in bladder cancer.

In respect to disease progression, CH did not reach significance as an independent prognostic factor in multivariate analysis. The small number in the cohort and the short follow-up period may partially explain this discrepancy.

In conclusion, CH proved to be an independent prognostic factor for tumor recurrence, and detection of CH may provide crucial prognostic information for tumor recurrence. A larger

**Table 4** Cox proportional hazard model for tumor recurrence in nonmuscle–invasive bladder cancer

<table>
<thead>
<tr>
<th>Factor</th>
<th>Category</th>
<th>Hazard ratio</th>
<th>95% confidence interval</th>
<th><em>P</em></th>
</tr>
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<tr>
<td>Univariate analysis</td>
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<td>CH</td>
<td>+ versus −</td>
<td>2.025</td>
<td>1.268−3.542</td>
<td>0.0025</td>
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<tr>
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<td>Positive versus negative</td>
<td>1.615</td>
<td>1.021−2.520</td>
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<tr>
<td>CIN</td>
<td>+ versus −</td>
<td>1.739</td>
<td>1.133−2.699</td>
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<td>20q13 gain</td>
<td>+ versus −</td>
<td>1.702</td>
<td>1.083−2.617</td>
<td>0.0222</td>
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<td>DNA ploidy</td>
<td>Aneuploid versus diploid</td>
<td>1.640</td>
<td>1.041−2.529</td>
<td>0.0336</td>
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<td>Multivariate analysis</td>
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<tr>
<td>CH</td>
<td>+ versus −</td>
<td>1.882</td>
<td>1.161−3.325</td>
<td>0.0094</td>
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<tr>
<td>Urine cytology</td>
<td>Positive versus negative</td>
<td>1.665</td>
<td>1.046−2.622</td>
<td>0.0324</td>
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</tbody>
</table>

Fig. 3 Progression-free survival in all of the patients stratified by the centrosome hyperamplification. The CH− group have a significantly higher progression-free survival than the CH+ group (*P* = 0.0070, log-rank test).
cohort and longer follow-up period may elucidate the relationship between CH and disease progression in bladder cancer.

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