Genetic Alterations in Primary Bladder Cancers and Their Metastases

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Abstract

Bladder cancer progression is thought to be associated with sequential genetic events. To search for the specific genetic changes associated with the metastatic process, comparative genomic hybridization was performed on 22 primary tumors and 24 metastases (10 distant and 14 nodal metastases) from 17 patients with stage pT2-4 bladder cancer. There was a striking similarity between the genetic alterations present in the primary and metastatic tumor samples from the same patient. The mean number of genetic changes/tumor was 12.2 for primary tumors and 11.7 for metastases. There was a strong concordance in the specific aberrations present in each patient’s primary and metastatic lesions (mean, 75%). Concordance was also high among multiple sites from an individual primary tumor (mean, 96%) and multiple metastases from the same patient (mean, 75%). There were no specific genetic changes overrepresented in the metastases compared with their primary tumors. Genetic alterations present in more than 40% of tumors included gains on 6p, 8q, 10q, and 17q and losses involving 8p, 10q, and Y. Two regions of high-level amplification were common: (a) 10q22.1-q23.1 (32.6%); and (b) 17q11-21.3 (23.9%); the locus of erbB-2. A summary statistic was developed to quantify the degree of clonal relationships between biopsies from the same patient. These data support a model in which minimal clonal evolution occurs in the metastatic tumor cell population after the metastatic event. When comparing primary cancers from patients with and without metastases, however, several unique genetic changes were identified in those cancers with metastases, suggesting that these loci may harbor genes important to the metastatic process.

Introduction

Bladder cancer is the fifth most common cancer in the United States, with over 50,000 new cases diagnosed each year and more than 11,000 deaths annually (1). Approximately 15–30% of bladder tumors show grade and stage progression (2), with metastasis being the main cause of death in bladder cancer patients. Despite radical local therapy, 50% of patients who present with muscle-invasive bladder tumors die from metastatic disease (3).

Identifying patients at greatest risk for harboring or developing metastases would help to optimize patient management. In addition, the identification of genes responsible for metastasis may allow the development of new targeted therapeutic regimens. Although many common chromosomal abnormalities have been implicated in bladder cancer, specific genetic changes leading to metastasis have not been identified. Several studies have compared chromosomal abnormalities between primary and metastatic tumors in nonbladder cancers (small cell lung cancer, soft tissue sarcoma, colon cancer, and breast cancer), demonstrating similarities between the primary tumors and metastases (4–7). However, there have been no published data directly comparing genetic alterations in primary bladder tumors and their metastases. There are also no data available comparing nodal and distant metastases from the same patient.

CGH is a technique that screens for chromosomal abnormalities in the entire genome in a single hybridization, without prior knowledge of where these changes occur, identifying gains and losses of DNA sequence copy number (8). In the present study, CGH was used to compare genetic alterations in primary muscle-invasive bladder tumors and their corresponding metastases to define the genetic pathway leading to metastasis.

Materials and Methods

Tumor Samples. Archival bladder cancer specimens were obtained from the University of California (San Francisco, CA) and the University of Basel (Basel, Switzerland). Tumor material consisted of 22 primary tumors (stage pt2-4 transitional cell carcinoma) and 24 of their associated metastases (14 nodal and 10 distant metastases) from 17 patients. Three patients had more than one region of the primary tumor analyzed, and seven patients had more than one metastasis analyzed. Six patients had grade II tumors, and 11 patients had grade III tumors. There were 12 men and 5 women (average age, 74.6 years; range, 57–86 years).

DNA. H&E-stained thin sections were reviewed to assure that tumor was mixed with less than 20% normal cells. High molecular weight DNA was isolated from twenty 10-μm sections as described previously (8). When possible, nontumor material was dissected away from tumors. Sections were deparaffinized, and DNA was isolated by 3-day proteinase K digestion followed by phenol-chloroform-isomyl alcohol extraction. Normal reference DNA was extracted from peripheral blood mononuclear cells from healthy volunteers. Tumor and normal reference DNA were labeled by nick translation using fluorescein-12-labeled dUTP (green) and Texas Red-labeled dUTP. After labeling, the size of the probes ranged from 300 bp to 3 kb.

CGH. Hybridization of differentially labeled tumor and normal DNA to normal metaphase chromosomes was performed as described previously (8, 9). Fluorescein-labeled tumor DNA (200 ng), Texas Red-labeled reference DNA (200 ng), and human Cot-1 DNA (20 μg) were denatured at 73°C for 5 min and applied to denatured normal metaphase slides. Hybridization was at 37°C for 2 days. After washing, the slides were counterstained with 0.15 μg/ml 4',6-diamidino-2-phenylindole in an antifade solution.

A second hybridization was performed for all tumor samples using fluorescein-labeled reference DNA and Texas Red-labeled tumor DNA (inverse labeling) to confirm the aberrations detected during the initial hybridization. In two select cases, simultaneous hybridization of primary and metastatic tumor DNA was performed to confirm the results obtained by standard CGH. Each CGH experiment also included a normal control hybridization using fluorescein-labeled and Texas Red-labeled normal DNA.

Digital Image Analysis. Digital image analysis was used to identify chromosomal regions with abnormal fluorescence ratios, indicating regions of DNA gains and losses (10). Three-color images were acquired using a Zeiss Axioplan microscope and a cooled charge-coupled-device camera. Changes in relative copy number of the DNA sequences were determined by a comparison of the relative intensities of the green and red fluorochromes along the length of the chromosomes. The average green:red fluorescence intensity ratio pro-

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files were calculated using four images of each chromosome (total, eight chromosomes) with normalization of the green:red fluorescence intensity ratio for the entire metaphase and background correction. A gain of DNA sequence copy number was defined as a tumor/reference ratio of $>1.2$, and a loss of DNA sequence copy number was defined as a tumor/reference ratio of $<0.8$, with changes present on both standard and inverse CGH hybridizations. High-level amplification was defined as a tumor/reference ratio of $>1.5$ in a chromosomal subregion, a ratio typically higher than that represented by a whole arm gain or loss. Chromosome identification was performed based on 4′,6-diamidino-2-phenylindole banding patterns. Only images showing uniform high-intensity fluorescence with minimal background staining were analyzed. All centromeres, $p$ arms of acrocentric chromosomes, and heterochromatic regions were excluded from the analysis.

**Statistics.** Differences in DNA copy number alterations between primary and metastatic bladder tumors were compared using contingency table analysis. A $P$ of less than 0.05 was considered significant. In addition, we formed a summary statistic to compare gains and losses among pairs of tumors at each of the chromosome arms. The summary statistic is a weighted sum of the results (i.e., gains, losses, or no changes) for each chromosome arm. The weights are based on the overall probabilities of gains and losses for each chromosome arm (derived from the data set being analyzed), with greater weight given to genetic changes that are more rare compared with those changes that are more common. The weights are proportional to the log likelihood of the observed difference.

The summary statistic is defined as follows: $X_{ij}$ is an indicator variable equal to 1 if there is a gain (or loss) at the $j$th chromosome arm in the $i$th member of the pair ($i = 1; j = 1 \ldots 82$), whereas $p_i$ is the overall probability of gain or loss at the $j$th chromosome arm. The statistic ($S$) is defined by:

$$S = \sum_r (-1)^{x_{ij} + s_{ij}} \left\{ (x_{ij} + s_{ij}) \ln \left( \frac{p_i}{1 - p_i} \right) + 2 \ln (1 - p_i) \right\}.$$  

Statistical simulation was used to establish that the distribution of this statistic is approximately Gaussian when nonrelated (different patient) pairings are analyzed. When the pairing is correlated (i.e., clonally related), the value of the summary statistic ($S$) is greater than it is when the pairing is random. Agreement in rare changes between chromosome pairs will result in a larger change in $S$ compared with those changes that are more common.

The summary statistic was calculated for paired samples from the same subject and compared to the distribution of values using pairs from two different subjects. The likelihood that a given $S$ statistic was from the same subject versus two different subjects was approximated by fitting normal (Gaussian) distributions to the two kinds of subject pairings and then forming the likelihood ratio from the two normal distributions. This comparison was performed on three types of pairings: (a) those between primary and metastatic tumors; (b) those between two metastatic tumors; and (c) those between two primary tumors.

**Results**

All primary and metastatic tumors showed at least one DNA copy number alteration (range, 1–22). The total number of DNA gains was slightly higher than the number of DNA losses (mean number of gains, 6.7; mean number of losses, 5.3). There was no significant difference in the number of aberrations/tumor between primary tumors (mean, 12.2 ± 6.6) and metastases (mean, 11.7 ± 3.6; Table 1). There was a slightly but not significantly higher number of aberrations/tumor in distant metastases compared with nodal metastases (mean, 12.6 ± 5.8 versus 11.1 ± 3.7).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>CGH aberrations in invasive bladder cancer</th>
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<tr>
<td></td>
<td>Average no. of changes/tumor</td>
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<tr>
<td>Primary tumors ($n = 22$)</td>
<td>12.2</td>
</tr>
<tr>
<td>All metastases ($n = 24$)</td>
<td>11.7</td>
</tr>
<tr>
<td>Nodal metastases ($n = 14$)</td>
<td>11.1</td>
</tr>
<tr>
<td>Distant metastases ($n = 10$)</td>
<td>12.6</td>
</tr>
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</table>

*A Twenty-two primary tumors from 17 patients.*

A summary of all DNA sequence number alterations present in the primary tumors is shown in Fig. 1. The most common genetic aberrations detected are summarized in Table 2. Several patients showed an associated gain of 5p and loss of 5q (found in five primary tumor samples and three metastases). There was high concordance between the aberrations present in the paired primary and metastatic tumors from each patient (mean, 75%; range, 25–100%). The genetic differences between each metastatic tumor and its respective primary are presented in Fig. 2. There were no specific genetic changes that were significantly overrepresented in the metastatic tumors. Losses of DNA sequences on 2q were overrepresented in the primary tumors compared with the metastases. Additional comparisons were made in patients with either multiple samples available from a single primary tumor or with multiple metastases. Concordance was highest among multiple sites from individual primary tumors (mean, 96%; range, 91–100%). Multiple metastases from the same patient were also similar but did not agree as completely as the primary tumors (mean concordance, 75%; range, 38–100%). This included both nodal and distant metastases.

Additional analyses were made using the summary statistic ($S$). This statistic was applied to the three types of tumor pairings (primary-metastasis, metastasis-metastasis, and primary-primary). Fig. 3 shows the distribution of the summary statistic for these pairings. These plots show that when the pairs were from the same subject, the summary statistic was significantly larger than it was when the pairs were from different subjects. A larger summary statistic represents a stronger correlation between the two samples in the pair. Table 3 further summarizes this analysis.

Several areas of high-level amplification (green:red fluorescence intensity ratio of $>1.5$) were identified in both primary and metastatic tumors. The most common regions involved were $10q22.1$–$q23.1$ (32.6% of tumors), $17q11$–$q21.3$ (23.9% of tumors; the locus of erbB-2), $1q12$–$q25$ (10.9% of tumors), $11q11$–$q14.1$ (8.7% of tumors; cyclin D1), and $20q11.2$–$qter$ (8.7% of tumors).

In the two cases for which simultaneous hybridization of primary and metastatic DNA was performed, the results confirmed those found using standard CGH. All regions of common genetic change showed flat ratio profiles. Changes that differed between the primary and metastatic tumors were shown as ratio profiles of either greater than or less than 1.0.

A comparison was made between the genetic aberrations present in this group of 22 stage pT2–4 bladder tumors with known metastases and aberrations detected by CGH in a previous series of 10 stage pT2–4 N0M0 primary bladder tumors (11). The primary tumors with associated metastases had a significantly higher number of aberrations/tumor than did those without metastases (mean, 12.2 versus 5.9, respectively). There were several genetic alterations that were significantly overrepresented in the tumors with metastases and were not present in those without metastases ($P < 0.05$). These include gains of 2p, 5p, 7q, 10q, 17q, and 20q and losses of 4p and 5q. The regions on 10q, 17q, and 20q were also common regions of high-level amplification.

**Discussion**

Bladder cancer development and progression is thought to result from an accumulation of multiple genetic events (12, 13). Some genetic changes have been associated with superficial tumors, whereas others are more commonly associated with invasion, suggesting that certain genetic abnormalities may be responsible for tumor initiation, whereas others are responsible for progression (13, 14). However, the genetic events leading to bladder cancer metastasis have not been clearly defined. Identifying the specific genetic pathways involved in the metastatic process would help us to better understand...
CGH IN PRIMARY AND METASTATIC BLADDER CANCERS

Fig. 1. Summary of gains and losses of DNA sequences detected by CGH in 17 primary bladder tumors. The lines to the left of the chromosomes represent DNA losses, and the lines to the right of the chromosomes represent DNA gains.

the natural history of this disease and lead to optimization of patient therapy, facilitating the development of more effective treatment modalities.

Primary invasive bladder tumors are genetically complex, as shown by the large number of genetic changes/tumor in our series (mean, 12.2). Genetic alterations were seen on every chromosome, with some regions showing especially frequent changes. Many of the common genetic changes detected in this series have been reported previously (11, 15–20). It is interesting that many of the alterations found in pT1 compared with pTa tumors (20) were also commonly found in our current series of pT2–4 and metastatic tumors. These alterations include gains at 1q, 3q, 5p, 6p, and 8q and losses at 2q and 8p. An associated gain of 5p and loss of 5q was found in eight tumor samples. This was recently reported in a case of invasive bladder cancer and interpreted as a possible iso(5p) (21). Loss of 10q was a common finding in our series (50% of primary tumors and 33% of invasive tumors), a finding that is consistent with a recent study showing a 45% rate of allelic loss involving 10q (22). The prevalence of chromosome 9 loss was lower than that reported previously using molecular analysis (23). This is likely due to the limitations of CGH in detecting small deletions. Also uncommon in our series was a loss of 14q, which has been previously associated with primary invasive bladder cancers (24). This finding is more surprising, because 14q deletions tend to be large. This may be due to the relatively small number of primary tumors in our study (22 primary tumor samples from 17 patients). Of interest, in the two patients that did show 14q loss, the loss was found in all specimens analyzed from those two patients (four primary tumors and three metastatic tumors).

Several common regions of high-level amplification were present, representing high-level copy number increases of chromosomal sub-regions. The regions of high-level amplification located at 1q, 11q, and 20q have been described previously in bladder cancer (11, 20). Regions of high-level amplification found on 1q, 10q, and 20q may represent locations of yet unknown oncogenes involved in bladder tumor progression and should be further investigated.

CGH is a whole-genome screening method that does not detect small regions of gains and losses. CGH is able to detect deletions of 5–10 mb and high-level amplifications as small as 100 kb in length. Tumor heterogeneity and normal cell contamination will decrease this Table 2  Frequent genetic alterations

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Primary tumors (%)</th>
<th>Metastatic tumors (%)</th>
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<tbody>
<tr>
<td>DNA gains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1q</td>
<td>72.7</td>
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</tr>
<tr>
<td>9p</td>
<td>45.5</td>
<td>41.7</td>
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<tr>
<td>1q</td>
<td>36.3</td>
<td>29.2</td>
</tr>
<tr>
<td>5p</td>
<td>36.4</td>
<td>37.5</td>
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<tr>
<td>10q</td>
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<tr>
<td>20q</td>
<td>36.4</td>
<td>33.3</td>
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<tr>
<td>3q</td>
<td>31.8</td>
<td>45.8</td>
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<td>7q</td>
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<td>2p</td>
<td>22.7</td>
<td>41.6</td>
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<tr>
<td>11q</td>
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<td>37.5</td>
</tr>
<tr>
<td>8q</td>
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<tr>
<td>1q</td>
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<tr>
<td>Y</td>
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<td>47.4</td>
</tr>
<tr>
<td>2q</td>
<td>45.5</td>
<td>12.5</td>
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<tr>
<td>5q</td>
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<tr>
<td>18q</td>
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<tr>
<td>6q</td>
<td>36.4</td>
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<tr>
<td>9p</td>
<td>31.8</td>
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sensitivity. CGH is unable to detect genetic alterations that do not involve an actual loss or increase of DNA copy number (e.g., point mutations, transcriptional gene activation, and chromosomal translocations).

The specific aberrations present in both the primary and metastatic tumors in each patient were very similar, demonstrating that minimal clonal evolution occurs in the metastatic tumor cell population after the metastatic event. The observation that both primary and metastatic tumors retain similar genetic changes suggests that these changes may be of biological importance.

A summary statistic was used to compare similarities and differences in the specific DNA gains and losses present in tumor pairs. This analytic tool was developed so that the relationships between paired biopsies could be interpreted relative to pairs in which no relationship existed (different patients). The analysis takes the background prevalence of alterations at each arm into consideration, so that more common alterations are not weighed too heavily. The \( S \) statistic computed for different biopsies of the same primary tumor shows a wide separation from the unrelated biopsies (Fig. 3c). This was a much greater separation that that seen in the primary-metastasis pairs (Fig. 3a). In fact, four of the primary-metastasis pairs overlapped with the distribution of unrelated pairs (i.e., the primary tumor and metastasis were as different from each other as the unrelated pairs), showing that the metastasis had undergone a significant clonal evolution away from the primary clone. Most of the primary-metastasis pairs showed a strong resemblance, however. Interestingly, pairs of metastases from the same patient (Fig. 3b) showed a distribution of the \( S \) statistic that was clearly separate from the unrelated pairs, but the values were not as high as the primary-primary pairs. It is remarkable that the different metastases showed such a strong similarity, because they were separated from each other during their entire growth of at least 20–30 cell doublings, yet their clonal divergence was minimal.

The extremely high congruence found in multiple sites analyzed from the same primary tumor demonstrates strong intratumor homogeneity with respect to DNA copy number. Intratumor heterogeneity in centromere 17 and in \( \text{erbB-2} \) copy number has been described previously using fluorescence in situ hybridization (25). Such intratumor heterogeneity cannot be detected by CGH, because CGH only detects the average chromosomal copy number. These results from multiple primary biopsies suggest that in these patients, whatever heterogeneity in absolute copy number that may have existed in the various cell populations still resulted in identical relative copy number changes in the different biopsies analyzed. One cannot rule out the possibility that minor cell populations with an altered copy number did exist in either the primary tumor or the metastasis, but that their proportion was too small to affect the overall observed copy number balance.

Although the lymph node and distant metastases shared common genetic changes, the distant metastases had, on average, more changes per tumor. This trend, however, was not statistically significant. Multiple metastatic tumors from the same patient are genetically similar and often identical by CGH analysis. This finding was especially interesting in a case showing identical CGH changes in a lymph node metastasis and a lung metastasis that were not found in the primary tumor, suggesting that both nodal and distant metastases share a common clonal past.

Genetic aberrations present in these muscle-invasive tumors with
known metastases were compared with the aberrations present in a small group of muscle-invasive tumors without metastases that we analyzed previously using CGH (11). The primary tumors with metastases showed significantly more changes than did those lacking metastases. This finding supports a model of tumor progression based on an accumulation of genetic events and illustrates the increased genetic instability in metastatic tumors. Although the number of tumors analyzed was relatively small, there were statistically significant genetic differences between the two groups of tumors. Several regions of common gains and losses of DNA sequences were present in the tumors with metastases that were not found in those tumors without metastases. Located in these regions are the oncogene erbB-2, found at locus 17q11.2–12, and other common regions of high-level amplification identified at 10q22.1–q23.1 and 20q11.2–qter, which could potentially represent the site of other oncogenes involved in bladder tumor metastasis. Additional studies of these regions using locus-specific probes could help identify the specific genes responsible for bladder cancer progression and metastasis, thus providing valuable prognostic and therapeutic information.

In conclusion, several commonly altered chromosomal regions were identified by CGH in muscle-invasive bladder tumors, including regions that have not been described previously. Clear genetic similarities were present between multiple tumors (primary and metastatic) from the same patient, confirming a common clonal past. The fact that these poorly differentiated primary tumors were genetically similar to their metastases indicates that the changes responsible for metastasis occurred earlier in the pathogenesis of these tumors. Models of tumor progression based on unique genetic changes leading to metastasis may need to be reexamined in light of these findings. Different genetic alterations were seen in invasive primary tumors with and without metastases, potentially representing the genes responsible for tumor progression and metastasis. Further refinement of these regions could identify chromosomal markers that would help predict the risk of metastasis, optimize initial therapy, and lead to the development of novel therapeutic regimens.

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References


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