Invasive (pT₁) Urinary Bladder Cancer

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Abstract

Many cytogenetic alterations are known to occur in urinary bladder cancer, but the significance of most of them is poorly understood. To define these chromosomal regions where clinically relevant genes may be located, a series of 54 pT₁ urinary bladder carcinomas with clinical follow-up information (median, 52 months; range, 5–167 months) were examined by comparative genomic hybridization. The most frequent alterations included DNA sequence copy number gains at 1q22-24 (33%), 20q11.2-ter (33%), 8q22 and 17q21 (28% each), and 6p22 (15%) as well as deletions at Y (37%), 9p (31%), 9q22-33 and 11p14-ter (28% each), 11q23 (26%), 8p (24%), 13q31 (19%), 2q35-ter (17%), and 2q22-33 (11%). Whereas the histological grade was unrelated to prognosis (P = 0.9752), the risk of tumor progression was significantly associated with the number of deletions per tumor (P = 0.0014). Individual cytogenetic alterations that were linked to subsequent tumor progression included gains of 3p22-24 (P = 0.0112) and 5p (P = 0.0003) as well as losses of 4p11-15 (P = 0.0052), 5q15-23 (P = 0.0410), 6q22-23 (P = 0.0090), 10q24-26 (P = 0.0232), and 18q12-23 (P = 0.0005). Genes with a role for bladder cancer progression may be located at these regions.

Introduction

Urinary bladder cancer is the fifth most common cancer in humans in Western societies. Most bladder neoplasms are transformational cell carcinomas that are either papillary noninvasive (stage pT₁) or have invasion into the muscular bladder wall (stage pT₂ or higher) at the time of diagnosis. Only 10–20% of bladder carcinomas present with tumor invasion limited to the lamina propria (pT₁). These carcinomas pose the greatest clinical problems. Most pT₁ bladder carcinomas can be cured by transurethral resection, but 20–30% will undergo local tumor progression into a potentially life-threatening muscle-invasive cancer after conservative surgical treatment. Therefore, early cystectomy is increasingly being considered in these patients. To identify patients that might benefit from an aggressive surgical therapy, a reliable prediction of the risk of progression is needed at the time of the initial diagnosis.

Development and progression of bladder cancer is driven by the malfunction of specific genes (i.e., overexpression of oncogenes or inactivation of tumor suppressor genes). Therefore, the identification of genetic alterations may provide clinical relevant information. Several genes are known to play a role in bladder cancer, including erbB-2, EGF, c-myc, Cyclin D1, h-RAS, p53, p16, and the retinoblastoma gene. Other critical genes may be located at these genomic regions and frequently deleted regions such as at 2q, 4q, 5q, 6q, 8p, 9p, 9q, 11p, 11q, and 13q may carry tumor suppressor genes, whereas typically overrepresented areas such as 1q, 3p, 5p, 6p, 8q, 17q, and 20q may carry oncogenes that are as yet unidentified. It is likely that alterations of the target genes at some of these loci may influence tumor aggressiveness or response to therapy, whereas other target genes may have less clinical importance. To identify genomic alterations that are linked to bladder cancer progression, we analyzed 54 pT₁ bladder carcinomas for which clinical follow-up information was available by CGH. CGH allows the detection of all relative DNA sequence copy number gains and losses of tumors in one examination. The results pinpoint several genomic regions that may carry genes with significance for bladder cancer progression.

Materials and Methods

Patients and Tumor Material. Formalin-fixed, paraffin-embedded primary bladder tumors were from the archives of the Institute of Pathology at the University of Basel and the Triemli Hospital Zurich, Switzerland. Tumor stage and grade were defined according to Union Internationale Contre le Cancer and WHO classifications by one pathologist (G. S.). Only tumors for which histological staging was unequivocal were included in this study. Unequivocal stroma invasion and presence of tumor-free muscularis propria were required to define a tumor as pT₁. Patients having muscle invasion in a follow-up biopsy taken <3 months after the initial biopsy were excluded from the study because a sampling error in the initial biopsy was considered more likely than a true progression in these cases. The series consisted of 54 pT₁ carcinomas, including 31 grade 2 and 23 grade 3 tumors. Thirty-eight patients were male and 16 were female. The CGH findings of 27 of our patients have been reported previously (1, 5). All 54 patients had undergone regular follow-up cystoscopies at 3, 9, and 15 months (or more frequently), and then annually until the end point of this study (progression, last control). The median follow-up period was 52 months (range, 5–167 months). Intravesical treatment had been performed in 30 patients (mitomycin in 17, BCG in 10, adriblastin in 1, and epirubicin in 2). Five patients underwent cystectomy during the course of their disease. Progression was defined as the presence of a histologically proven muscle invasion in a follow-up biopsy taken at least 3 months after the initial biopsy.

DNA Preparation. Tissue preparation and DNA extraction were as described (5). Tumors having an average tumor cell content of <75% were excluded. Tumor DNA (1 µg) was nick translated using a commercial kit (BioNick kit; Life Technologies, Gaithersburg, MD) and Spectrum Green dUTPs (Vysis Inc, Downers Grove, IL) for direct labeling of tumor DNA. Spectrum Red-labeled normal reference DNA (Vysis) was used for cohybridization. CGH. The hybridization mixture consisted of 200 ng of Spectrum Green-labeled tumor DNA, 200 ng of Spectrum Red-labeled normal reference DNA, and 20 µg of Cot-1 DNA (Life Technologies) dissolved in 10 µl of hybridization buffer (50% formamide, 10% dextran sulfate, 2× SSC, pH 7.0). Hybridization, image acquisition, image analysis, and control experiments were as described previously (1, 5). At least four observations per autosome and two observations per sex chromosome were included in each analysis. Each CGH experiment included a tumor cell line (Spectrum Green MPE-600;...
Vysis) with known aberrations (positive control) and a hybridization of two
differentially labeled sex-mismatched normal DNAs to each other (negative
control). A gain of DNA sequences was assumed at chromosomal regions
where the hybridization resulted in a tumor to normal ratio >1.20. Overrep-
resentations were considered amplifications when the fluorescence ratio values
exceeded 1.5 in a subregion of a chromosome arm. A loss of DNA sequences
was presumed where the tumor-to-normal ratio was <0.80. Definition of a
change as an aberration also required that the first SD be above (gain) or below
(deletion) 1.00. Because some false aberrations were detected in normal tissues
at 1p, 16p, 19, and 22, these G-C-rich regions, known to produce false-positive
results by CGH, were excluded from all analyses.

Statistics. Contingency table analysis and Student’s t tests were used to
analyze the relationship between individual cytogenetic changes or the number
of genomic alterations and the histological grade. Survival curves were plotted
according to Kaplan-Meier. A log-rank test was applied to test the statistical
significance of differences between tumor grades and genomic alterations with
progression. Thirty-nine patients were censored at the time of their last
significance of differences between tumor grades and genomic alterations with
according to Kaplan-Meier. A log-rank test was applied to test the statistical
univariate analysis was used to test for independent prognostic information.

Results

CGH Findings. The mean number of aberrations per tumor was
6.7 (range, 0–23). On average, there were 3.4 deletions (range, 0–13),
2.9 gains (range, 0–10) and 0.4 amplifications (range, 0–3) per tumor.
Seven pT1 carcinomas lacked detectable aberrations. All aberrations
are summarized in Fig. 1. Typical examples of CGH alterations are
given in Fig. 2. A gain of 1q was the most frequent aberration (37%),
with a minimal common region at 1q22-25. Other frequently gained
loci included 20q11.2-ter (33%), 17q21 and 8q22 (28% each), and
6p22 (15%). Deletions were most frequently seen at Y (37%), 9p
(31%), 9q22-33 and 11p14-ter (28% each), 11q23 (26%), 8q (24%),
and 13q31 (19%) as well as at 2q35-ter (17%) and 1q22-33 (11%). There
were 19 amplifications found at nine different loci, including 1q22-24
(n = 1), 3p12 (1), 3p22-24 (2), 5p15 (1), 6p22 (3), 8q22 (2), 8q24 (1),
10p13 (2), 11q13 (3), 12q15 (1), 13q33-34 (1), and Xp21 (1).

Histopathological Correlations. Grade 2 carcinomas had
3.3 ± 3.0 deletions, 2.3 ± 2.6 gains (including high-level amplifica-
tions), and 5.6 ± 5.2 total aberrations per tumor. There were more
alterations in grade 3 than in grade 2 carcinomas, but this difference
reached significance only for the number of gains. Grade 3 carcino-
mas had 3.7 ± 2.8 deletions (P = 0.5898 for grade 2 versus Grade 3),
4.5 ± 3.6 gains (P = 0.0137), and 8.2 ± 5.8 total aberrations per
(tumor (P = 0.0898). A comparison of the prevalence of the most
frequent changes between tumors of different grades is shown in
Table 1. Overrepresentation of 6p22 was the only individual alteration
that was significantly linked to high tumor grade (P = 0.0054).

Tumor Progression. A subsequent tumor progression occurred in
15 of our 54 pT1 carcinomas. The risk of progression was significantly
associated with the number of deletions that were detectable by CGH
(P = 0.0014; Fig. 3B) but not with the number of gains (P = 0.3030),
the total number of genetic alterations (P = 0.1701), or the histologi-
cal grade of the tumors (P = 0.9752; Fig. 3A). The 31 most frequent
genomic alterations (all changes that were found in at least five
tumors) were evaluated for their prognostic significance. Gains at
3p22-24 (P = 0.0112; Fig. 3C) and 5p (P = 0.0003; Fig. 3E), as well
as losses at 4p11-15 (P = 0.0052; Fig. 3D), 5q15-23 (P = 0.0410;
Fig. 3F), 6q22-23 (P = 0.0090; Fig. 3G), 10q24-26 (P = 0.0232; Fig.
3H), and 18q12-23 (P = 0.0005; Fig. 3I) were significantly linked to
an increased risk of tumor progression. For gain of 5p (P = 0.0063)
and losses at 4p11-15 (P = 0.0308), 10q24-26 (P = 0.0037), and
18q12-23 (P = 0.0015), this hold also true if the analysis was limited
to the small group of 30 patients who had undergone intravesical
chemotherapy.

The level of significance was not achieved for a gain at 3p22-24
(P = 0.7038) and losses at 5q15-23 (P = 0.1158) and 6q22-23
(P = 0.1191) in the subgroup of treated patients despite a clear
tendency toward a poor prognosis in tumors having losses at 5q15-23
or 6q22-23.

CGH findings that were not significantly associated with poor
prognosis included losses at 1q22-24 (P = 0.8588), 2q22-33
(P = 0.1108), 4q25-ter (P = 0.0594), 4q (P = 0.3212), 8p
(P = 0.0532), 9p (P = 0.6131), 9q22-33 (P = 0.1310), 11p14-ter
(P = 0.2880), 11q23 (P = 0.1827), 13q31 (P = 0.9841), 14q23-31
(P = 0.3800), 17p (P = 0.3486), Xp (P = 0.3486), Xq11-22.3

Fig. 1. Summary of all relative DNA sequence copy
number changes detected by CGH in 54 pT1, bladder
carcinomas. The vertical lines on the right of the
chromosome idiomats indicate gains; vertical lines on the
left indicate losses of the corresponding chromosomal
regions. Amplifications are indicated as solid bars. 1p,
16p, 19, and 22 were not analyzed.
(P = 0.2320), and Y (P = 0.1176) as well as gains at 6p22 (P = 0.8278), 7q22 (P = 0.1055), 8q22 (P = 0.4596), 10p12-13 (P = 0.6757), 11q13 (P = 0.4843), 12q15-21 (P = 0.7140), 17q21 (P = 0.7592), 20p (P = 0.1781), 20q11.2-ter (P = 0.5650), and Xp21 (P = 0.3062).

A multivariate analysis that included all individual chromosomal alterations that were significantly associated with tumor progression in univariate analysis suggested that gains at 5p (P = 0.0090) are independently predictive of poor prognosis, whereas gains at 3p22-24 (P = 0.1002) and losses at 4p11-15 (P = 0.7599), 5q15-23 (P = 0.4683), 6q22-23 (P = 0.0712), 10q24-26 (P = 0.2057), and 18q12.2-23 (P = 0.0963) yielded no significant results.

**Discussion**

In this study, 54 urinary bladder carcinomas with invasion limited to the lamina propria (stage pT1) were examined by CGH to determine whether genomic alterations were associated with poor prognosis. CGH is a powerful tool to retrospectively evaluate the prognostic significance of genomic alterations because it can be applied to archival tissue and because alterations of all chromosome arms can be related to clinical endpoints in one study. Moreover, the total number of alterations can be determined, a parameter that may define a "genetic grade" of a tumor. It has been suggested that an increasing number of CGH aberrations is linked to poor prognosis in several tumor types (6, 7). The results of this study suggest associations between the number of gains and the histological grade as well as between the number of deletions and a poor prognosis. These observations are consistent with an increasing number of genomic alterations occurring during tumor progression.

Several individual changes were significantly linked to an increased risk of subsequent progression. These findings must be interpreted with caution because the high number of statistical analyses performed in this study may have lead to an increased risk of false significant P values. In addition, it cannot be excluded that heterogeneity of treatment could have influenced the analyses because some results could not be confirmed in a separate analysis of patients that had undergone intravesical treatment. However, this latter observation could also be explained by the small number of patients in this subgroup (n = 30). Despite these limitations, our results suggest that genes playing a role in bladder cancer progression may be located at 5p and 6q—belong to these six aberrations that previously were found to be significantly more frequent in muscle-invasive (pT2-4) than in minimally invasive (pT1) tumors by CGH (1). On the basis of the assumption that alterations that cause progression will accumulate in high stage tumors, this was interpreted as evidence for a role of genes at these locations for progression. Thus, the findings of the present study provide additional and independent evidence for a possible role of genes at 5p and 6q for the progression of invasive bladder neoplasms.

Few previous studies have investigated the possible role of 6q— and 5p+ in bladder cancer. Loss of heterozygosity at 6q has been reported in <10% of a series of bladder tumors that predominantly consisted of noninvasive tumors (8). Only a few microsatellite probes were used in that study, resulting in a large minimal common region of deletion spanning 6q22-27. Loss of heterozygosity at 6q was found to be clearly more frequent in several other tumor types, such as carcinomas of the breast (9), cervix uteri (10), prostate (11), ovary (12), and the kidney (13) as well as in lymphomas (14) and melanoma (15). Some of these studies have defined minimal regions of deletion...
at 6q21, 6q25, and 6q27. Further studies are needed to determine whether one or several of these regions have importance for urinary bladder cancer.

Overrepresentations of 5p are well known in bladder cancer. Cytogenetic studies have suggested that isochromosomes at 5p may represent primary alterations in urinary bladder cancer (16). The strong association between 5p gains and 5q deletions found in this study is consistent with isochromosome formation as a major mechanism for overrepresentation of 5p sequences in bladder cancer. Because 5p gains were much more strongly (and independently) linked to poor prognosis than 5q deletions, it could be speculated that the link between 5q- and poor prognosis might be driven by the 5p gains that are often associated with 5q deletions. Nevertheless, the observation of isolated 5q deletions argues for an independent role of genes on 5q in urinary bladder cancer, although a malfunction of these genes may be less significant for patient prognosis than overrepresentations at 5p. Currently there is no strong candidate oncogene on 5p. However, because 5p DNA sequence copy number gains and high-level amplifications were often found by CGH in various other tumor types, such as carcinomas of the lung, head, and neck and the uterine cervix as well as osteosarcoma (reviewed in Ref. 17), this region is likely to carry at least one oncogene with general importance.

Other cytogenetic alterations associated with poor prognosis in this study included gains at 3p22-24 and losses at 4p11-15, 10q24-26, and 18q12-23. The finding of circumscribed high-level amplifications at 3p12 and 3p22-24 in this and other CGH studies (3) and a previous report of amplifications of \( \text{RAF1} \) (at 3p25; Ref. 18) provide evidence for a role of multiple 3p oncogenes in urinary bladder cancer. This is also supported by the frequent involvement of 3p in translocations detected in cytogenetic analyses of bladder cancer (19). Our data raise the possibility that at least the distal one of the putative 3p oncogenes may be linked to tumor progression. Although there are no strong bladder cancer tumor suppressor gene candidates on 4p and 18q, it is possible that 10q deletions lead to a growth advantage of bladder cancer cells through inactivation of \( \text{PTEN} \). \( \text{PTEN} \), the gene that codes for a putative protein tyrosine phosphatase, has been found mutated in a fraction of bladder carcinomas (20). Further studies are needed to

Fig. 3. Chromosomal alterations associated with tumor progression. Whereas the histological grade was not associated with prognosis (A), the risk of progression was significantly linked to the number of deletions (B) and to several individual genomic alterations including gain of 3p22-24 (C), loss of 4p11-15 (D), gain of 5p (E), loss of 5q15-23 (F), loss of 6q22-23 (G), loss of 10q24-26 (H), and loss of 18q12-23 (I).
determine whether PTEN inactivation may be a predictor of poor prognosis in urinary bladder cancer.

In summary, these data suggest that gains at 3p22-24 and 5p as well as losses at 4p, 5q, 6q, 10q, and 18q are linked to the progression of pT3 bladder carcinomas. Further studies are now needed not only to identify target genes at these loci and to determine whether these genes will be suited as therapeutic targets but also to investigate whether fluorescence in situ hybridization detection of these cytogenetic changes may provide clinically useful information for predicting imminent disease progression. Our recently developed tissue array approach (21) will tremendously facilitate fluorescence in situ hybridization analyses of multiple regions of the genome in a sufficiently large set of patients to test the prognostic significance of the alterations described in this study.

Acknowledgments

We thank Carole Egenter, Martina Mirlacher, Hedvika Novotny, Heidi Oggier, Martina Storz, and the staff of the Institute of Pathology, University of Basel for excellent technical support.

References

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