Chromosomal Imbalances in Noninvasive Papillary Bladder Neoplasms (pTa) 1

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ABSTRACT

Almost 70% of urinary bladder neoplasms present as low-grade papillary noninvasive tumors (stage pTa). To determine which genomic alterations can occur in pTa tumors of different grades and to evaluate the prognostic significance of chromosomal imbalances, we analyzed 113 pTa tumors (40 grade 1, 55 grade 2, 18 grade 3) by comparative genomic hybridization. pTaG1 (1.9 ± 2.0) and pTaG2 (3.1 ± 2.9) tumors had only few genomic alterations with Yq (44%), 9p (36%), and −Y (21%) being most prevalent. Neither the total number of aberrations nor any individual alteration was linked to the risk of recurrence in 95 pTaG1/G2 tumors with clinical follow-up information. pTaG3 tumors were characterized by a high number of alterations (7.7 ± 4.5; P < 0.0001 for G3 versus G2). Several chromosomal imbalances that have previously been reported to be typical for invasive bladder neoplasms were significantly more frequent in pTaG3 than in pTaG2 tumors, including 2q+, 5p+, Yq−, 6q−, 9p−, 10q+, 18q−, and 24q−. A dysfunction of genes at these loci may contribute to the development of high-grade urothelial neoplasias. However, there is no evidence for a direct role of these alterations for development of invasive tumor growth.

INTRODUCTION

Almost 70% of urinary bladder neoplasms present as low-grade papillary noninvasive tumors (stage pTa, grade 1 or 2). About 60% of these tumors recur after transurethral resection, but <5% progress into life-threatening muscle-invasive carcinomas (1). Several individual cytogenetic alterations were recently shown to occur significantly more frequently in early invasive (pT1) carcinomas than in low-grade pTa tumors, including 1q+, 2q−, 5p+, 5q−, 6q−, 8q+, 10q+, 18q−, and 24q−. A dysfunction of genes at these loci may indicate the development of high-grade urothelial neoplasias. However, to our knowledge, there are no published studies describing unequivocal transition of pTa tumors into invasive carcinomas including evidence for a histological-proven pTa1 carcinoma arising at the exact site of a previously incompletely resected pTa tumor. It is, therefore, possible that pT1 carcinomas do not, or only exceptionally, develop from low-grade pTa tumors. In this case, the cytogenetic alterations found in pT1 carcinomas could be part of a set of genetic alterations that is characteristic of a bladder cancer subtype that is distinct from low-grade pTa tumors and, therefore, independent of tumor invasion. A better understanding of the genomic alterations in these rare papillary bladder neoplasms that are noninvasive but have a high degree of nuclear atypia, as typically seen in invasive carcinomas (pTaG3), could clarify the relationship between cytogenetic changes and tumor invasion. Alterations that are linked to invasive tumor growth should be absent or rare in pTaG3 tumors that have not yet been systematically analyzed by molecular methods.

Phenotypically, minimally invasive bladder tumors develop from pTa tumors, these results raised the possibility that a malfunction of genes at these loci could play a direct role for invasive tumor growth. However, to our knowledge, there are no published studies describing unequivocal transition of pTa tumors into invasive carcinomas including evidence for a histological-proven pTa1 carcinoma arising at the exact site of a previously incompletely resected pTa tumor. It is, therefore, possible that pT1 carcinomas do not, or only exceptionally, develop from low-grade pTa tumors. In this case, the cytogenetic alterations found in pT1 carcinomas could be part of a set of genetic alterations that is characteristic of a bladder cancer subtype that is distinct from low-grade pTa tumors and, therefore, independent of tumor invasion. A better understanding of the genomic alterations in these rare papillary bladder neoplasms that are noninvasive but have a high degree of nuclear atypia, as typically seen in invasive carcinomas (pTaG3), could clarify the relationship between cytogenetic changes and tumor invasion. Alterations that are linked to invasive tumor growth should be absent or rare in pTaG3 tumors that have not yet been systematically analyzed by molecular methods.

In this study, we examined 18 pTaG3 tumors and 95 pTaG1/G2 tumors with clinical follow-up information by CGH. 2 CGH allows detection of all relative DNA sequence copy number gains and losses of a tumor in one examination (4). The specific aims were to determine which genomic alterations can occur in noninvasive high-grade bladder neoplasias (pTaG3) and to evaluate the prognostic significance of chromosomal imbalances in pTaG1/G2 tumors.

MATERIALS AND METHODS

Tumors. All tumors were from a series of 2919 bladder carcinomas, the tumor stage and grade of which were assessed by one pathologist (G. S.) according to Union International Contre Cancer (5) and WHO (6) criteria. Among these tumors were 85 pTaG3 tumors (2.9%). Follow-up information was available from 37 pTaG3 tumors. The risk of subsequent progression into muscle invasion was significantly lower in these 37 pTaG3 tumors, of which only one progressed, than in 59 carcinomas with minimal stromal invasion not extending beyond the lamina muscularis mucosae (pTa1a), of which 11 progressed (P = 0.0042). This argues against an inclusion of understaged pTaG3 tumors in our group of pTaG3 tumors. Eighteen pTaG3 tumors having evident high-grade atypia, unequivocal histological staging, and sufficient (at least 1 cm2) and pure tumor tissue (at least 75% tumor cells) were selected for this study. Clinical end points were not evaluated for pTaG3 tumors because follow-up information was only available from eight of these patients. In addition, 40 pTaG1 and 55 pTaG2 tumors were randomly selected among those tumors having follow-up information and at least 1 cm2 of pure enough tumor tissue for molecular analysis (>75% tumor cells). Nontumorous, flat urothelium was present in 33 of 113 biopsies. These showed CIS in 7 cases, mild or moderate dysplasia in 12 cases, and normal urothelium in 14 cases. Five of seven patients with a CIS had a pTaG3 tumor, and two patients had a pTaG2 tumor.

Patients. All 95 patients with pTaG1/G2 tumors had undergone regular follow-up cystoscopies at least at 3, 9, and 15 months, then annually until the end point of this study (recurrence, last control). The medium follow-up period was 38 months (range, 3–115). Intravesical treatment had been performed in 21 patients (mitomycin in 14 patients, Bacillus Calmette-Guérin in 4 patients, adriblastin in 2 patients, and epirubicin in 1 patient). Recurrences were defined as cystoscopically visible tumors. Tumor progression could not be evaluated as an end point because only two tumors progressed. In the entire patient set (including patients with pTaG3 tumors) there were 94 males and 19 females.

DNA Preparation. All tumor blocks were trimmed to enrich for tumor. Twenty 10-μm thick sections were taken for DNA extraction. The first and the last sections were stained with H&E. These sections were, again, carefully reviewed by a pathologist to exclude presence of stroma invasion and to determine the percentage of tumor cells. Tumors having an average tumor cell content of <75% in these sections were excluded. DNA extraction and labeling was as described (2). Tumor DNA (1 μg) was nick-translated by using a commercial kit (BioNick kit; Life Technologies, Inc., 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 ng of Cot-1 DNA (Life Technologies, Inc.) dissolved in 10 μl of hybridization buffer [50% formamide, 10% dextran sulfate, and 2× SSC (pH 7.0)]. Hybridization, image acquisition, image analysis, and control experimen-
ments were exactly as described previously (2, 7). At least four observations/autosome and two observations/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:normal ratio >1.20. Overrepresentations 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:normal ratio was <0.80. To define an aberration, it was additionally required that the first SD was above (gain) or below (deletion) 1.00. Because some false aberrations were detected in normal tissues at 1p, 16q, 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 compare the number of aberrations and the frequency of individual changes between tumors of different grades. Survival curves were plotted according to Kaplan-Meier (8). A log rank test was applied to examine the relationship between genetic alterations and the time to tumor recurrences. Patients were censored at the time of their last clinical control, showing no evidence of disease.

**RESULTS**

**CGH Findings.** The CGH findings of all 113 tumors examined in this study are shown in Fig. 1A (pTaG1), B (pTaG2), and C (pTaG3). In all tumors, losses of DNA sequences were most prevalent at 9q13-33 (44%), 9p (38%), Y (24%), 18q12-21 (13%), 2q35-pter (10%), and 11p12-pter (10%). DNA sequence copy number gains occurred most frequently at 17q (14%), 20q (13%), and 1q21-22 (11%). Five amplifications were found at four different loci. There was one 10p13 amplification in a pTaG3 tumor. Four amplifications were seen in pTaG2 tumors at 8p12, 11q13 (*n* = 2), and Xq21. Amplifications were not seen in pTaG1 tumors.

**Histopathological Correlations.** The number of deletions, gains, and the total number of aberrations in tumors having different grades is shown in Table 1. There was a slight, but statistically significant, increase of the number of aberrations/tumor from grade 1 to grade 2 tumors (*P* = 0.0261). This difference was mainly driven by an increase of the number of gains from grade 1 to grade 2. A much stronger difference was seen between grade 2 and grade 3 tumors. A comparison of the frequency of individual alterations between tumors of different grades is shown in Table 2. Gains of DNA sequence copy numbers at 7q and 17q were significantly more frequent in grade 2 than in grade 1 tumors. Almost all aberrations were clearly more frequent in grade 3 than in grade 2 tumors. This difference reached significance for 2q−, 5p+, 5q−, 6q−, 8p−, 10q−, 18q−, and 20q+.

**Tumor Recurrences.** Within the 95 pTaG1/G2 tumors with clinical follow-up information, the histological grade (*P* = 0.83) was not linked to the risk of subsequent tumor recurrences. Also, the CGH findings had no prognostic significance. This held true for both the number of aberrations and any of the individual changes found in our pTaG1/G2 tumors (Fig. 2).

**DISCUSSION**

The vast majority of high-grade bladder neoplasms grow invasively. pTaG3 tumors are a rare bladder cancer subtype. Because of its frequent association with CIS of the surrounding urothelium, it might be speculated that pTaG3 tumors represent papillary outgrowths of flat CIS. Particular emphasis was, therefore, placed on a correct staging of our pTaG3 tumors because these tumors can be difficult to distinguish from minimally invasive carcinomas. Arguments suggesting that the tumors selected for this study are true pTaG3 tumors include: (a) consistent lack of evidence for invasion in repeated reviews of the slides; (b) low overall frequency of pTaG3 tumors in our entire tumor set (2.9%); and (c) significant difference in prognosis as compared with carcinomas with only minimal stromal invasion (pT1a). The number of genomic aberrations in our pTaG3 tumors was similar, as previously found in pT1 (2) and in pT2–4 bladder carcinomas (7, 9), but much higher than in low-grade pTa tumors. This suggests a considerable degree of genetic instability in pTaG3 tumors. A comparison of the most common individual alterations between high-grade (G3) and low-grade (G1/G2) pTa tumors revealed several changes being significantly more frequent in high-grade tumors, including 2q−, 5q−, 6q−, 8p−, 10q−, 18q−, and 20q+. Because the same alterations were previously found in early invasive, but not in noninvasive, bladder neoplasms, it seemed possible that these loci might carry genes that, in case of a malfunction, can contribute to invasive tumor growth (7, 9). However, the high frequency of these
changes in noninvasive high-grade tumors strongly suggests that they are not directly linked to invasive tumor growth.

In a previous study, we had found that loss of 5q, 6q, and 15q and gains of 5p, 7p, and Xq were significantly more frequent in pT2–4 than in pT1 carcinomas, and we concluded that these changes may be linked to the progression of invasive carcinomas (7). Interestingly, 5p+, 5q−, and 6q− were also significantly more frequent in pTaG3 tumors than in our previously analyzed set of 37 pT1 carcinomas where we found 5p+ in 5% of cases, 5q− in 8% cases, and 6q− in 5% of cases (7). Although these results are contradictory at first sight, they are consistent with these alterations being late events in bladder carcinomas. Although invasively growing carcinomas are either resected early or lead to the patient’s death, it is possible that pTaG3 tumors can be chronologically much older than early invasive carcinomas (pT1). Because pTaG3 tumors do not have the potential to kill their hosts as long as they have not become invasive, they can grow within the bladder for a long time. Accordingly, these genetic unstable tumors may accumulate molecular alterations that usually occur late in bladder cancer development. It seems likely that late alterations occurring in noninvasive tumors may not be related to metastasis or invasion.

The CGH findings in low-grade pTa tumors (pTaG1/G2) confirmed the results of previous studies. These tumors were characterized by a low number of alterations with 9p−, 9q−, and −Y being most prevalent. Minimal deleted regions were repeatedly found at 9q34 and 9p21 (10–12). The p15/p16 genes are candidates on 9p21, although mutations are rare in primary bladder neoplasms (13–16). Currently, there are no candidate tumor suppressor genes on 9q for which mutations were detected in bladder tumors (12). The biological significance of Y losses in bladder cancer is unclear especially because Y losses can be found in a variety of normal tissues, including nonneoplastic urothelium (17, 18). A slight, but significant, increase in the number of detectable alterations from G1 to G2 tumors is consistent with an increasing number of genomic changes going along with cellular dedifferentiation. Interestingly, the increase in the total number of alterations was driven by a distinct increase in the number of chromosomal gains, suggesting a role of gene overexpression for an increased level of cytological atypia in G2 tumors. Some of the involved genes may be located on 7q and 17q, which were significantly more frequently overrepresented in G2 tumors than in G1 tumors. Several additional changes occurred somewhat more frequently in pTaG2 tumors than in pTaG1 tumors, including almost all changes that are frequent in pTaG3 or in invasively growing tumors (2, 7). This could suggest that several of the genes that are typically altered in advanced bladder tumors may be affected in a small fraction of low-grade pTa tumors. It could be speculated that an increased level of cytological atypia occurs in noninvasive bladder neoplasms having acquired one or a few of these changes that are typically present in the more aggressive high-grade/invasive bladder cancer subtype. Their low degree of genetic instability may prevent pTaG1/G2 tumors from the acquisition of a higher number of critical alterations.

CGH potentially is a powerful tool to retrospectively evaluate the prognostic significance of genomic alterations because it can be
applied to archival tissue and alterations of all chromosome arms can be related to clinical end points in one study. Moreover, the total number of alterations can be determined, a parameter that may define a “genetic grade” of a tumor. It was suggested that the number of alterations frequently including changes on specific chromosomes 1p, 3p, 8p, 10q, 17q, and 20q. This tumor subtype includes most, if not all, invasively growing bladder neoplasms (pT1G3). Noninvasive high-grade tumors (pTaG3) and CIS may also belong to this second group of tumors based on the results of this study and on the findings of Rosin et al. (25) showing a high frequency of loss of heterozygosity at various loci in CIS. Because these genetically unstable tumors will be more likely than low-grade pTa tumors to accumulate additional genetic alterations required for invasive tumor growth, most high-grade tumors may ultimately grow invasively, and the risk of subsequent tumor progression is high in the rare noninvasive high-grade tumors. The data from molecular and cytogenetic studies currently do not provide strong clues on the possible location of genes with significance for invasive growth of bladder neoplasms.

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