

Patterns of Chromosomal Imbalances in Advanced Urinary Bladder Cancer Detected by Comparative Genomic Hybridization

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To identify genetic changes linked to bladder cancer progression we analyzed 90 invasive transitional cell carcinomas (37 pT1 and 53 pT2–4) by comparative genomic hybridization. The most frequent alterations included 1q+ (37%), 5p+ (24%), 6q– (19%), 8p– (29%), 8q+ (37%), 9p– (31%), 9q– (23%), 11p– (24%), 11q– (22%), 17q+ (29%), and 20q+ (28%). Interestingly, there were three groups of alterations that frequently occurred together (9p– and 11q13+/20q+ and 11q13+ or 17q+/1q+ and 3p+ or 11q–). These loci might carry genes that interact with each other in specific molecular pathways. There were remarkable genetic similarities between minimally and deeply invasive tumors of different histological grades, including a similar number of aberrations per tumor and an equal frequency of most individual alterations. However, deletions of 5q, 6q, and 15q and gains of 5p, 7p, and Xq were significantly more frequent in pT2–4 than in pT1 carcinomas. These loci may harbor genes that are important for bladder cancer progression. (Am J Pathol 1998, 153:1615–1621)

Urinary bladder cancer is the fifth most common malignancy in men in Western societies. The majority of bladder neoplasms are noninvasive (stage pTa). Only approximately 30 to 40% of tumors show invasion at the time of initial diagnosis. Invasion depth is of utmost clinical importance. Tumors having invasion limited to the lamina propria (stage pT1) can be cured by transurethral resection and intravesical therapy in most instances. Prognosis is clearly worse if cancer cells reach the muscular bladder wall (stages pT2 and greater). Approximately 50% of these patients die from their cancer despite aggressive surgery.

It appears likely that progression from minimally invasive to deeply invasive cancer goes along with the acquisition of genomic alterations increasing the malignant potential of cancer cells. Previous studies investigating

specific alterations have suggested that at least a few molecular changes are more frequent in pT2–4 than in pT1 carcinomas, including epidermal growth factor receptor (EGFR) overexpression and loss of heterozygosity (LOH) at 3p.^{1,2} A systematic genome-wide screening for differences between the stages pT1 and pT2–4 has not been performed. Previous studies attempting a comprehensive analysis of the entire genome by either allelotyping studies or comparative genomic hybridization (CGH) have focused on early-stage tumors^{3,4} or analyzed only a small number of tumors.^{5,6} In this study, we analyzed a series of 90 invasive urinary bladder carcinomas by CGH to search for genomic alterations that are associated with tumor progression. CGH allows detection of all relative DNA sequence copy number gains and losses of a tumor in one examination.⁷ The results implicate several genomic alterations that may play a role in bladder cancer progression.

Materials and Methods

Tumor Material

Formalin-fixed, paraffin-embedded primary bladder tumors were from the archives of the Institute of Pathology, University Hospital Basel, Basel, Switzerland. Thirty-seven papillary pT1, twenty-four papillary pT2–4, and twenty-nine solid pT2–4 carcinomas were randomly selected from a series that had been reviewed by a single pathologist (G. Sauter). The CGH findings of 27 of our pT1 carcinomas have previously been published.⁴ Tumor stage and grade were defined according to Union Internationale Contre le Cancer (UICC) and World Health Organization classifications.^{8,9} Only tumors for which histological staging was unequivocal were included in this study. A papillary tumor growth was assumed if at least one unequivocal papilla with similar atypia as in the invasive tumor area was present. The histological tumor

Supported by Schweizerische Krebsliga (SKL 137-7-1995), Krebsliga Beider Basel, Schweizerischer Nationalfonds (NF 3200–043969.95.1), and Stiftung für Klinische Krebsforschung der Onkologischen Abteilung des Kantonsspitals Basel.

Accepted for publication August 3, 1998.

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grade was grade 2 in 15 and grade 3 in 22 pT1 tumors and grade 2 in 14 and grade 3 in 39 pT2–4 carcinomas. Of the 53 muscle-invasive tumors, 24 had a papillary and 29 had a solid growth pattern. Sixty-six patients were male, and twenty-four were female. Tumor-specific survival data were available from 67 patients. Tumor-specific 5-year survival was markedly shorter in 30 patients with pT2–4 carcinomas (50%) than in 37 patients with pT1 carcinomas (89%, $P < 0.0001$).

DNA Preparation

All tumor blocks were trimmed to enrich for tumor. Twenty 10- μ m-thick sections were taken for DNA extraction. The first and the last sections were stained with hematoxylin and eosin. Tumors having an average tumor cell content of less than 75% in these sections were excluded. DNA extraction and labeling was as described.⁴ One microgram of tumor DNA was nick translated by using a commercial kit (BioNick kit, Life Technologies, Gaithersburg, MD) and Spectrum Green-dUTPs (Vysis, Downers Grove, IL) for direct labeling of tumor DNA. Spectrum Red-labeled normal reference DNA (Vysis) was used for cohybridization.

CGH and Digital Image Analysis

The hybridization mixture consisted of 200 ng of Spectrum Green-labeled tumor DNA, 200 ng of Spectrum Red-labeled normal reference DNA, and 20 μ g of Cot-1 DNA (GIBCO, Gaithersburg, MD) dissolved in 10 μ l of hybridization buffer (50% formamide, 10% dextran sulfate, 2X SSC, pH 7.0). Hybridization was over 3 days at 37°C to normal metaphase spreads (Vysis). Posthybridization washes were as described.⁴ Digital images were collected from six to seven metaphases using a Photometrics cooled CCD camera (Microimager 1400, Xillix Technologies, Vancouver, British Columbia, Canada) and a Sun workstation. The VYSIS software program was used to calculate average green-to-red ratio profiles for each chromosome. At least four observations per autosome and two observations per sex chromosome were included in each analysis.

Controls and Threshold Definition

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). Sex-mismatched normal controls were also used to test the ability of each metaphase batch to allow for a linear relationship between fluorescence intensities and DNA sequence copy numbers. Metaphases were used only if the color ratio of sex-mismatched normal DNAs was ≤ 0.66 at the X chromosome. Thresholds used for definition of DNA sequence copy number gains and losses were based on the results of CGH analyses of formalin-fixed normal tissues. A gain of DNA sequences was assumed at chromosomal regions where the hybrid-

ization resulted in a tumor-to-normal ratio of ≥ 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 at chromosomal regions where the tumor-to-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. As 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 was used to analyze the relationship between genomic alterations, grade, stage, and growth pattern. A Student *t*-test was applied to compare the number of genomic alterations between tumors of different grades and stages. A log rank test was applied to examine the relationship between tumor stage and tumor-specific survival.

Results

CGH Findings

The total number of aberrations per tumor ranged from 0 to 24 (mean, 7.5). Deletions (3.8 ± 2.7) were slightly more frequent than gains of chromosomal material (3.4 ± 2.8). Eight tumors had no detectable aberrations. One pT1G2 carcinoma had a 9p deletion as a sole aberration. The CGH findings of all 90 tumors examined in this study are shown in Figure 1, A (37 papillary pT1 carcinomas), B (24 papillary pT2–4 carcinomas), C (29 solid pT2–4 carcinomas). Overall, deletions were most prevalent at 2q33-ter (17%), 4q25–31 (10%), 5q (18%, consensus regions 5q12–13 and 5q22–23), 6q21–24 (19%), 8p (29%), 9p (31%), 9q21–33 (23%), 10q25–26 (17%), 11p14–15 (24%), 11q23–25 (22%), 13q (11%, 13q21 and 13q31–34), 17p (19%), 18q21-ter (12%), Xp (11%), Xq24-ter (8%), and Y (22%). A gain of chromosomal material was most commonly seen at 1q (37%, consensus regions 1q22–24 and 1q32-ter), 3p23–24 (13%), 5p14–15 (24%), 6p21–22 (16%), 7p15–22 (20%), 8q (37%, 8q21.3–22 and 8q24), 10p13–15 (19%), 12q (14%, 12q15–21.2 and 12q24), 17q (29%, 17q21 and 17q25), 20p (9%), and 20q13 (28%).

There were 30 amplifications found at 14 different loci in these 90 carcinomas. Most of these amplification sites were at loci that also had frequent, circumscribed low-level gains including 1q22–24 ($n = 3$), 3p24 ($n = 3$), 6p22 ($n = 5$), 8q21–22 ($n = 2$), 8q24 ($n = 1$), 10p13–14 ($n = 2$), 11q13 ($n = 2$), 12q15 ($n = 3$), 17q21 ($n = 2$), and 20q13 ($n = 2$). At 13q33–34, 18p11, and Xp21 there were only rarely gains of large chromosomal fragments but high-level amplifications in two tumors, one tumor, and one tumor, respectively. On chromosome 5 the entire short arm (5p) was overrepresented at a high level in three tumors, but a circumscribed amplification (according to

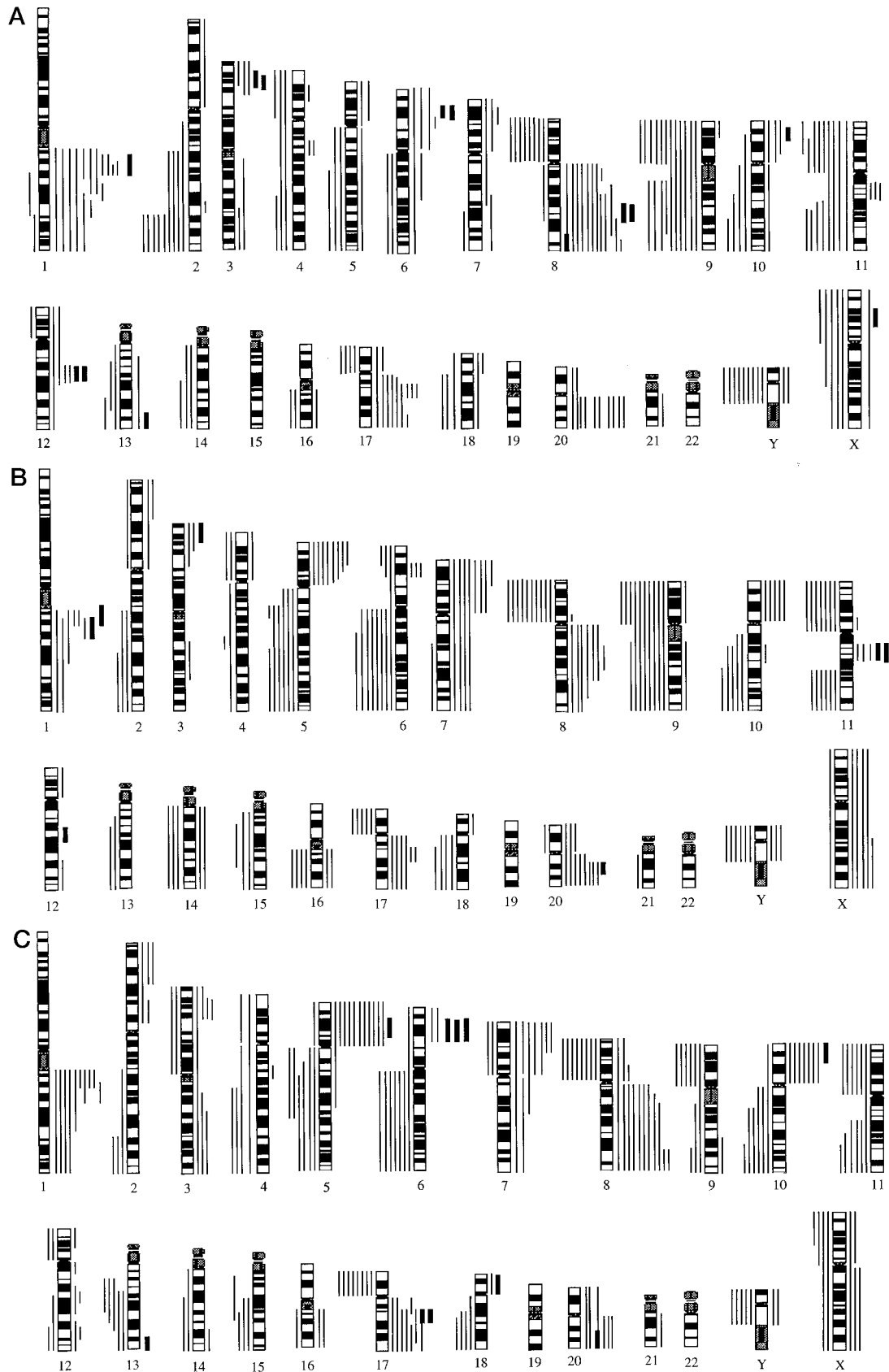


Figure 1. Summary of all relative DNA sequence copy number changes detected by CGH in 37 papillary pT1 (A), 24 papillary pT2-4 (B), and 29 solid pT2-4 carcinomas (C). The vertical lines on the right side of the chromosome ideograms indicate gains, those on the left losses of the corresponding chromosomal regions. Amplifications are indicated as solid bars. *1p, 16p, 19, and 22 were not analyzed.

Loci being frequently affected together may harbor genes that interact with each other in specific pathways

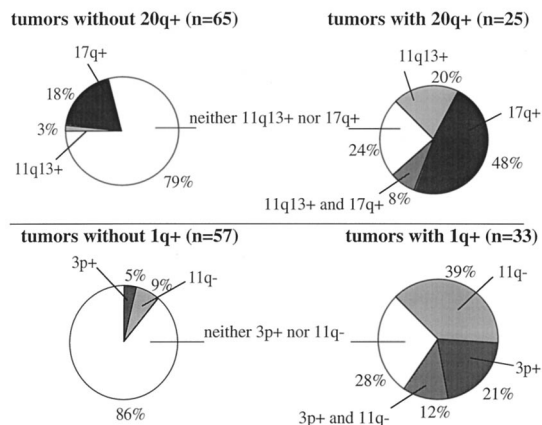


Figure 2. Relationship between individual alterations. Tumors with 20q gains had an increased likelihood to have also 11q13 gains or 17q gains. Carcinomas with 1q gains had frequent 3p gains and 11q deletions.

our definition) could be found at 5p14–15 in only one tumor.

Interestingly, there were strong associations between some individual alterations. For example, gains of 11q13 were associated with 9p deletions. 9p deletions were seen in 6 of 8 tumors (75%) with an 11q13 gain but in only 22 of 82 tumors without 11q gain (27%, $P = 0.005$). Gains of 20q were linked to gains of both 17q and 11q13 (Figure 2). An 11q13 gain was observed in 6 of 25 tumors (24%) with a 20q gain but in only 2 of 65 tumors without a 20q gain (3%, $P = 0.0018$). 17q gains were present in 13 of 25 tumors (52%) with a 20q gain but in only 12 of 65 tumors without 20q gain (18%, $P = 0.0015$). An additional gain of 11q13 or 17q was present in 68% of all tumors with a 20q gain, as 11q13 gains and 17q gains coincided rarely (only two tumors had both alterations). Similarly, 1q gains were associated with 3p gains and 11q deletions (Figure 2). An 11q deletion was present in 15 of 33 tumors (45%) with 1q gain as compared with only 5 of 57 tumors without 1q gain (9%, $P = 0.0001$). And 9 of 33 tumors (27%) with 1q gain had a 3p gain as compared with only 3 of 57 tumors without 1q gain (5%, $P = 0.0031$). There was no association between 3p gains and 11q deletions ($P = 0.32$). Sixty-one percent of all tumors with a 1q gain had an additional 11q deletion or a 3p gain.

Histopathological Correlations

There was no significant increase in the number of detectable aberrations with stage and grade of the tumors. pT1 tumors had 3.0 ± 2.3 deletions, 2.7 ± 2.8 gains, and 0.4 ± 0.8 amplifications per tumor resulting in a total of 6.1 ± 4.9 alterations. This was only insignificantly less than found in pT2–4 tumors (3.8 ± 2.9 deletions, 3.8 ± 2.7 gains, 0.4 ± 0.7 amplifications, and 7.9 ± 4.9 total aberrations). Grade 2 carcinomas had 3.0 ± 2.6 deletions, 2.6 ± 2.7 gains, 0.3 ± 0.8 amplifications, and 5.8 ± 5.0 alterations in total. These numbers were somewhat higher in grade 3 carcinomas (3.7 ± 2.7 deletions, 3.7 ± 2.7 gains, 0.4 ± 0.7 amplifications, and 7.8 ± 4.8 total

aberrations), but the difference between grade 2 and grade 3 did not achieve significance. To screen for specific alterations that might be linked to tumor progression, we compared the CGH findings of 37 papillary pT1 and 24 papillary pT2–4 carcinomas (Table 1). This analysis showed that deletions of 5q, 6q, and 15q as well as gains of 5p, 7p, and Xq were significantly linked to high stage. These aberrations were also common in solid pT2–4 carcinomas. The relationship between tumor grade and specific alterations was clearly less prominent. Only gains of 6p were significantly more frequent in grade 3 than in grade 2 tumors ($P = 0.02$). 9q deletions and 11q13 gains were the only alterations showing significant differences in frequency between papillary and solid muscle-invasive carcinomas (Table 1). Deletions of 5q ($P < 0.0038$) and 10p gains ($P < 0.0353$) were linked to poor outcome within these 67 patients with tumor-specific survival data. 1q+, 5p+, 6q-, 7p+, 8p-, 8q+, 9p-, 9q-, 11p-, 11q-, 15q-, 17q+, 20q+, and Xq were not significantly associated with the tumor-specific survival of our patients.

Discussion

To detect genetic changes associated with bladder cancer progression we compared the CGH findings in minimally invasive (pT1) and deeply invasive (pT2–4) transitional cell carcinomas. Stringent criteria were applied for the diagnosis of stage pT1 carcinomas so as not to include understaged pT2–4 carcinomas in our pT1 group. Marked survival differences between patients having pT1 and pT2–4 carcinomas argue for a correct staging of these tumors. Despite clear-cut prognostic differences, the genetic alterations detected by CGH were comparable in carcinomas with minimal and deep invasion. Not only was the number of alterations similar in pT1 and in pT2–4 carcinomas, but also most individual alterations were independent of the depth of invasion. Genetic alterations being frequent in all invasive bladder carcinomas (papillary pT1 and papillary and solid pT2–4) included 1q+, 8p-, 8q+, 9p-, 11p-, 11q-, 17q+, 20q+, and -Y. Given their high frequency it is not surprising that all of these changes were already known to occur frequently in bladder cancer.^{3–6,10–13} Although pT1 carcinomas are frequently grouped together with noninvasive papillary bladder tumors (pTa) as superficial bladder cancer, these genetic similarities between pT1 and pT2–4 carcinomas suggest that invasively growing bladder carcinomas represent a bladder cancer subtype that is distinct from pTa tumors in which only few genomic alterations can be found by CGH.⁴

The statistical analyses for comparison of the CGH findings in carcinomas having limited (pT1) and extensive (pT2–4) invasion was restricted to papillary carcinomas because previous studies had suggested genetic differences between solid and papillary tumors.¹⁴ Several alterations were significantly more frequent in pT2–4 than in pT1 carcinomas, including deletions of 5q, 6q, and 15q as well as gains of 5p, 7p, and Xq. Most of these changes have only rarely been reported in bladder car-

Table 1. Most Frequent Losses and Gains of DNA Sequences in Invasive Bladder Cancer

	pT1 papillary (n = 37)	pT2-4 papillary (n = 24)	pT2-4 solid (n = 29)	P values	
				Papillary pT1 versus papillary pT2-4	Papillary pT2-4 versus solid pT2-4
1q+	41%	33%	34%	NS	NS
5p+	5%	33%	41%	0.004	NS
7p+	8%	33%	24%	0.0123	NS
8q+	35%	29%	38%	NS	NS
10p+	11%	21%	28%	NS	NS
11q+	8%	21%	0%	NS	0.0098
17q+	24%	25%	34%	NS	NS
20q+	27%	38%	21%	NS	NS
Xq+	3%	22%	12%	0.0188	NS
Xp+	6%	17%	8%	NS	NS
2q-	24%	13%	10%	NS	NS
5q-	8%	29%	21%	0.03	NS
6q-	5%	33%	24%	0.004	NS
8p-	22%	42%	28%	NS	NS
9p-	32%	42%	21%	NS	NS
9q-	27%	33%	10%	NS	0.04
11p-	24%	29%	21%	NS	NS
11q-	22%	25%	21%	NS	NS
17p-	11%	21%	28%	NS	NS
10q-	11%	21%	21%	NS	NS
18q-	8%	17%	14%	NS	NS
13q-	8%	8%	17%	NS	NS
15q-	0%	13%	14%	0.0274	NS
Y-	25%	26%	19%	NS	NS

NS, not significant.

Only male patients were analyzed (pT1, n = 26; pT2-4 papillary; n = 22; pT2-4 solid, n = 18).

cinomas, probably because previous studies have either examined specific loci not including these regions or focused on early-stage tumors.³⁻⁵ It is tempting to speculate that these loci might carry genes of which a malfunction can contribute to bladder cancer progression. It cannot be excluded that one or several of these loci carry genes with relevance for invasive tumor growth, as alterations that are necessary for invasion may be present only in subpopulations of pT1 carcinomas, not detectable by CGH, but in a much higher fraction of the tumor cells if a cancer is predominantly growing invasively (stage pT2-4). Deletions of 5q and gains of 10p were significantly related to poor outcome in our patients. However, these alterations were strongly associated with advanced stage, and prognosis was markedly different between pT1 and pT2-4 tumors. Additional studies on larger sets of patients are required to evaluate whether cytogenetic changes are linked to the risk of progression in pT1 and to tumor-specific survival and metastasis in pT2-4 carcinomas.

To screen for genetic differences between tumors with different growth patterns, we compared the CGH results of 24 papillary and 29 solid muscle-invasive carcinomas. Deletions of 9q and gains of 11q13 were the only two alterations showing significant differences between papillary and solid tumors. The higher frequency of 9q deletions in papillary than in solid carcinomas is in line with previous studies suggesting that chromosome 9 deletions may be characteristic for papillary tumors.^{14,15} As 9p deletions were not significantly linked to papillary growth pattern, our data would be consistent with the location of a gene linked to papillary tumor growth on 9q

rather than on 9p. This is in agreement with the findings of Simoneau et al¹⁶ showing that 9q deletions were more frequent than 9p deletions in papillary pTa tumors. Interestingly, gains of 11q13 were also associated with a papillary growth pattern. A role for cyclin D1 (located at 11q13) overexpression has recently been described in low-grade and low-stage bladder tumors.^{17,18} In a previous study we analyzed 393 bladder neoplasms for cyclin D1 expression and found that papillary tumor growth but not low grade or low stage was independently linked to cyclin D1 expression.¹⁹

The high number of tumors examined in this study enabled us to search for alterations that frequently occur together. Loci that are frequently affected together might carry genes that interact in specific molecular pathways. The significant association between 11q13 gains and 9p deletions is an example of a combination of alterations that might involve genes with known interaction. Although expression analyses have not been performed in this study, it is tempting to speculate that 11q13 amplification, most likely leading to cyclin D1 overexpression,¹⁷ may give tumor cells a particularly significant growth advantage if the cyclin D1 inhibitors p16/p15 on 9p are also affected. Even more striking associations could be found for two other groups of alterations: 20q gains were strongly linked to both gains of 17q and 11q13, and 1q gains were associated with both 3p gains and 11q deletions. Although there are currently no clues on the potentially involved genes, it appears possible that genes at these loci could interact with each other. It could be speculated that, for example, overexpression of a gene on 1q will exert a particularly strong oncogenic role if

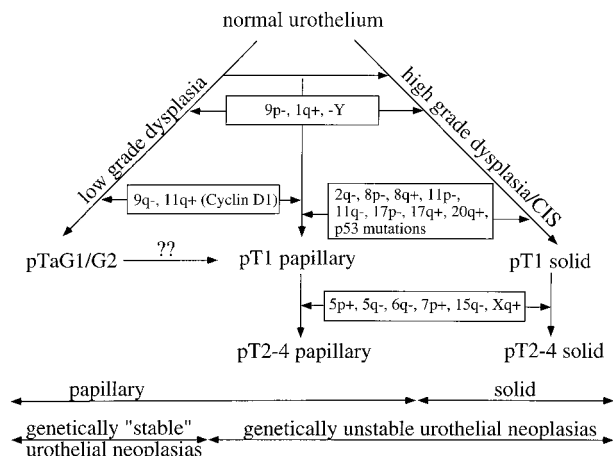


Figure 3. A putative model of bladder cancer development and progression based on molecular cytogenetic findings. For explanations, see text.

either a tumor suppressor gene on 11q is inactivated or another gene on 3p is co-overexpressed.

It is assumed that circumscribed high-level amplifications are linked to the overexpression of oncogenes. Previous studies using Southern blotting, fluorescence *in situ* hybridization, conventional cytogenetics, or CGH have described 21 regions of the genome that can be amplified in urinary bladder cancer including the sites of the known oncogenes erbB-2, EGFR, cyclin D1, and c-myc as well as 1q21-31, 2q13, 3p22-24, 6p22, 8p11, 8q21, 9p21, 10p13-14, 12q13-15, 13q13, 13q31-33, 18p11, 20q, 21p11, 22q11-13, Xp11-13, and Xq21-22.2.^{4-6,20-24} In this study, 30 high-level amplifications were found at 14 different loci, including 13 regions where amplifications had previously been described in bladder cancer. The only amplification site that was newly detected in this study was located at 5p14-15. Although gains involving the entire 5p occur frequently in bladder cancer, this finding is consistent with a target gene in the 5p14-15 region. The shortage of new amplification sites found in this set of advanced tumors suggests that the list of genomic regions that can be amplified in urothelial neoplasms is more or less complete. Additional investigations will now have to identify overexpressed target genes at these sites.

Taken together, the results of this and other studies suggest that several groups of chromosomal alterations may be associated with specific steps along bladder cancer progression (Figure 3). Deletions of 9p, 1q gains, and Y losses are frequent in bladder tumors of all grades and stages and may therefore occur early in bladder cancer development.^{4,25,26} The significantly higher frequency of 9q deletions and 11q13 (cyclin D1) gains in papillary than in solid tumors is consistent with a role of genes at these loci for papillary tumor growth. Cytogenetic alterations such as 2q-, 8p-, 8q+, 9p-, 11p-, 11q-, 17q+, and 20q+ can be found at a comparable frequency in papillary and solid invasive carcinomas irrespective of the invasion level. Several of these alterations are also frequent in carcinoma *in situ*,^{27,28} suggesting a considerable similarity between noninvasive and invasive high-grade neoplasias on the level of gross

genomic alterations. These cytogenetic changes as well as 17p deletions, which are difficult to detect by CGH because of the small size of 17p, and p53 mutations may be characteristic for a genetically unstable bladder neoplasm that is distinct from low-grade pTa tumors. Cytogenetic changes that are more frequent in pT2-4 than in pT1 carcinomas, such as 5p+, 5q-, 6q-, 7p+, 15q-, and Xq+, may carry genes that play a role for tumor progression. Additional studies are now required to evaluate which are the target genes of cytogenetic lesions and to translate our knowledge on the genetic background of bladder cancer into clinical applications.

Acknowledgments

We thank Ms. Carole Egenter, Ms. Hedvika Novotny, Ms. Martina Storz, Ms. Martina Mirlacher, and the staff of the Institute of Pathology, University of Basel, for their excellent technical support. We thank Prof. Robert McGandy for helpful discussions and proofreading the manuscript.

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