[CANCER RESEARCH 57, 2860–2864, July 15, 1997]

Advances in Brief

Marked Genetic Differences between Stage pTa and Stage pT1 Papillary Bladder Cancer Detected by Comparative Genomic Hybridization[1]

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Abstract

Little is known about the genetic changes underlying invasive tumor growth in bladder cancer. Because alterations that are linked to invasive tumor growth may be detectable in minimally invasive (stage pT1) but not in noninvasive (stage pTa) tumors, we searched for genetic differences between 28 pTa and 28 papillary pT1 bladder tumors by comparative genomic hybridization. Losses of 9q (54%), 9p (39%), and Y (28%) and gains of 1q (14%) were most prevalent in pTa tumors. These changes may play a role in the initiation of noninvasive papillary bladder cancer. The total number of aberrations was higher in pT1 tumors (6.5 ± 5.4) than in pTa tumors (2.3 ± 2.1; $P = 0.0003$), suggesting an increased genetic instability at stage pT1. Specific alterations, which were significantly more frequent in pT1 than in pTa tumors ($P \leq 0.05$), included deletions at 2q (36% of pT1 tumors), 8p (32%), and 11p (21%) and gains at 1q (54%), 8q (32%), 3p, 3q, 5p, 6p, and 10p (18% each). These loci are candidates for carrying genes involved in invasive tumor growth in bladder cancer. High-level amplifications at 1q22–24, 3p24–25, 6p22, 8p12, 8q21–22, 10p12.1–14, 11q13, 12q15–21, 13q31–33, Xp11–13, and Xq21–22.2 may pinpoint the location of oncogenes with relevance for bladder cancer.

Introduction

More than 60% of bladder neoplasms present as noninvasive papillary tumors (stage pTa). Another 10–20% of bladder carcinomas show minimal invasion, involving the suburothelial stroma but not the muscular bladder wall (stage pT1), at initial presentation. The stages pTa and pT1 are often grouped together as superficial bladder cancer because treatment is similar and prognosis does not differ markedly between these stages in many studies (1). Despite clinical similarities, pTa and pT1 tumors must feature fundamental biological differences that enable pT1 but not pTa tumors to grow invasively. It is likely that invasive tumor growth is linked to the overexpression or inactivation of one or several genes. Oncogenes and tumor suppressor genes known to be relevant for bladder cancer include *p53*, the retinoblastoma susceptibility gene, *MTS1/2, H-ras, c-myc, erbB-2,* and the *epidermal growth factor receptor* gene. Frequent deletions at 2q, 3p, 8p, 9p, 9q, 11p, and 13q and frequent gains of 1q, 3q, chromosome 7, 8q, 17q, and 20q may pinpoint the location of other, yet unidentified tumor suppressor genes and oncogenes with a role in bladder cancer initiation and progression (2–4). The relationship between most of these genetic aberrations and histological tumor phenotype, especially tumor stage, is currently unknown.

It was the aim of this study to identify genomic alterations linked to invasive tumor growth in bladder cancer. On the basis of the hypothesis that alterations that can induce invasive tumor growth may be detectable in minimally invasive but not in noninvasive tumors, we searched for genetic differences between stages pTa (noninvasive) and pT1 (minimally invasive). A total of 56 superficial bladder carcinomas (28 pTa and 28 pT1) were screened by CGH.[4] CGH allows the detection of all relative DNA sequence copy number gains and losses (>10 Mb) in previously formalin-fixed tumors in a single examination (5). The results show marked genetic differences between pTa and pT1 tumors and pinpoint several loci that are candidates for harboring genes with a role in invasive tumor growth.

Materials and Methods

Tumor Material. Formalin-fixed, paraffin-embedded primary bladder tumors were from the archives of the Institute of Pathology (University Hospital, Basel, Switzerland). Twenty-eight pTa tumors and 28 papillary pT1 carcinomas were randomly selected from a series that had been reviewed by a single pathologist (G. Sau.). Nonpapillary pT1 tumors were not examined to avoid a possible bias due to postulated genetic differences between solid and papillary bladder tumors (6). Forty-three patients were male and 13 were female. Tumor stage and grade were defined according to Union International Contre Cancer and WHO classifications. Only tumors for which histological staging was unequivocal were included in this study. The histological tumor grade was grade 1 in 13 pTa tumors, grade 2 in 14 pTa tumors, grade 3 in 1 pTa tumor, grade 2 in 11 pT1 carcinomas, and grade 3 in 17 pT1 carcinomas.

DNA Preparation. All tumor blocks were trimmed to enrich for tumor. Fifteen 10-μm-thick sections were taken for DNA extraction. The first and the last sections were stained with H&E. Tumors having an average tumor cell content of less than 75% in these sections were excluded. DNA extraction and labeling was as described (7). Sections were deparaffinized and suspended in DNA extraction buffer containing 0.5 mg/ml proteinase K. Additional proteinase K was added at 24 and 48 h later, for a total incubation time of 72 h. One μg of tumor DNA was nick translated by using a commercial kit (BioNick kit; Life Technologies, Inc., Gaithersburg, MD), fluorescein-12-labeled dUTPs, and Texas Red-5-labeled dUTPs (DuPont, Boston, MA) for direct labeling of tumor and normal DNA (extracted from mononuclear blood cells of healthy volunteers).

CGH and Digital Image Analysis. All tumors were analyzed twice, with the tumor DNA labeled once in green (initial hybridization) and once in red ("inverse control"). The hybridization mixture consisted of 200 ng of fluorescein-labeled DNA (tumor or normal), 200 ng of Texas Red-labeled DNA (normal or tumor), and 20 μg of unlabeled human 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 was performed over 3 days at 37°C to normal metaphase spreads (VYSIS Inc., Downers Grove, IL). Posthybridization washes were as described previously (7). Digital images (4,6-diamidino-2-phenylindol, FITC, and Texas Red) were collected from six or seven metaphases using a Photometrics cooled CCD camera (Microimager 1400; Xillix Technologies, Vancouver, British Columbia, Canada) and a Sun workstation. The VYSIS program was used to calculate average green:red ratio

Received 4/4/97; accepted 5/28/97.

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[1] This study was supported by Schweizerische Krebsliga Grant SKL 137-7-1995, Krebsliga Beider Basel, Schweizerischer Nationalfonds Grant NF 3200-043969.95.1, Wilhelm-Sander Stiftung Grant 97.005.1, and Stiftung für Klinische Krebsforschung der Onkologischen Abteilung des Kantonsspitals Basel.

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[4] The abbreviations used are: CGH, comparative genomic hybridization; FISH, fluorescence *in situ* hybridization; HPV, human papillomavirus.

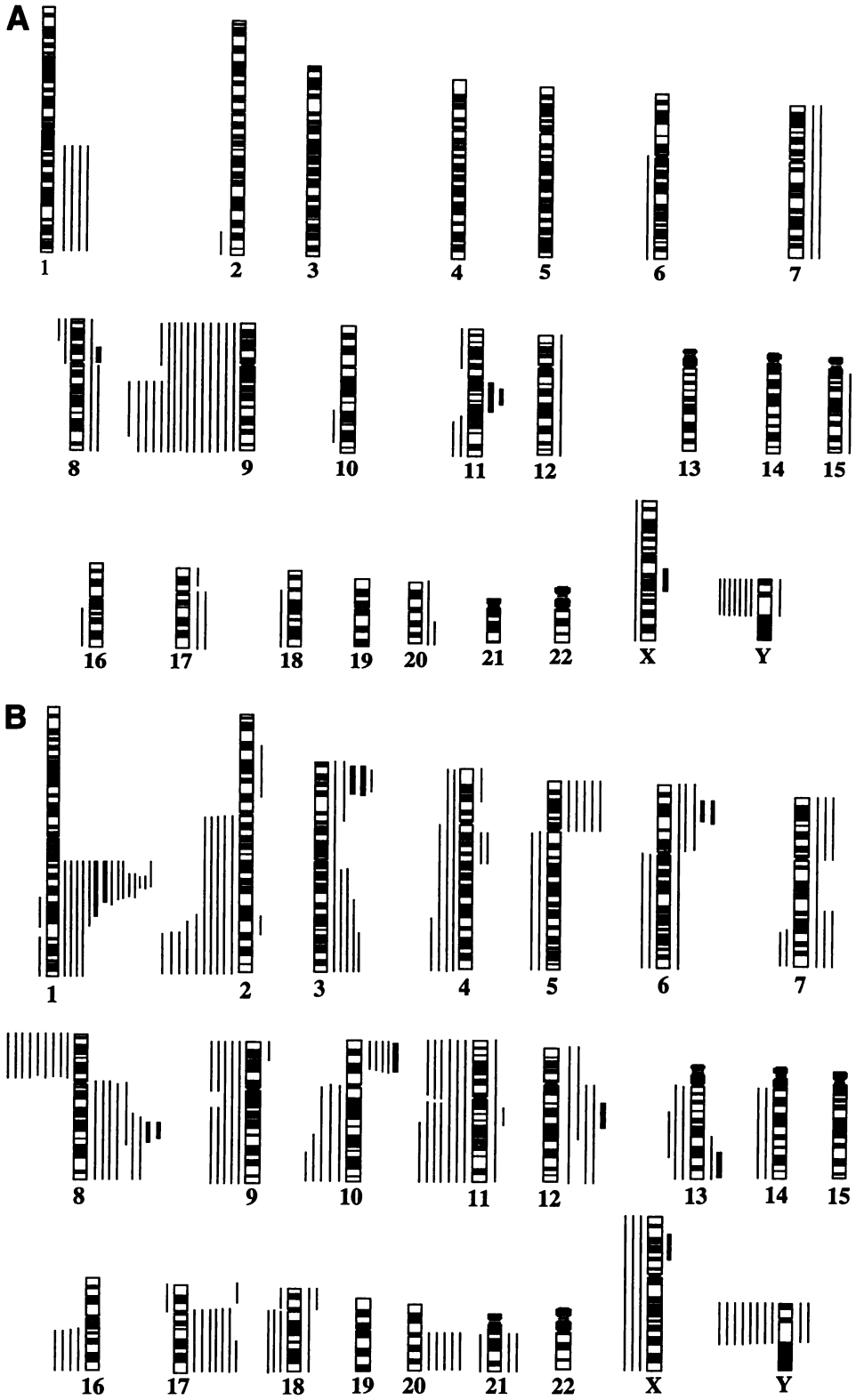

Fig. 1. Summary of all relative DNA sequence copy number changes detected by CGH in 28 pTa tumors (*A*) and 28 pT1 tumors (*B*). The *vertical lines* on the *right* of the chromosome ideograms indicate gains of the corresponding chromosomal regions; those on the *left* indicate losses. *Solid bars*, amplifications. 1p, 16p, 19, and 22 were not analyzed.

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 normal cell line 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 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 only used 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 10 formalin-fixed normal tissues. A gain of DNA sequences was assumed at chromosomal regions where both hybridizations (initial and inverse) resulted in a tumor: normal ratio of >1.15 and at regions where one hybridization yielded a ratio of >1.20. Overrepresentations were considered amplifications when the fluorescence ratios exceeded 1.5 in a subregion of a chromosome arm. A loss of

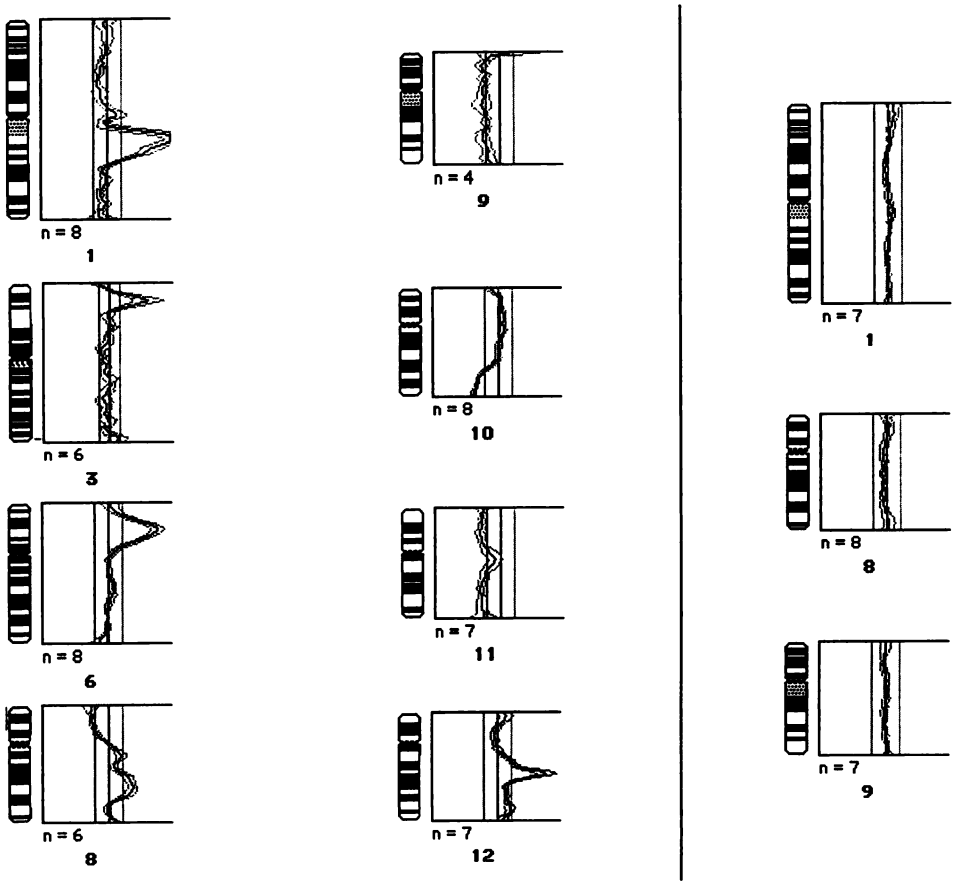

Fig. 2. Examples of green:red ratio profiles for selected chromosomes reflecting DNA sequence copy number changes in different pTa−/pT1− bladder tumor samples. The mean green:red fluorescence ratio profile (*thick line*) and its SD (*thin lines*) are shown for each chromosome from pter to qter. The chromosome identification and the number of observations are shown at the *bottom* of each profile. *Dark, straight line*, baseline ratio (1.0); *dotted lines*, ratio values of 1.2 and 0.8. Profiles on the *left* illustrate the following changes: amplification of 1q12–24, amplification of 3p22–24, amplification of 6p21.2–22, concomitant loss of 8p and gain of 8q21.1–23, loss of 9, loss of 10q25.1-qter, loss of 11, and amplification of 12q21.1–21.3. Profiles on the *right* were obtained from chromosomes that did not show evidence of DNA copy number changes.

DNA sequences was presumed at chromosomal regions where both hybridizations resulted in a tumor:normal ratio of <0.85 and at regions where one hybridization yielded a ratio of <0.80. To define an aberration, it was additionally required that the first SD was above (gain) or below (deletion) 1.00. Despite inverse controls, some false aberrations were detected in normal tissues using these definitions. These were always located at G-C-rich regions known to produce false positive results by CGH including 1p, 16p, 19, and 22. These regions were excluded from all analyses.

Statistics. Contingency table analysis was used to analyze the relationship between the frequency of genomic alterations between tumors of different grades and stages. Student's *t* test and ANOVA were applied to compare the number of genomic alterations between tumors of different grades and stages.

Results

CGH and Tumor Stage

Stage pTa. pTa tumors had a comparatively low number of aberrations (mean, 2.3; range, 0–8). On average, there were 1.4 deletions (range, 0–7), 0.9 gains (range, 0–5), and 0.1 amplifications (range, 0–2) per tumor. Eight tumors had no detectable aberrations. One tumor showed only one aberration, *i.e.*, a deletion of 9p. All aberrations found in pTa tumors are shown in Fig. 1*A*. A relative loss of chromosome 9 material was the most frequent aberration, occurring in 16 of 28 pTa tumors. Ten of these tumors had a loss of the entire chromosome 9, 1 had a loss of only 9p, and 5 had a loss of only 9q. One tumor with a 9q deletion had an interstitial deletion spanning 9q21–9q34. Other alterations occurring in more than two pTa tumors included losses of the Y chromosome (in 7 of 25 tumors) and 1q gains (4 of 28). Two amplifications were found at 11q13, including the site of cyclin D1/PRAD1. One tumor each showed an amplification at 8p12 and Xq21–22.2. Representative CGH profiles are shown in Fig. 2.

Stage pT1. pT1 carcinomas had a strikingly higher number of aberrations than pTa tumors (mean, 6.5; range, 0–17; $P = 0.0003$). On average, there were 3.1 deletions (range, 0–9), 3.5 gains (range, 0–15), and 0.5 amplifications (range, 0–3) per pT1 carcinoma (Table 1). Five pT1 tumors lacked detectable aberrations. pT1 tumors with single aberrations were not found. All aberrations of pT1 tumors are summarized in Fig. 1*B*. A gain of 1q was the most frequent alteration at this stage (54%). Although all 1q gains involved the entire long arm in pTa tumors, most 1q gains (11 of 15) in pT1 carcinomas involved only part of 1q, with a minimal common region of gain at 1q22–24. Also, two high-level amplifications included this commonly gained region at 1q22–24. Other loci that were frequently gained included 3p22–26 (5 of 28), 6p22 (5 of 28), 8q21–22 (10 of 28), 10p12.1–14 (5 of 28), 17q22-pter (8 of 28), and 20q (5 of 28). Deletions were most frequently seen at the Y chromosome (8 of 18), 2q34-qter (10 of 28), 8p (9 of 28), 11q14.1-qter (7 of 28), and 11p (6 of 28). A total of 10 pT1 tumors had a loss of chromosome 11 material. Three of these tumors had lost only 11p, four had lost only 11q, and three had lost the entire chromosome 11. Chromosome 9 losses involved 9p and 9q in five tumors each. There were 12 amplifications found at eight different loci in pT1 carcinomas. Six amplification sites were at loci that also had frequent low-level gains, including 1q22–24 ($n = 2$), 3p24–25 ($n = 2$), 6p22 ($n = 2$), 8q21–22 ($n = 2$), 10p12.1–14, and 12q15–21 ($n = 2$). Other amplification units were found at 13q31–33 and Xp11–13.

A comparison of the prevalence of the most frequent alterations in pTa and pT1 tumors is given in Table 2. Only losses of 9q were significantly less frequent in stage pT1 than in pTa tumors ($P = 0.0053$). Changes occurring significantly more frequently in pT1 than in pTa tumors included deletions of 2q, 8p, and 11p and gains at 1q, 8q, 3p, 3q, 5p, 6p, and 10p.

Table 1 *Number of aberrations and histological phenotype*

ANOVA was used for the statistical evaluations.

	No.	Deletions[a]	Gains[a]	Amplifications[a]	Total number of aberrations[a]
pTa	28	1.4 ± 1.5	0.9 ± 1.3	0.1 ± 0.4	2.3 ± 2.1
pT1	28	3.1 ± 2.8[b]	3.5 ± 3.6[c]	0.5 ± 1.0[d]	6.5 ± 5.4[e]
Grade 1	13	0.9 ± 1.0	0.4 ± 0.7	0.0	1.3 ± 1.2
Grade 2	25	1.9 ± 2.1	1.7 ± 1.9	0.3 ± 0.7	3.6 ± 3.5
Grade 3	18	3.6 ± 2.8[f]	4.2 ± 3.9[e]	0.6 ± 1.0[g]	7.8 ± 5.3[h]

[a] Aberrations per tumor (mean ± SD).
[b] $P = 0.0061$.
[c] $P = 0.0008$.
[d] $P = 0.0807$.
[e] $P = 0.0003$.
[f] $P = 0.0037$.
[g] $P = 0.00824$.
[h] $P = 0.0001$.

CGH Findings and Histological Grade

The number of aberrations was strongly linked to the histological grade (Table 1). Grade 2 tumors had more aberrations than grade 1 tumors, although this difference reached significance only for the number of gains ($P = 0.0209$) and the total number of aberrations ($P = 0.0318$), not for the number of deletions or amplifications. The genetic differences were even greater between grade 2 and grade 3 tumors than between grades 1 and 2. Grade 2 and grade 3 tumors differed significantly in their number of deletions ($P = 0.0266$), gains ($P = 0.0072$), and total aberrations ($P = 0.0029$).

Discussion

Losses of the chromosomes 9 and Y and gains of 1q were the most frequent alterations found in stage pTa tumors. A disturbed function of genes in these regions of the genome may therefore contribute to the initiation of noninvasive papillary bladder tumors. The high frequency of chromosome 9 losses in pTa tumors is in agreement with previous studies examining loss of heterozygosity or applying cytogenetics and FISH (8–10). The predominance of chromosome 9 losses as compared to other detectable alterations in pTa tumors argues for a decisive role of a disturbed function of one or several genes on chromosome 9 for initiation of papillary bladder tumors as previously suggested (6). The observation of more isolated 9q than 9p deletions is consistent with the assumption that inactivation of a gene on 9q could precede the inactivation of a 9p gene in bladder cancer initiation (11). A high prevalence of Y chromosome losses and 1q gains was expected in pTa tumors because both alterations have previously been found in early bladder cancer by FISH (12, 13). Poddighe *et al.* (12) found an overrepresentation of the 1q telomere in 6 of 13 diploid bladder tumors. In another study, we detected a Y loss in 6 of 21 pTa tumors (13).

Table 2 *Specific genomic alterations and tumor stage*

Type of aberration	Locus	pTa ($n = 28$)	pT1 ($n = 28$)	P[a]
Deletion	2q	1	10	**0.0025**[b]
	8p	2	9	**0.0186**
	9p	11	5	0.0759
	9q	15	5	**0.0053**
	10q	1	5	0.084
	11p	1	6	**0.0434**
	11q	2	7	0.0689
	16q	1	4	0.1548
	Y	7[c]	8[d]	0.2693
Gain	1q	4	15	**0.0019**
	3p	0	5	**0.0191**
	3q	0	5	**0.0191**
	5p	0	5	**0.0191**
	6p	0	5	**0.0191**
	7p	2	3	0.6393
	7q	2	3	0.6393
	8q	3	9	0.0507
	10p	0	5	**0.0191**
	12q	1	5	0.084
	17q	2	6	0.1266
	20q	2	5	0.2254
	Y	1[b]	2[c]	0.3665

[a] χ^2 test.
[b] Significant results ($P < 0.05$) are in boldface.
[c] 25 male patients.
[d] 18 male patients.

It is currently unclear how invasive papillary bladder carcinomas evolve. They may either develop from noninvasive papillary precursors (*i.e.*, stage pTa tumors) or else they may, from the very beginning, represent an entirely different tumor type with little relationship to pTa G_1-G_2 tumors. Some evidence for this latter hypothesis comes from our observation that 9q deletions were significantly more frequent in pTa than in pT1 tumors. Because all of our pT1 tumors were papillary, this difference cannot be explained by the inclusion of solid pT1 carcinomas, which were postulated to have fewer chromosome 9 deletions than did solid tumors (6). The higher frequency of 9q deletions in pTa than in pT1 tumors may suggest that progression does not often occur in pTa tumors with 9q deletions. However, all alterations that were frequent in pTa tumors (1q+, 9p−, 9q−, −Y) were also seen in pT1 carcinomas. This is consistent with a development of at least a fraction of papillary pT1 tumors from pTa precursors. Nevertheless, the clearly higher number of genetic alterations in papillary pT1 (mean, 6.5) than in pTa bladder cancer (mean, 2.3) indicates remarkable genetic differences between these tumor stages. Biological differences between pTa and pT1 bladder tumors are likely to include a higher degree of genetic instability in stage pT1 tumors as compared to stage pTa tumors. Genetic instability in pT1 tumors could facilitate the development of DNA sequence copy number gains and losses, some of which may contribute to a malfunction of specific genes, resulting in invasive tumor growth.

Specific alterations that were rare or absent in pTa tumors but frequent in pT1 carcinomas included deletions at 2q, 8p, and 11p and gains at 3p, 3q, 5p, 6p, 8q, and 10p. Independent of whether pT1 tumors arise *de novo* or through progression of pTa tumors, these loci are candidates for carrying genes with a role in bladder cancer invasion. With the exception of 3p gains, all of these alterations have already been found in bladder cancer, but mostly in more advanced tumor stages (pT2–pT4; Refs. 3 and 4). The observation of major differences between pTa and pT1 tumors is consistent with the results of a previous FISH study, in which we had found a significantly higher prevalence of 8p deletions in pT1 (8 of 19) than in pTa (3 of 34) tumors in a different set of patients.[5] Some of the loci that are

[5] U. Wagner, personal communication.

frequently altered in pT1 tumors may carry genes of which a disturbed function can contribute to the development of invasive tumor growth. Interestingly, gains of 3q have recently been implicated in the development of invasive tumor growth in carcinomas of the uterine cervix having an infection with HPV (14). It has been suggested that HPV may also be involved in some bladder carcinomas (15). Further studies will have to show whether HPV can be detected in bladder tumors with 3q gains.

The strongest difference in frequency between pTa and pT1 tumors was found for gains of 1q in this study, thus making 1q another candidate region for carrying a gene involved in bladder cancer invasion. Interestingly, not only the frequency but also the extent of 1q gains differed between stage pTa and stage pT1. Whereas all 4 pTa tumors with an 1q gain had an overrepresentation of the entire long arm, 11 of 15 pT1 carcinomas had a partial 1q gain. The minimal common region of overrepresentation at 1q22–24 was also included twice in a highly amplified DNA sequence. Although it cannot be excluded that gains of the entire 1q arm lead to an overexpression of more genes than partial 1q gains, it is also possible that all 1q gains activate the same gene(s) in the commonly overrepresented region between 1q22 and 1q24. In that case, the higher frequency of partial gains in pT1 tumors could be due to a secondary loss of previously gained distal parts of chromosome 1q during pTa-pT1 progression or else due to an increased tendency of genetically more instable pT1 tumors to gain shorter DNA fragments. Oncogenes on 1q22–24 that are potentially involved include *SKI* and *TRK* (16, 17). The expression of these genes has not been studied in bladder cancer.

Although the biological significance of low-level gains of large DNA fragments is still unclear, it is assumed that circumscribed high-level amplifications are linked to the overexpression of oncogenes. In this study, high-level amplifications were found at 11 different loci, including 8 regions where amplifications had previously been described in bladder cancer [1q22–24, 3p24–25, 6p22, 8p12, 8q21–22, 10p12.1–14, 11q13, and 12q15–21 (3, 4, 10)]. Amplification sites that were newly detected in this study were located at 13q31–33, Xp11–13, and Xq21–22.2. Although all amplifications occurred infrequently, they may pinpoint the location of important oncogenes for bladder cancer with overexpression in a higher number of tumors. It is known for a number of oncogenes that overexpression is clearly more frequent than gene amplification in bladder cancer (18, 19). Some loci that were found to be amplified in this study harbor oncogenes known to be involved in bladder cancer, such as 11q13 (*cyclin D1/PRAD1* and *EMS1*) or 12q12–13 (*MDM2*; Refs. 20 and 21). According to the Genome Database, other amplification units harbor defined or putative oncogenes that have previously not been linked to bladder carcinoma, such as 1q22–24 (*SKI* and *TRK*), 3p24–25 (*RAB5, RAF1, RARB,* and *THRB*), 6p22 (*cyclin D3*), 8p12 (*heregulin* and *FGFR1*), 8q21–22 (*MYBL1* and *ETO/MTG8*), and Xp11–13 (*ELK1, RAFA1,* and *TIMP*). Further studies will have to evaluate the expression of these genes in bladder cancer.

In summary, these data show that marked genetic differences exist between pTa and papillary pT1 bladder cancer. This challenges the concept of grouping these tumors together as "superficial bladder cancer."

Acknowledgments

We thank Hedvika Novotny, Martina Storz, 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 for proofreading the manuscript.

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Cancer Res 1997;57:2860-2864.