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Superimposed histologic and genetic mapping of chromosome 9 in progression of human urinary bladder neoplasia: implications for a genetic model of multistep urothelial carcinogenesis and early detection of urinary bladder cancer

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The evolution of alterations on chromosome 9, including the putative tumor suppressor genes mapped to the 9p21-22 region (the MTS genes), was studied in relation to the progression of human urinary bladder neoplasia by using whole organ superimposed histologic and genetic mapping in cystectomy specimens and was verified in urinary bladder tumors of various pathogenetic subsets with long-term follow-up. The applicability of chromosome 9 allelic losses as non-invasive markers of urothelial neoplasia was tested on voided urine and/or bladder washings of patients with urinary bladder cancer. Although sequential multiple hits in the MTS locus were documented in the development of intraurothelial precursor lesions, the MTS genes do not seem to represent a major target for p21-23 deletions in bladder cancer. Two additional tumor suppressor genes involved in bladder neoplasia located distally and proximally to the MTS locus within p22-23 and p11-13 regions respectively were identified. Several distinct putative tumor suppressor gene loci within the q12-13, q21-22, and q34 regions were identified on the q arm. In particular, the pericentromeric q12-13 area may contain the critical tumor suppressor gene or genes for the development of early urothelial neoplasia. Allelic losses of chromosome 9 were associated with expansion of the abnormal urothelial clone which frequently involved large areas of urinary bladder mucosa. These losses could be found in a high proportion of urothelial tumors and in voided urine or bladder washing samples of nearly all patients with urinary bladder carcinoma.

Keywords: bladder cancer; chromosome 9; MTS; microsatellites; multistep carcinogenesis; superimposed histologic and genetic mapping

Introduction

Alterations of chromosome 9 may be a common denominator in human neoplasia, playing an important role in the growth promotion and clonal expansion of various tumors including carcinoma of the bladder (Cairns *et al*., 1993a; Kishimoto *et al*., 1995; Miyao *et al*., 1993; Simoneau *et al*., 1994; Solomon *et al*., 1991). An area corresponding to 9p21-22 is of particular interest, as the putative tumor suppressor genes (MTS 1 and 2) were mapped to this region (Kamb *et al*., 1994). The MTS genes may not, however, represent ultimate targets of deletions in the 9p21-22 region and other distinct loci on chromosome 9 could be altered in specific pathogenetic subsets of human tumors (Coleman *et al*., 1994; Olopade *et al*., 1993; Cairns *et al*., 1994). Some of these alterations may play a role in the development of clinically occult precursor conditions such as dysplasia and carcinoma *in situ* (Kishimoto *et al*., 1995; Simoneau *et al*., 1994). More recent studies with the use of hypervariable DNA markers have identified several additional chromosome 9 regions on the p and q arms containing putative tumor suppressor genes that could play a role in urothelial tumorigenesis (Devlin *et al*., 1994; Habuchi *et al*., 1995, 1997; Simoneau *et al*., 1996). More specific identification of critical chromosome 9 regions involved in bladder neoplasia by comparing allelic patterns of tumor versus non-tumor tissue is hampered by the fact that a significant number of urothelial tumors show either evidence of chromosome 9 monosomy or large portions of both arms of the chromosome are deleted (Spruck *et al*., 1994b; Cairns *et al*., 1993b; Linnenbach *et al*., 1993; Tsai *et al*., 1990).

We studied allelic losses of chromosome 9 on multiple DNA samples extracted from invasive bladder cancer and from the microscopically identified adjacent intraurothelial precursor conditions of the entire organ (Chaturvedi *et al*., 1997). As a result, we were able to match the site of genetic alterations to progressive histologic alterations that parallel the natural history of the disease. The markers identified as significantly altered by this approach, including alterations of the MTS 1 and 2 genes, were subsequently tested on multiple bladder tumors and related to various clinico-pathological parameters including the follow-up. Finally, the applicability of chromosome 9 allelic losses as non-invasive markers of urothelial neoplasia was tested in voided urine and bladder washings of patients with urinary bladder carcinoma. The MTS genes were included in this study to disclose their pattern of involvement in the evolution of urothelial neoplasia from precursor changes to

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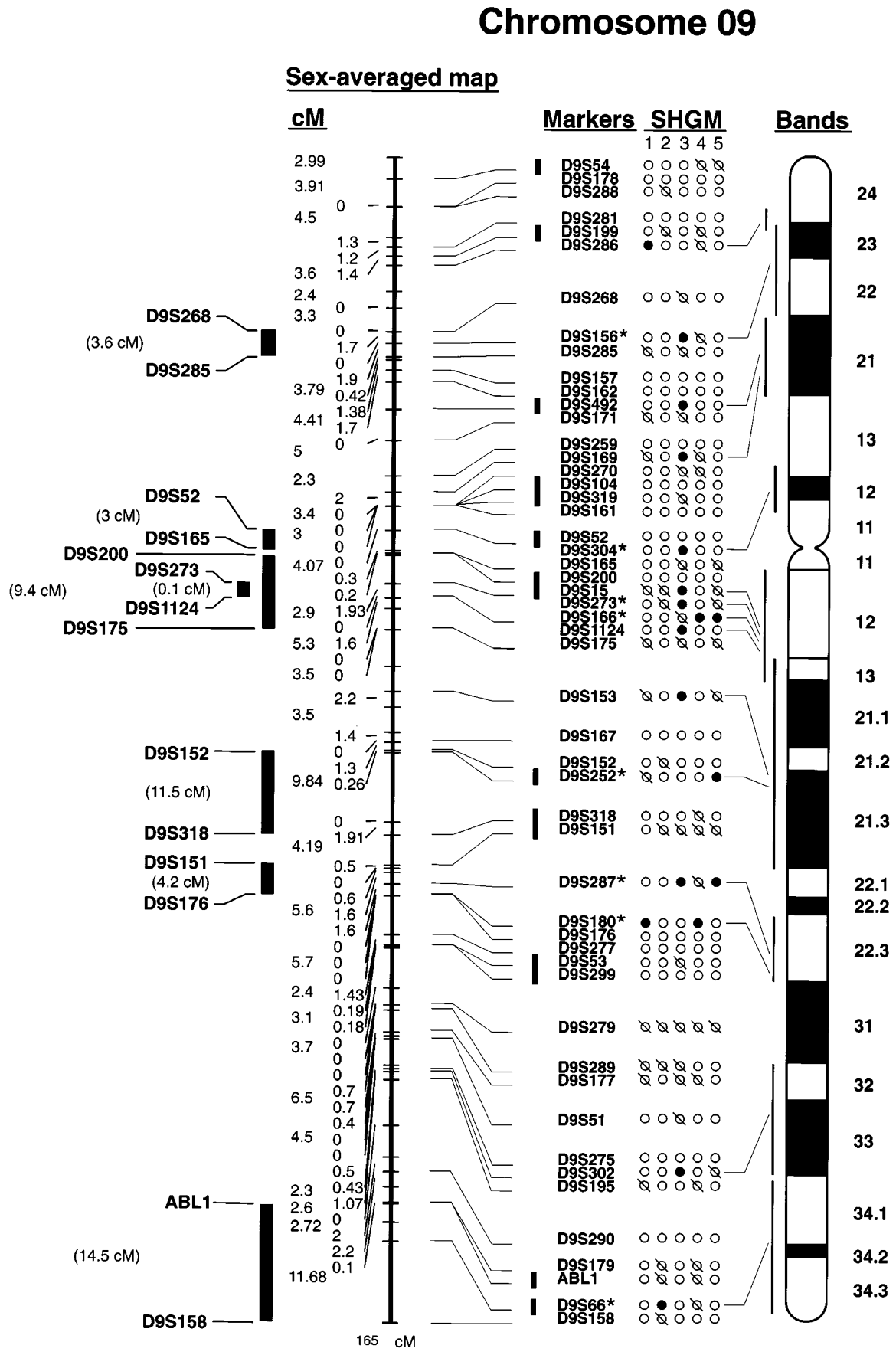


Figure 1 Genethon vector of chromosome 9 with a list of tested markers and their distances. Chromosomal locations are provided for altered markers only. Markers with solid bars on the left were added to the vector. All the markers are positioned on the vector and on chromosomal bands according to the human genome database (version Oct. 16, 1995). Asterisks on the right side of the markers indicate the statistically significant association between an altered marker and urothelial neoplasia as established by LOD scores. Bars on the left side of the chromosomal vector identify the lost regions associated with the development and progression of

invasive cancer. Moreover, a controversy exists whether these genes represent one of the major targets for deletions on 9p in urinry bladder neoplasia.

This study, together with the previously reported results of chromosome 17 alterations, represents a step in our efforts to generate a genome-wide genetic model of multistep urothelial carcinogenesis and to assess the clinical applicability of the findings (Chaturvedi *et al*., 1997).

Results

Superimposed histologic and genetic mapping

The initial testing of paired normal and tumor DNA samples from the same patient revealed loss of heterozygosity (LOH) in 15 out of 52 tested markers. No shortening or expansion of the repetitive regions was identified. None of the cystectomy cases used for superimposed histologic and genetic mapping showed evidence of chromosome 9 monosomy, i.e. none of the cases showed LOH of all informative markers indicating complete loss of chromosome 9. The list of tested markers, their alterations, and chromosomal location is illustrated in Figure 1. Testing of alterations on multiple samples from the same patient revealed the same pattern of allelic loss, i.e. the same allele was always altered (lost), indicting a clonal relationship among the samples with an altered marker (Figure 2a). The superimposition of distributions of marker alterations over the histologic maps disclosed two basic patterns of chromosome 9 deletions: scattered and plaque-like (Figure 2b). Some of the plaque-like alterations involved large areas of urinary bladder mucosa encompassing various precursor conditions and even some areas of morphologically normal urothelium.

The three-dimensional superimposed histologic and genetic maps generated by the nearest neighbor analysis visualized the patterns of alterations of the entire chromosome in relation to neoplastic progression (Figure 2c). This analysis disclosed that scattered foci of alterations were in fact loaded within the field change in which other chromosomal regions were deleted and involved larger areas of the urinary bladder mucosa. An example of the nearest neighbor analysis in a case of multifocal TCC shown in Figure 2c discloses LOH involving a large area of urinary bladder mucosa in locus D9S273 (q12-13) and a somewhat smaller area in locus D9S153 (q21). Marker D9S273 (q12-13) shows significant LOD scores in relation to all phases of neoplasia. It is evident that in this case the two separate foci of superficial papillary TCC developed in association with extensive losses of multiple markers on chromosome 9. Invasive non-papillary TCC in the same bladder does not show accumulation of multiple allelic losses of chromosome 9 and is distinct from two synchronous papillary lesions. However, both types of the lesions (superficial papillary and invasive non-papillary) have originated from the same large pre-existing field change exhibiting LOH of D9S273.

For the purpose of statistical analysis the intraurothelial precancerous changes were classified into two major groups: low grade intraurothelial neoplasia (mild and moderate dysplasia; LGIN) and high grade intraurothelial neoplasia (severe dysplasia and carcinoma *in situ*; HGIN). The analysis of LOD scores revealed that the markers with a statistically significant relationship to the development and progression of urothelial neoplasia were located in several distinct chromosomal regions: *p21-23* (*D9S156*); *p11-13* (*D9S304*); *q12-13* (*D9S273, D9S166*); *q21* (*D9S252*); *q22* (*D9S287, D9S180*); *q34* (*D9S66*). Markers *D9S156*, *D9S304*, *D9S166*, *D9S252*, *D9S180*, and *D9S66* were altered early in LGIN and also involved some adjacent areas of morphologically normal urothelium (Figure 3). None of the alterations could be exclusively related to the later phases of urothelial neoplasia, i.e. invasive carcinoma. Overall, the number of markers with statistically significant LOD scores did not increase with progression of intraurothelial neoplasia from low to high grade and with development of the invasive phenotype (data not shown).

It appears that a pericentromeric region on a q arm (q12-13) flanked by the markers *D9S15* and *D9S175* spanning approximately 9.4 cM represented the critical region deleted in early urothelial neoplasia. Allelic losses in this area involving markers D9S273 and D9S166 were found as significant changes of early phases of intraurothelial neoplasia (Figure 3) in three of five cases tested by the superimposed histologic and genetic maps. The smallest deleted region in this area was restricted to 0.1 cM and was flanked by markers *D9S273* and *D9S1124*. Additional regions on chromosome 9 potentially involved in early urothelial neoplasia are shown and defined in Figure 1.

Superimposed histologic and genetic mapping of the MTS locus

Superimposed histologic and genetic mapping of homozygous deletions in the MTS locus was performed with sequence tagged sites (STS) primers in a single cystectomy specimen that on preliminary testing of normal versus tumor DNA exhibited homozygous deletions of the STS's. In addition, the

urothelial neoplasia. These regions are defined by flanking markers and a size of deleted segment in cM. In general, the diagram shows scattered regions of LOH on both arms of chromosome 9 that may contain putative tumor suppressor genes involved in urinary bladder carcinogenesis. The regions defined by the nearest markers flanking the microsatellites exhibiting LOH with significant LOD scores as defined in Figure 3 are as follows: *D9S268-D9S285*, (p22-23); *D9S52-D9S165*, (p11-13); *D9S200-D9S175*, (q12-13); *D9S152-D9S318*, (q21); *D9S151-D9S277*, (q22); *ABL-1-D9S158*, (q34). These regions show alternating involvement in the development and evolution of urothelial neoplasia as established by superimposed histologic and genetic mapping and the LOD score analysis. The markers and deleted regions implicated to play a role in the development and progression ofneoplasia are shown without designation of particular phases of urothelial neoplasia. The statistically significant relationship of individual altered markers/chromosomal regions to various stages of neoplasia can be obtained from the LOD score table shown in Figure 3. (cM, centimorgans; SHGM, superimposed histologic and genetic mapping of individual cystectomy specimens consecutively numbered 1 through 5. ○ nonaltered marker, ● altered marker, and ∅ noninformative marker)

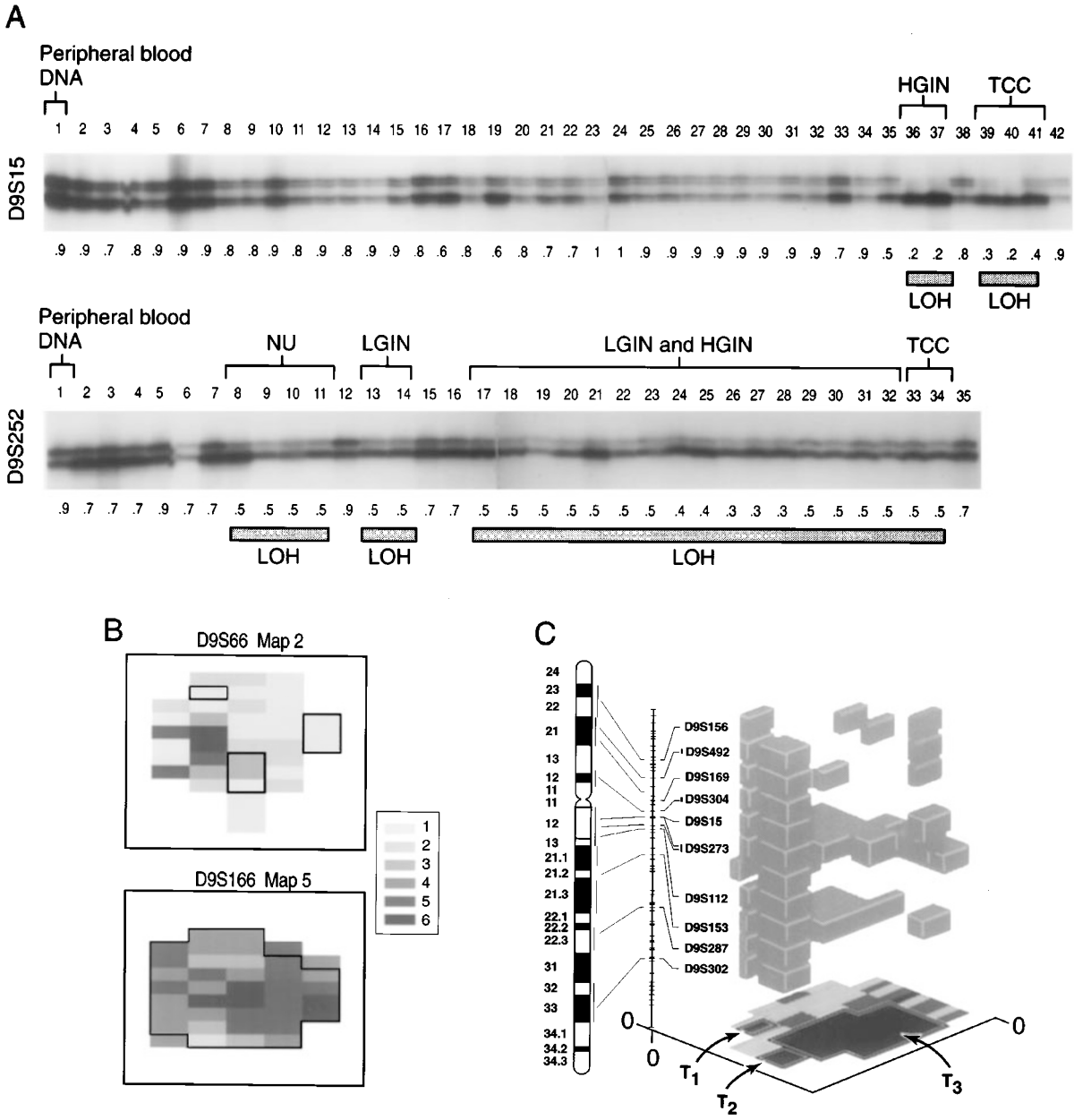


Figure 2 Assembly of superimposed histologic and genetic maps. (**a**) Examples of two markers tested on multiple mucosal samples from the same cystectomy specimen. Marker D9S15 shows LOH in five samples. Samples 36 and 37 correspond to HGIN. Samples 39–41 represent invasive TCC. Marker D9S252 shows LOH in multiple samples corresponding to TCC (samples 33 and 34) as well as involving large areas of urinary bladder mucosa exhibiting changes consistent with LGIN and HGIN (samples 13–14 and 17–32). Samples 8–11 correspond to microscopically normal urothelium with evidence of LOH. Samples #1 in both panels represent allelic patterns of the same marker from peripheral blood of the same patient and serves as control. In summary, marker D9S15 shows LOH restricted to invasive TCC and a small area of mucosa corresponding to advanced precursor intraurothelial conditions, i.e. severe urothelial dysplasia/flat transitional cell carcinoma *in situ* (HGIN). On the other hand, marker D9S253 shows LOH involving a large area of urinary bladder mucosa with invasive carcinoma and virtually all stages of intraurothelial precursor conditions as well as adjacent microscopically normal urothelium. The presence of LOH in all samples was confirmed by densitometry and is expressed as O.D. ratio below each sample in both panels. O.D. $\leqslant 0.5$ is indicative of LOH. (**b**) Examples of superimposed histologic and genetic maps of two cystectomy specimens. Marker D9S66 (top panel) shows scattered foci of LOH. Marker D9S166 (lower panel) shows a plaque-like LOH involving almost the entire urinary bladder mucosa. Open boxes delineated by black lines indicate areas of urinary bladder mucosa with alterations in a given locus. The background shadowed area represents a histologic map of cystectomy specimen depicting distribution of various intraurothelial precursor conditions and TCC. Histologic map code; (1) normal urothelium; (2) mild dysplasia; (3) moderate dysplasia; (4) severe dysplasia; (5) carcinoma *in situ*; (6) transitional cell carcinoma. (**c**) Example of chromosome 9 allelic losses in a single cystectomy specimen with multifocal TCC and assembled by nearest neighbor analysis. The vertical axis represents a chromosome 9 vector with positions of markers and their chromosomal locations. Only altered markers are shown. The shaded blocks represent areas of urinary bladder mucosa with LOH as they relate to progression of neoplasia presented by a histologic map of cystectomy in the background. An area designated at T_1 represents a focus of invasive non-papillary TCC. Areas of T_2 and T_3 represent foci of non-invasive papillary TCC. The histologic map code is the same as in **b**. Note that allelic losses of chromosome 9 show wide involvement of urinary bladder mucosa in loci D9S153 and D9S15. There is accumulation of allelic losses involving multiple loci in areas of mucosa corresponding to the foci of non-invasive papillary TCC (T_1 and T_2) but not in the area of invasive TCC (T_3). Scattered apparently separate loci of allelic losses occur within areas of urinary bladder mucosa with wide field type allelic losses in other chromosomal regions

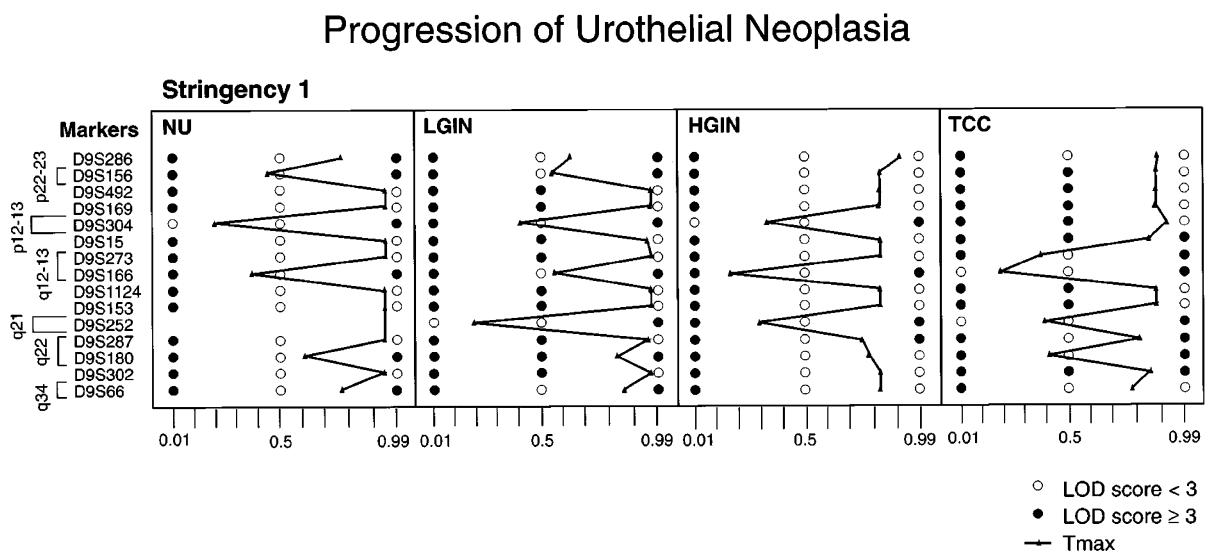


Figure 3 Cumulative LOD scores of chromosome 9 markers calculated at variable $\theta = (0.01, 0.5$ and $0.99)$ and tested against T_{max} were analysed for normal urothelium (NU); low-grade intraurothelial neoplasia (LGIN); high-grade intraurothelial neoplasia (HGIN); and transitional cell carcinoma (TCC). (○, LOD score <3; ●, LOD score $\geqslant 3$). To simplify the data, only stringency 1 calculations are presented. The patterns of statistically significant LOD scores are as described in Materials and methods. Note that significant patterns of LOD scores typically parallel the high T_{max} values. Chromosomal locations for markers with significant LOD scores is provided. In general, this LOD score analysis provides the data on the relationship between LOH of a tested marker and the development of intraurothelial precursor lesions, its evolution to severe dysplasia/flat carinoma *in situ* (HGIN) and progression to invasive carcinoma (TCC)

marker *D9S492* (located between exon 1 and 2 of MTS 1) and the nearest flanking marker *D9S169* showed LOH in this case. Homozygous deletions of STS clustered in the region corresponding to exon 2 and flanking the 5′ region of the MTS 1 gene. Early alterations involving homozygous deletions of one STS (C5.1) were mapped to morphologically normal mucosa adjacent to LGIN. Gradual expansion of the deleted region with eventual homozygous deletions of four adjacent STS occurred in the course of LGIN development and subsequent progression to HGIN and TCC. Moreover, the development of non-invasive papillary high grade TCC was associated with allelic loss of two adjacent hypervariable markers, *D9S492* and *D9S169*, spanning an approximate 10 cM segment (Figure 4a). Superimposition of homozygous deletions in the MTS locus over the histologic maps disclosed that areas of urinary bladder mucosa with precursor conditions ranging from LGIN to HGIN and exhibiting progressively widening homozygous deletions in the MTS locus were adjacent to each other and formed plaque-like areas corresponding to the distribution of preneoplastic intraurothelial changes (Figure 4b). This analysis disclosed that a relatively small focus of deletions in the MTS locus is unstable and may expand in progression of urothelial neoplasia from intraurothelial precursor conditions to TCC.

Allelic losses of chromosome 9 and alterations of MTS1 and 2 in relation to clinicopathological parameters of urinary bladder tumors

The chromosomal regions which were identified as significantly altered in relation to development of urothelial neoplasia by superimposed histologic and genetic mapping were tested with the use of hypervariable markers for potential allelic losses in 98 urinary bladder tumors of various histologic grades, growth patterns, invasiveness, and in relation to long-term follow-up data (Tables 1 and 2). Alterations of MTS 1 and 2 such as homozygous deletions in the MTS locus as well as structural alterations (mutations or deletions) of their coding sequences were also analysed (Table 3). Allelic losses of six regions, i.e. p21-23, p11-13, q12-13, q21, q22 and q34 identified by superimposed histologic and genetic mapping were present in 18.3–67.1% of all tumors. Alterations involving only one of the above listed regions as the sole chromosome 9 allelic loss were identified in 31.5% of all tumors. The extensive allelic losses defined as involvement of three or more regions (including chromosome 9 monosomy, i.e. LOH of all informative markers tested) were present in 59.7% of all tumors. The allelic losses in the six tested regions of chromosome 9 seemed to be ubiquitous in bladder tumors and could not be related to any specific pathogenetic subsets (papillary, non-papillary) histologic grade, invasion, or clinical aggressiveness of TCC.

Allelic losses of p21-23 and homozygous deletions in the MTS locus were documented in 57.5 and 67.6% of the cases respectively. However, the mutations or large deletions directly involving the coding sequences of the MTS 1 and 2 genes were less frequent and could be documented in only 13.7 and 6.8% of the cases respectively. In addition, when the molecular data on the MTS 1 gene were related to patterns of p16 protein expression, it was determined that the presence of a mutation that was associated with LOH in the MTS 1 locus (only one mutant allele of the gene was present) no staining for p16 could be identified by immunohistochemistry (Figure 5). However, when a mutation within the MTS 1 gene

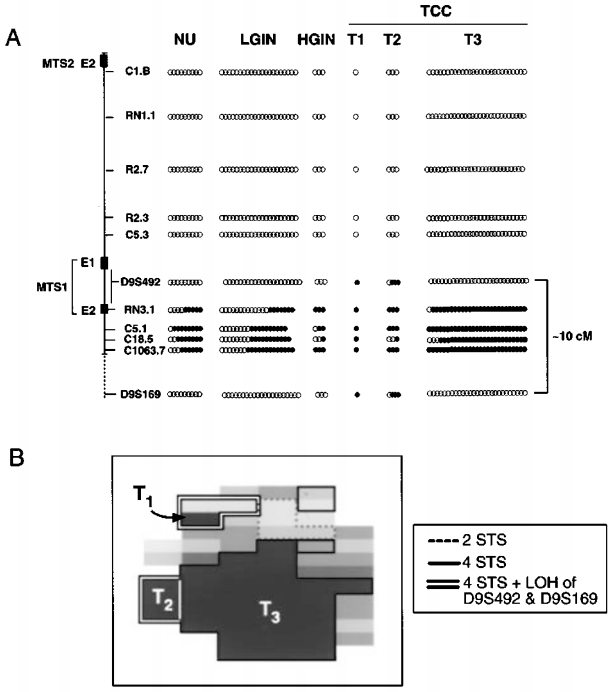


Figure 4 Evolution of alterations in the MTS locus during progression of urothelial neoplasia in a single cystectomy specimen. (**a**) Homozygous deletion within the MTS genomic cosmid depicting the positions of STS sites and MTS coding sequences according to Kamp *et al*. In addition, allelic losses of a D9S492 marker located with the MTS 1 gene and the flanking marker D9S169 were tested. Urothelial samples are grouped according to progression of neoplasia from NU through LGIN and HGIN to TCC. T_1 and T_2 represent foci of non-invasive papillary TCC. T_3 represents invasive non-papillary TCC. Note that a homozygous deletion of a single STS (C5,1) is present in normal urothelium. Loss of three adjacent STSs is evident with the development of low-grade intraurothelial neoplasia (LGIN). Allelic losses of two adjacent markers (D9S492 and D9S169) approximately 10 cM apart is present with the development of non-invasive papillary TCC. ○, no evidence of homozygous deletion. ●, homozygous deletion. In three representative samples corresponding to the T_1, T_2 and T_3 foci, the presence of homozygous deletions have been confirmed by genomic Southern blotting using the DNA fragments amplified by the given set of STS primers as the DNA probe (data not shown). (**b**) Superimposed histologic and genetic map of the same case showing the distribution of homozygous deletions in MTS locus revealed by STSs. Boxed delineated by lines depict mucosal areas exhibiting homozygotic deletions of 1 STS, 3 STSs, or more than 3 STSs. Note that there is a relationship between extent of the deleted region and progression from precursor conditions to TCC. Samples with the three STSs deleted correspond to areas of three foci of TCC. Further expansion of the deleted region, i.e., allelic losses of two adjacent hypervariable markers is associated with the development of non-invasive papillary TCC (foci T_1 and T_2)

occurred in the absence of LOH, it was associated with a normal heterogenous staining pattern indicating the presence of at least one normally functioning MTS 1 allele. These studies provided confirmation that the coding sequence alterations of the MTS 1 gene identified by single strand conformational polymorphism (SSCP) and sequencing studies represent real mutations of the gene that alter its expression pattern as well as further confirming the accuracy of our molecular data. Detailed analysis of the relationship between immunohistochemical and molecular data on the MTS 1 gene is a subject of separate study (Benedict *et al*., in press).

Identification of chromosome 9 allelic losses in voided urine samples

The clinical data of 26 patients whose urine samples were tested for LOH on chromosome 9 are summarized in Table 4. Alterations of at least one of the selected markers could be documented in 25 of 26 patients with urinary bladder carcinoma (Table 5). In the vast majority of cases, LOH of multiple markers were present. Moreover, alterations of multiple hypervariable markers were present in six of seven patients 1 to 60 months after the removal of grade 2-3 transitional cell carcinoma (TCC) even though disease was not clinically or microscopically detectable at that time, i.e. there was no tumor seen cystoscopically and urinary bladder wall biopsies as well as urine cytologies were negative for TTC and/or urothelial dysplasia at the time of testing (cases 1-7 with current status T_0). Two of these patients experienced prior recurrences of the tumor. They could also be identified in patients after the transurethral resection of invasive TCC with evidence of residual flat carcinoma *in situ* (T_{is}) only (cases 24–26). No allelic losses were identified in voided urine samples of ten healthy individuals.

Discussion

Our studies of chromosome 9 in human urinary bladder neoplasia have focused on three specific issues: (1) Localization of multiple putative tumor suppressor gene loci that play a role in early preneoplastic clonal expansion of urothelial cells; (2) The potential role of MTS 1-2 as model tumor suppressor genes mapped to chromosome 9, and (3) Usefulness of allelic losses on chromosome 9 as non-invasive markers for the preclinical phases of urinary bladder neoplasia in voided urine samples.

The strategy of superimposed histologic and genetic mapping disclosed multiple allelic losses of chromosome 9, which occurred in early preinvasive phases of urothelial neoplasia. Some of these alterations could be identified in microscopically normal urothelium adjacent to areas of the urinary bladder mucosa exhibiting early precursor changes, i.e. LGIN, indicating they precede the development of microscopically recognizable changes within the urothelium. None of the allelic losses could be exclusively linked to the later phases of urothelial neoplasia such as invasive TCC. Several of the losses involved large areas of urinary bladder mucosa further supporting the concept that they represent early events associated with clonal expansion of abnormal urothelial cells. Although no specific patterns of marker alterations could be identified that applied to all of the cases, certain regions of chromosome 9 were involved in early urothelial neoplasia, including p22-23, p11-13, q12-13, q21-22, and q34.

Chromosome 9 is considered the most frequently altered chromosome in bladder cancer. The alterations range from monosomies as sole cytogenetic abnormalities to allelic losses that were initially reported to be present in 67% of advanced bladder cancers (Gibas *et al*., 1984; Smeets *et al*., 1987; Vanni *et al*., 1988; Atkin and Baker 1985; Babu *et al*., 1987; Berger *et al*., 1986).

Table 1 Distribution of chromosome 9 and MTS gene alterations in relation to pathologic features of transitional cell carcinoma[a] (analysis of 98 cases)

	Evidence of LOH in different regions of chromosome 9								*Homozygotic deletions in MTS locus*[b]	*Alterations of MTS genes coding sequences*[c] *MTS1*	*MTS2*
	p21-23	*p11-13*	*q12-13*	*q21*	*q22*	*q34*	*0–2*	*⩾3*			
Growth pattern:											
(2) papillary	55.6	29.1	63.0	19.6	64.9	67.3	36.4	63.6	71.7	16.7	7.3
(1) non-papillary	63.2	15.8	36.8	14.3	50.0	71.4	57.9	42.1	55.6	5.0	5.3
Histological Grade:											
1–2	51.3	25.0	59.0	18.2	65.1	66.7	40.0	60.0	76.9	15.0	7.5
3	64.7	26.5	52.9	18.5	55.9	70.6	44.1	55.9	56.2	11.8	5.9
Superficial	53.8	30.8	65.8	12.5	70.0	66.7	33.3	66.7	75.0	17.9	7.7
Invasive	60.6	18.2	48.5	25.0	52.9	67.6	48.5	51.5	60.0	8.8	5.7
Total	57.5	25.7	56.2	18.3	60.3	67.1	40.3	59.7	67.6	13.7	6.8

[a]The numbers indicate percentage of cases with alterations in a given category of tumors. The following markers were used to test allelic losses on chromosomes 9; pter, D9S178; p21-23, D9S492, D9S171, D9S169, D9S270; p12-13, D9S52, D9S304, D9S200; q12-13, D9S273, D9S166, D9S1124, D9S175; q21, D9S167, D9S152, D9S252; q22, D9S151, D9S287, D9S180, D9S176; q34, D9S179, AB11, D9S66; qter, D9S158. [b]Homozygotic deletions in the MTS locus were tested with STS primers. The positions of STS sites as reported by Kamb *et al*. are shown in Figure 4. [c]Alterations of coding sequences of the MTS genes were tested by SSCP and direct sequencing. The specific data are listed in Table 3

Table 2 Summary of statistical analysis among alterations of chromosome 9 and clinico-pathologic parameters (analysis of 98 cases)

	Chromosome 9 regions with evidence of LOH (P value)						*# of chromsome 9 regions with evidence of LOH (*P *value)*
Feature	*p21-23*	*p11-13*	*q12-13*	*q21*	*q22*	*q34*	*0–2 versus ⩾3*
Growth pattern	0.56	0.25	0.48	0.65	0.24	0.73	0.10
Histologic grade	0.25	0.89	0.60	0.97	0.41	0.72	0.72
DNA ploidy	0.44	0.50	0.51	0.47	0.51	0.10	0.94
Invasion	0.56	0.22	0.14	0.21	0.13	0.93	0.19
Recurrence	0.07	0.78	0.64	0.61	0.69	0.29	0.28
Metastasis	0.018	0.22	0.02	0.40	0.12	0.76	*
Alive or dead	0.57	0.19	0.64	0.95	0.86	0.34	0.83
Recurrence free interval	0.41	0.70	0.96	0.93	0.33	0.10	0.52
Metastasis free interval	*	0.23	0.03	0.44	0.09	0.73	*
Overall disease free interval	0.52	0.63	0.93	0.99	0.25	0.08	0.64
Overall survival	0.36	0.26	0.87	0.64	0.65	0.38	0.91

Relationship between chromosome 9 regions with evidence of LOH and growth pattern, histologic grade, DNA ploidy, invasion, recurrence, metastasis, and alive or dead status was analysed by Gehan-Wilcoxon and Peto log rank tests. Recurrence, metastasis, and overall disease free intervals as well as overall survival were tested by Kaplan-Meier analysis. *Insufficient data to perform the analysis

Table 3 Summary of sequencing data of MTS 1 and 2 genes (analysis of 98 cases)

Gene/exon	*Case*	*Codon*	*Alteration*[a]	*Function*
MTS1/ exon 1	1	27	G(del)	Glu→Arg (frameshift)
	2	4	T(ins)	Frameshift to stop codon
	3	24	G(del)	Stop codon
MTS1/ exon 2	4	148	A(ins)	Ala→Thr
	5	148	G→A	Ala→Thr
	6	113	C→A	Leu→Met
	7	148	G→A	Ala→Thr
	8	144	G→T	No change
	9	145	C(ins)	Asp→Thr (frameshift)
	10	106	T(del)	Frameshift to stop codon
	11	147	G→A	Ala→Thr
	12	53	G→A	Met→Ile
	13	72	25 nucleotide deletion	Large deletion
MTS2/ exon 1	14	intron	C(ins)	No change
MTS2/ exon 2	15	63	G→C	Glu→Gln

[a]Alterations: del, deletion; ins, insertion; G→A, G to A mutation

Some studies have shown that allelic losses on 9q are the most frequent and appear in tumors of all histologic grades and stages (Olumi *et al*., 1990). The concepts of molecular progression of urothelial neoplasia postulate early involvement of chromosome 9 in the urothelial hyperplasia/noninvasive low-grade papillary tumor pathway and its late involvement in the dysplasia, carcinoma *in situ*, invasive nonpapillary carcinoma pathway (Simoneau *et al*., 1994). Our data indicate that allelic losses of chromosome 9 are associated with early events of urothelial dysplasia and may even precede the development of microscopically identifiable urothelial abnormalities. Moreover, such genetic changes could be identified in voided urine samples of patients with a history of TCC in the absence of clinically detectable disease at the time of testing.

The results of this study are generally consistent with recently published data indicating the involvement of several loci on the p and q arms. We identified two additional putative tumor suppressor gene loci located proximally and distally to the MTS locus on the p arm. The q arm appears to contain four distinct loci of putative tumor suppressor genes involved in urothelial neoplasia. A recently identified locus of putative tumor suppressor gene in q32-33 region (Habuchi *et al*., 1997) does not seem to be significantly involved in our studies. However, we placed one of the potential tumor suppressor gene loci more distally in the q34 region. The pericentromeric region on a q arm flanked by the *D9S15* and *D9S175* markers showed evidence of LOH in three of five cases tested by the superimposed histologic and genetic mapping with particularly significant LOD score patterns indicating that it may contain a critical gene or genes for initiation of the process.

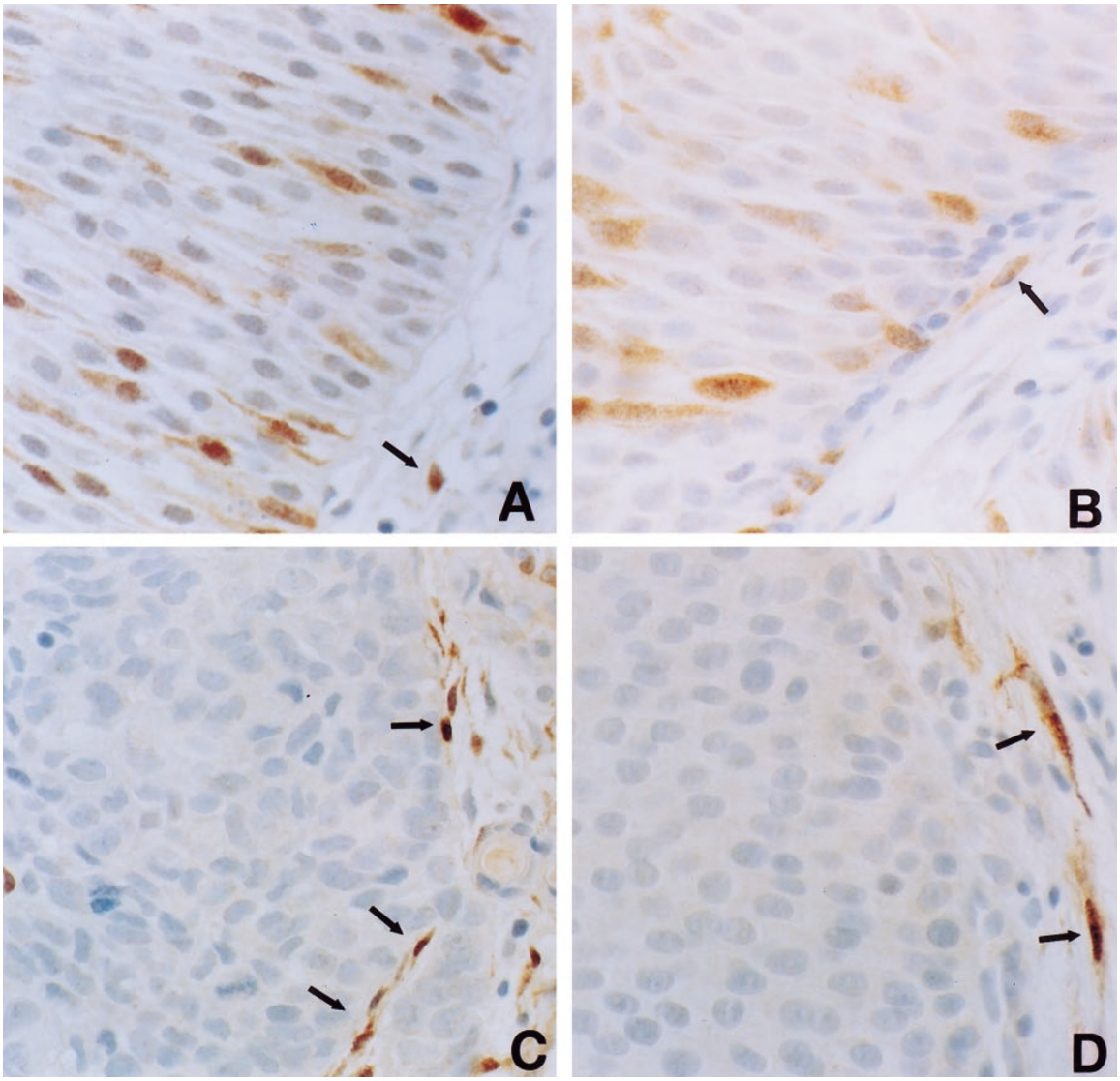


Figure 5 Expression patterns of p16 in relation to molecular status of MTS 1. (**a**) Heterogenous (normal) pattern of p16 expression in a TCC with no LOH in 9p21 and a wild type sequence of the MTS 1 gene. (**b**) Retention of heterogenous pattern of p16 expression in a TCC with mutation of MTS 1 but no LOH in 9p21, (one wild type allele of the gene is present). (**c**) Absence of p16 expression in a TCC with mutation generating stop codon and LOH in 9p21, (one mutant allele of the gene is present). (**d**) Absence of p16 expression in TCC with large homozygous deletion of MTS 1 and LOH in 9p21. Note the presence of individual stromal fibroblasts with p16 expression that serve as internal positive control (arrows). (Toluidine blue counterstain; magnification $\times 400$)

The development of non-invasive papillary tumors was associated with extensive allelic losses on chromosome 9. The analysis of 98 bladder tumors disclosed that extensive allelic losses on chromosome 9 were indeed more often seen in superficial tumors than on invasive bladder carcinomas. These observations suggest that putative tumor suppressor genes on chromosome 9 are more likely responsible for clonal expansion of superficial lesions and are unlikely the contributors to the invasive clinically aggressive growth. Clearly multiple putative tumor suppressor genes on chromosome 9 play a role in the development of urothelial neoplasia. The loci on chromosome 9 lack the consistency of involvement, i.e. some tumors exhibit LOH in several regions while other show different patterns of alteration. Although there are regions on chromosome 9 that are preferentially involved, each tumor evolves via its own distinct pattern of allelic losses. The possibility that we were unable to identify allelic losses in some urothelial samples as a result of contamination with stromal DNA cannot be entirely excluded. Therefore, the patterns of chromosome 9 allelic losses in the development and progression of urothelial neoplasia can be even more complex and less consistent than disclosed by our study. Moreover, there are additional genetic alterations, e.g. mutations of the transforming genes and various mechanisms of gene inactivation such as hypermethylation additionally complicating the issues which were not addressed in our approach. Valuable information can also be obtained if alterations of an individual chromosome are analysed in correlation with losses on other chromosomes on a whole genomic scale.

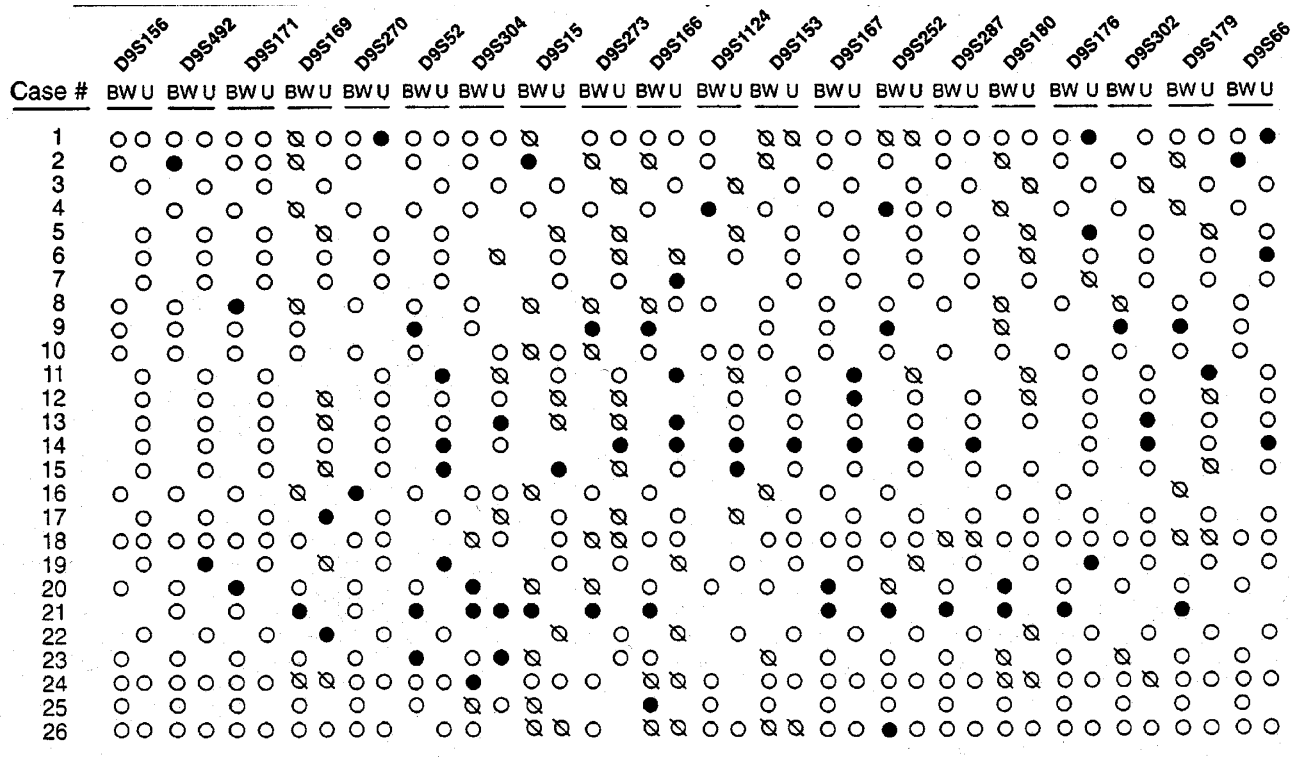
The MTS 1 gene was postulated as a potential major target of allelic losses involving the p21 region in bladder cancer (Williamson *et al*., 1995). Others indicate that LOH on p21 less frequently results in the absence of functional MTs 1 product in bladder cancer (Packenham *et al*., 1995; Orlow *et al*., 1995). It

appears that the frequency of the MTS 1 gene involvement in TCC is much lower than that reported for cell lines (Cairns *et al*., 1994; Spruck *et al*., 1994). An alternative mechanism of transcriptional silencing of the MTS 1 gene by hypermethylation has been postulated, but it appears to play a minor role in bladder cancer (Merlo *et al*., 1995; Reed *et al*., 1996; Wong *et al*., 1997). In our studies the presence of homozygous deletions as revealed by STS's could be documented in 67.6% of TCC's. It has to be

Table 4 Clinico-pathologic data of patients whose voided urine and/or bladder washing samples were tested for allelic losses of chromosome 9 (analysis of 26 cases)

	Current status			*Follow-up*	*Primary tumor*		
Case no	*Growth*	*Grade*	*Stage*	*Months*	*Growth*	*Grade*	*Stage*
1			T_0	60	P	2	T_a
2			T_0	3	NP	3	T_2
3			T_0	15	P	2	T_a
4			T_0	1	NP	3	T_2
5			T_0	6	P	2	T_2
6			T_0	2	NP	3	T_{is}
7			T_0	1	NP	3	T_{is}
8				0	P	2	T_1
9	P	2	T_a	100	P	2	T_a
10	P	2	T_a	0	P	2	T_a
11	P	2	T_a	805	P	2	T_1
12	P	2	T_a	149	P	2	T_a
13	P	2	T_a	140	P	2	T_a
14	P	2	T_a	55	P	1	T_a
15	NP	3	T_1	2	NP	3	T_1
16	NP	3	T_2	0	NP	3	T_2
17	NP	3	T_2	3	NP	3	T_2
18				0	NP	3	T_3
19	NP	3	T_{3a}	25	NP	3	T_1
20	NP	3	T_{3a}	1	NP	3	T_2
21	NP	3	T_{3b}	1	NP	3	T_1
22	NP	3	T_4	4	NP	3	T_2
23	NP	3	T_a	120	NP	3	T_3
24	NP	3	T_{is}	1	NP	3	T_2
25	NP	3	T_{is}	1	NP	3	T_1
26	NP	3	T_{is}	1	NP	3	T_2

Table 5 Allelic losses of chromosome 9 identified on voided urine samples of patients with urinary bladder tumors (analysis of 26 cases)[a]



[a]Clinicopathologic data of these patients are summarized in Table 4. ○, no evidence of LOH; ●, LOH; and ∅, noninformative marker

mentioned, however, that the identification of homozygous deletions in the MTS locus is not synonymous with the presence of structural alterations within the MTS genes. In fact, in the vast majority of these cases, no alterations of MTS 1 and 2 could be documented by SSCP and sequencing. Structural alterations involving the MTS 1 and 2 genes could be documented in only 13.7 and 6.8% of TCC's respectively. In summary, our data indicate that the MTS genes are less frequently involved in bladder tumorogenesis than previously reported but they still represent one of the alternative target genes on chromosome 9 associated with urinary bladder neoplasia.

Our studies indicate that allelic losses of chromosome 9 were ubiquitous in urinary bladder cancer and none of the chromosome 9 regions could be related to specific pathogenetic subsets, histologic grade or invasive phenotype of TCC. However, allelic losses of chromosome 9 can serve as markers of urothelial neoplasia and were identified in voided urine of nearly all patients with TCC. Moreover, they could be identified in patients with the history of TCC but no clinical or microscopic evidence of tumors at the time of testing. This suggests that alterations of chromosome 9 may represent evidence of persistent and recurrent neoplasia antecedent to the development of clinically and microscopically identifiable lesions.

The strategy of superimposed histologic and genetic mapping used in this study expands the applicability of polymorphic markers to studies of the events of multistep carcinogenesis, i.e. the progression of neoplasia from clinically occult precursor conditions to invasive cancer. The use of this approach helped to define more specifically the regions of chromosome 9 involved in early phases of urothelial neoplasia. A similar approach on a whole genomic scale can be used to generate more accurate data than currently available on genetic models of multistep carcinogenesis in a variety of human cancers. This data will in turn serve as important landmarks for subsequent more specific studies of mechanisms of cancer development and progression. Moreover, such an approach can identify genetic alterations evident in early clonal expansion of preneoplastic changes that could serve as markers of clinically occult neoplasia.

Materials and methods

Tumor samples and clinico-pathological data

Five cystectomy specimens containing TCC were used to create superimposed histologic and genetic maps. Fresh samples of urinary bladder tumors from 98 patients and their follow-up data were used to analyse the relationship of genetic alterations to histologic grade, invasiveness, growth pattern and to the clinical behavior of the tumor. Allelic losses in those regions of chromosome 9 that were identified as significantly altered by the superimposed histologic and genetic mapping were tested in voided urine and/or bladder washings of 26 patients with TCC. The intraurothelial precancerous changes were microscopically classified as mild, moderate, or severe dysplasia, or as carcinoma *in situ*. The TCCs were classified according to the three-tier histologic grading system of the World Health Organization (Koss, 1995). Their growth pattern (papillary versus nonpapillary) and depth of invasion were also recorded. The histologic sections were evaluated independently by two pathologists (Ro and Czerniak). DNA was extracted from mucosal samples of cystectomy specimens, individual bladder tumors and sediments of voided urine samples and/or bladder washings as previously described (Chaturvedi *et al*., 1997). For controls, DNA was also extracted from the peripheral blood lymphocytes and/or normal tissue in the resected specimens from each patient.

Superimposed histologic and genetic maps

Cystectomy specimens were prepared as previously described (Chaturvedi *et al*., 1997). We obtained 49, 39, 65, 42 and 39 mucosal samples respectively from each bladder. In four cases (maps 1, 2, 4, and 5), a single focus of grade 3, nonpapillary TCC invading the muscularis propria was present. It was accompanied by extensive precancerous lesions ranging from mild dysplasia to carcinoma *in situ*. In one case (map 3), multiple foci of TCC were present. One focus represented a grade 3 nonpapillary TCC with transmural invasion of the bladder wall and involvement of the perivesical adipose tissue. Two additional foci of carcinoma represented grade 3 papillary TCC without invasion. Like the other four cases, extensive areas of the urinary bladder mucosa in this case exhibited changes ranging from mild dysplasia to carcinoma *in situ*. The results were recorded as histologic maps. Subsequently, DNA was extracted from all mucosal samples and corresponded to microscopically verified urothelial lesions or normal bladder mucosa.

Microsatellites

A set of primers for 52 microsatellite loci on chromosome 9 based on an updated Genethon microsatellite map was purchased from Research Genetics (Huntsville, AL, USA), (Gyapay *et al*., 1994). Several markers located within or flanking the MTS genes were also included in this study. The markers selected for testing exhibited high levels of heterozygosity and relatively uniform distribution, i.e. covered all regions of chromosome 9. The allelic patterns of markers were resolved on polyacrylmide gels after their amplification using the polymerase chain reaction as previously described (Chaturvedi *et al*., 1997). A minimum 50% reduction in signal intensity was required to be considered evidence of LOH. Tests with questionable results were repeated. In such cases the densitometric measurements were performed to ensure objective reading of the data. Testing of markers was performed in two phases. Initially, all 52 markers were tested on paired non-tumor versus tumor DNA samples. This revealed LOH of 15 markers which were subsequently tested on all mucosal samples to generate superimposed histologic and genetic maps.

Alterations of MTS

Allelic losses in the MTS locus were tested with marker *D9S492*, located between exons 1 and 2 of the MTS 1 gene (Liu *et al*., 1995). Homozygous deletions within the MTS locus were tested with the following sequence-tagged site (STS) primers: 1063.7, c18.b, c5.1, RN3.1, C5.3, R2.3, R2.7, and c1.b (Kamb *et al*., 1994). The presence of homozygous deletions in the MTS locus as revealed by PCR using STS primers was confirmed by Southern blotting. The probes used for Southern blotting represented the DNA fragments amplified by the STS primers that exhibited homozygous deletions in a given site. The probes were labeled by the random priming method, and the hybridization was carried out using standard conditions (Maniatis *et al*., 1989). The presence of homozygous

deletions was verified by Southern blotting in five cases of bladder tumor samples and in representative tumor samples of cystectomy specimens corresponding to three foci of TCC in a cystectomy specimen used for superimposed histologic and genetic mapping of the MTS locus. The hybridization signal was compared between tumor and non-tumor DNA samples (data not shown).

Alterations within coding sequences of MTS 1 and 2 genes were tested by SSCP and direct sequencing of the PCR-amplified gene fragments using the following primers:

MTS 1 (exon 1)	5′-GAA GAA AGA GGA GGG GCT G-3′
	5′-GCG CTA CCT GAT TCC AAT TC-3′
MTS 1 (exon 2)	5′-GGA AAT TGG AAA CTG GAA GC-3′
	5′-TCT GAG CTT TGG AAG CTC T-3′
MTS 1 (exon 3)	5′-TTC TTT CTG CCC TCT GCA-3′
	5′-GCA GTT GTG GCC CTG TAG GA-3′
MTS 2 (exon 1)	5′-CCA GAA GCA ATC CAG GCG CG-3′
	5′-AAT GCA CAC CTC GCC AAC G-3′
MTS 2 (exon 2)	5′-TGA GTT TAA CCT GAA GGT GG-3′
	5′-GGG TGG GAA ATT GGG TAA G-3′

For SSCP analysis, 100 ng of genomic DNA was amplified by PCR using 1 μM each of the primers, as previously described (Chaturvedi *et al*., 1997). To confirm the presence of alterations identified by SSCP, direct sequencing of PCR-generated MTS gene fragments were performed using the Sequenase PCR Product Sequencing kit (United States Biochemical Corp., Cleveland, OH, USA), according to the protocol supplied by the manufacturer. All sequence modifications that represented polymorphic sites were not considered as sequence alterations and were excluded from the analysis.

In order to confirm that the structural alterations of the coding sequence of the MTS 1 gene affected the gene expression, the results of molecular analysis, i.e. LOH in p21, homozygous deletions, as well as gene mutations identified by SSCP/sequencing studies were compared with the expression patterns of its product, the p16 protein, identified by immunohistochemistry (Larsen, 1997). Staining for p16 was performed on formalin fixed paraffin-embedded tissue sections. Briefly, after hydrogen peroxide treatment to block the endogenous peroxide activity, the slides were washed in distilled water and placed in 0.01 M sodium citrate buffer (pH 6.0) for 15 min at 95°C, which was followed by rinsing in distilled water and PBS (Phosphate buffer saline, pH 7.4). The slides were then processed for staining of p16 using the anti-p16 antibody, NCL-p16, clone DCS-50 (Vector Laboratories, Burlingame, CA, USA) at a 1:25 dilution. The primary antibody was visualized using ABC Elite Kit (Vector Elite Kit; Vector Laboratories, Burlingame, CA, USA) with 0.05% 3,3′-diaminobenzidine in Tris-HCl, buffer containing 0.01% hydrogen peroxide and counterstained with 0.01% toluidine blue. In addition, all cut sections were kept at 4°C prior to staining. Tumors were considered to have a a normal heterogenous p16 if they expressed relatively weak nuclear staining with considerable differences in nuclear intensity, including many negative cells. A tumor was termed p16 negative if no malignant cells had positive staining and at least several contiguous p16 positive non-tumor stromal cells were present as internal controls. Each section was submitted by pathology number and the scorer did not know the status of 9p21 LOH or MTS 1 with SSCP and sequencing studies.

Identification of chromosome 9 allelic losses in voided urine samples

Twenty hypervariable markers corresponding to regions of chromosome 9 disclosed as significantly altered by our superimposed histologic and genetic mapping studies were tested on DNA extracted from the sediments of voided urine samples and/or bladder washings of 26 patients with TCC of the bladder. The current and past clinico-pathologic data were used to evaluate the status of these patients utilizing the TNM staging system (Fleming *et al*., 1997). DNA extracted from sediments of voided urine samples of ten healthy individuals with no clinical signs of urinary bladder tumors were used as controls.

Analysis of data

Three-dimensional displays of chromosomal alterations in relation to progression of the neoplasia from a precursor intraurothelial condition to invasive cancer were generated and initially analysed by the nearest-neighbor analysis (Hartigan, 1975). A nearest neighbor analysis was performed on the three-dimensional stacks of maps consisting of plots of marker alterations by location on the histologic bladder maps and on chromosomal vectors. An altered region was considered a neighbor of another altered region if the two were side by side in the same marker plot of above and below each other. An altered region was also considered to be connected to another altered region if there was a continuious string of altered regions between them. Since the bladder was laid open and pinned flat, the left-most and right-most regions were also neighbors.

The relationship between altered markers and progression of urothelial neoplasia from precursor conditions to invasive carcinoma revealed by superimposed histologic and genetic mapping were tested by a modified LOD score analysis as previously described (Chaturvedi *et al*., 1997). Cumulative LOD scores were calculated at variable θ (0.01, 0.5, and 0.99). Stringency level one designated LOD scores for specific stages of neoplasia. Stringency level two designated LOD scores for progression to higher stages of neoplasia. The patterns of LOD scores $\geqslant 3$ at $\theta = 0.01$ or 0.99 and LOD scores < 3 at $\theta = 0.5$ for the same marker were considered significant. The strongest association between an altered marker and neoplasia was when a LOD score was $\geqslant 3$ at $\theta = 0.99$ and 0.5 and < 3 at $\theta = 0.01$. The use of LOD scores in this analysis was not the same as that commonly used in linkage analysis of familial genetic predisposition for diseases (Ott, 1991). Rather, it was intended to be used in its generic mathematical sense as a likelihood test of events (Brownlee, 1965). We used the LOD score variant of the likelihood test, as many researchers are more familiar with approximate levels of significance when expressed in this form.

The relationship among altered markers, the MTS genes, and various clinico-pathological parameters were tested by Gehan's generalized Wilcoxon, log-rank tests, and Kaplan-Meier analysis.

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