

Two Target Regions of Allelic Loss on Chromosome 9 in Urinary-bladder Cancer

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Allelic losses on chromosome 9 are common in a wide variety of human tumors; moreover, two predisposing loci for some inherited cancer syndromes, i.e., familial malignant melanoma and Gorlin syndrome, have been identified on this chromosome. To define the location of putative tumor suppressor genes involved in cancer of the urinary bladder, 85 bladder cancers were examined for allelic loss at 18 microsatellite loci on chromosome 9. Correlations were also sought between loss of heterozygosity on chromosome 9 and several clinicopathological parameters. Allelic loss was observed in 54 of the tumors (64%) and deletion mapping identified two target regions; one at an interval on 9p21 flanked by D9S736 and D9S165, and the other at an interval on 9q31–34 flanked by D9S58 and D9S61. No subtle mutation was detected in the *PTCH* gene which lies in the latter interval. Allelic loss on chromosome 9 was observed frequently in low grade and non-invasive tumors as well as in tumors of more advanced phenotype. Inactivation of tumor suppressor genes lying in either of two regions of common deletion identified on chromosome 9 might affect carcinogenic mechanisms at an early stage of tumor development in the urinary bladder.

Key words: Urinary bladder cancer — Chromosome 9 — Loss of heterozygosity — Tumor suppressor gene

Bladder cancer is the most common cancer of the upper urinary tract. Carcinogenesis in human tissues and progression of those tumors are considered to require a series of genetic alterations involving activation of dominant oncogenes and inactivation of tumor suppressor genes. Inactivation of tumor-suppressor functions usually occurs as a consequence of deletion of one allele of a gene involved in control of cell growth, followed by mutation of the other. Therefore, when allelic losses at specific chromosomal loci (loss of heterozygosity, LOH) are observed frequently in a particular type of human cancer, one can infer that putative tumor suppressors are present in normal alleles of those loci and are inactivated by the cancer-associated events causing LOH.¹ In cancers of the urinary bladder, LOH has been reported on chromosomes 8, 9, 10, 11, 17, and 18.^{2–9}

Germline mutations of tumor suppressor genes confer predispositions to several dominantly inherited cancer syndromes. Two genes in this category have been localized to chromosome 9. On the short arm, cyclin-dependent kinase inhibitor 2 (*CDKN2*), a gene associated with familial malignant melanoma,¹⁰ was isolated recently from 9p21. Homozygous or hemizygous deletions of *CDKN2* and/or nearby p15 are also common in cell lines or primary

tissues derived from various other types of cancer^{11,12} including cancers of the esophagus,¹³ pancreas¹⁴ and urinary bladder.¹⁵ On the long arm, the human homologue of the *Drosophila* “patched” gene (*PTCH*), whose mutant alleles predispose carriers to nevoid basal cell cancer (Gorlin syndrome), was recently isolated from 9q22.3.¹⁶ Somatic mutations and losses of *PTCH* also have been found in medulloblastomas and meningiomas.¹⁷ LOH at yet another region of chromosome 9, q32–33,¹⁸ is also frequent in urinary-bladder cancers.

In the study reported here, we undertook to construct a deletion map of chromosome 9 in 85 urinary-bladder cancers by taking advantage of a high-resolution chromosomal map of the region consisting of 18 microsatellite loci. We searched for mutations of *PTCH* by PCR-single-strand conformation polymorphism (SSCP) analysis, and also compared the frequencies of allelic losses on chromosome 9 with several clinicopathological parameters.

MATERIALS AND METHODS

Samples and DNA preparation Specimens of 85 urinary bladder cancers were obtained, along with paired blood samples as a source of constitutional normal DNA, at Kyoto University Hospital, Yokohama City University Hospital, and Nippon Medical School Hospital. Informed consent of all participating patients were obtained prior to

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surgery for urinary-bladder cancer during hospitalization. All of the tumors were classified as transitional-cell cancers. Genomic DNAs were extracted from frozen tissues by a method described previously.¹⁹⁾

LOH analysis Eighteen microsatellite markers (D9S178, D9S286, D9S157, D9S736, D9S165, D9S153, D9S287, D9S180, D9S176, D9S58, D9S154, D9S302, D9S60, D9S61, D9S62, D9S113, D9S66, D9S158) were described in the Genethon comprehensive human linkage map.^{20,21)} Each polymerase chain reaction (PCR) was performed in a Gene Amp PCR system 9600 (Perkin-Elmer Cetus, Norwalk, CT) using 10 ng of template DNA in a volume of 10 μ l, with 30 cycles of 94°C for 30 s, 54–60°C for 30 s, 72°C for 30 s. A 3- μ l aliquot of each product was electrophoresed, after which the gels were dried and exposed to X-ray film for 16–48 h.²²⁾ Signal intensities of polymorphic alleles were quantified by a Hoefer GS-300 scanning densitometer; peak areas corresponding to each signal were calculated by electric integration using the GS-370 electrophoresis data system (Hoefer Scientific Instruments, San Francisco, CA). Reductions in signal intensities >50% were judged to reflect LOH. We also distinguished LOH from chromosome multiplication by normalizing each signal to the signal obtained when the same DNA was analyzed with markers for loci on other chromosomes.

PCR-single strand conformation polymorphism (SSCP) analysis of the *PTCH* gene *PTCH* gene was examined for sequence variations in all 45 tumors that showed allelic losses on 9q. PCR-SSCP analysis was performed for all coding exons, using PCR designed to amplify 24 overlapping segments. PCR primers for amplification of each exon and all exon-intron boundaries of the *PTCH* gene were described previously by Hahn *et al.*¹⁶⁾ and are presented in Table I. PCR was carried out according to the procedures described previously.²³⁾ In brief, each PCR was carried out using 20 ng of genomic DNA extracted from lymphocytes, in a 10- μ l solution containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 200 μ M each of dNTP, 2 μ Ci of [α -³²P]-dCTP (3000 Ci/mmol, 10 mCi/ml), 2.5 pmol of each primer, and 0.25 units of *Taq* polymerase. Each of the 35 PCR cycles consisted of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C. Reaction mixtures were diluted with 50 μ l of 95% formamide dye and 20 mM EDTA, incubated at 85°C for 5 min, and applied (2 μ l/lane) to 6% polyacrylamide gels containing 0.5 \times TBE (90 mM Tris-borate/2mM EDTA) and 5% glycerol. Electrophoresis was performed overnight at 100 to 150 V for 16 to 20 h both at room temperature and at 4°C. The gels were dried and autoradiographed with intensifying screens.

Table I. Sequences of *PTCH* Primers Used for PCR-SSCP Analysis

Exon	Sense primer (5'–3')	Antisense primer (5'–3')	Position (bp)	Size (bp)
1	GAAGGCGAGCACCCAGAC	TCTTCCCTCCTCTCCCTTC	1–189	189
2	GTGGCTGAGAGCGAAGTTTC	TTCCACCCACAGCTCCTC	190–382	193
3	CTATTGTGTATCCAATGGCAGG	ATTAGTAGGTGGACGCGGC	383–582	200
4	GAGAAATTTTTGTCTCTGCTTTTCA	CCTGATCCATGTAACCTGTTTC	583–642	60
5	GCAAAAATTTCTCAGGAACACC	TGGAACAAACAATGATAAGCAA	643–734	92
6	CCTACAAGGTGGATGCAGTG	TTTGTCTCCACCCTTCTGA	735–933	199
7	GTGACCTGCCTACTAATTCCC	GGCTAGCGAGGATAACGGTTTA	934–1055	122
8	GAGGCAGTGGAAACTGCTTC	TTGCATAACCAGCGAGTCTG	1056–1203	148
9	GTGCTGTCTGAGGCTTGTG	ACGGACAGCAGATAAATGGC	1204–1335	132
10	GTGCTGTCTGAGGCTTGTG	ACGGACAGCAGATAAATGGC	1336–1492	157
11	GTGTTAGGTGCTGGTGGCA	CTTAGGAACAGAGGAAGCTG	1493–1591	97
12	TCTGCCACGTATCTGCTCAC	CATGCTGAGAATTGCAGGAA	1591–1835	245
13	GGCCTACACCACACACAC	TTTTTTTGAAGACAGGAAGAGCC	1836–2238	403
	GTCAGCAGACTGATTCAGGT	AAGATGAGAGTGTCCACTTCG		
14	GACAGCTTCTCTTTGTCCAG	ACGCAAAAGACCGAAAGGACGA	2239–2548	310
15	AGGGTCCTTCTGGCTGCGAG	TCAGTGCCCAGCAGCTGGAGTA	2549–2691	143
16	AACCCATTCTCAAAGGCCTCTGTTC	CACCTCTGTAAGTTCCCAGACCT	2692–2875	185
17	AACTGTGATGCTCTTCTACCCTGG	AAACTTCCCGCTGCAGAAAGA	2876–3156	281
18	TTTGATCTGAACCGAGGACACC	CAAACAGAGCCAGAGGAAATGG	3157–3294	138
19	TAGGACAGAGCTGAGCATTTACC	TACCTGACAATGAAGTCG	3295–3437	143
20	TAGGACAGAGCTGAGCATTTACC	TACCTGACAATGAAGTCG	3437–3537	101
21	AACAGAGGCCCTGAAAAAT	GATCACTTGGTGGGCAGG	3538–3792	255
22	TCTAACCCACCCTCACCCCTT	ATTGTTAGGGCCAGAATGCC	3793–4330	537
	AGAAAAGGCTTGTGGCCAC	TCACCCTCAGTTGGAGCTG		

Table II. LOH at Loci on Chromosome 9 in 85 Urinary Bladder Cancers

Locus	Allelic loss/informative cases (%)
D9S178	30/47 (64)
D9S286	26/47 (55)
D9S157	29/60 (48)
D9S736	17/29 (59)
D9S165	26/55 (47)
D9S153	27/56 (48)
D9S287	26/48 (54)
D9S180	21/34 (62)
D9S176	28/52 (54)
D9S58	34/65 (52)
D9S154	34/66 (52)
D9S302	29/61 (48)
D9S60	29/60 (48)
D9S61	26/58 (45)
D9S62	26/57 (45)
D9S113	22/50 (44)
D9S66	18/43 (42)
D9S158	16/39 (41)
Total	54/85 (64)

Clinicopathological parameters Tumors were graded according to WHO (World Health Organization) recommendations²⁴⁾ and staged according to the TNM classification of UICC (Union Internationale Contre le Cancer).²⁵⁾ The χ^2 test and Fisher's exact test were used for statistical analysis of the results. *P* values of less than 0.05 were considered statistically significant.

RESULTS

LOH was detected in 54 (64%) of the 85 urinary bladder cancers analyzed with 18 polymorphic markers on chromosome 9. The marker loci and their frequencies of LOH are listed in Table II, in descending order from the 9p telomere. Among the 54 tumors with LOH in this chromosome, 38 had lost alleles at all informative loci. Of the others, ten showed partial or interstitial deletions on 9p and seven on 9q.

Representative autoradiograms of cases that showed interstitial or partial deletions of 9p21 or 9q31–34 are presented in Fig. 1. Tumor 64 showed LOH at D9S736 but retained alleles at D9S157 and D9S165. Tumor 68 showed LOH at D9S165 but retained alleles at all informative loci distal to D9S736. Tumor 236 showed LOH at D9S302, but retained alleles at D9S58 and D9S61. Tumor 58 retained alleles at all informative loci proximal to D9S58, but lost alleles at D9S154 and more distal loci.

The results of LOH analysis of the 17 that showed partial or interstitial deletions of chromosome 9 are summa-

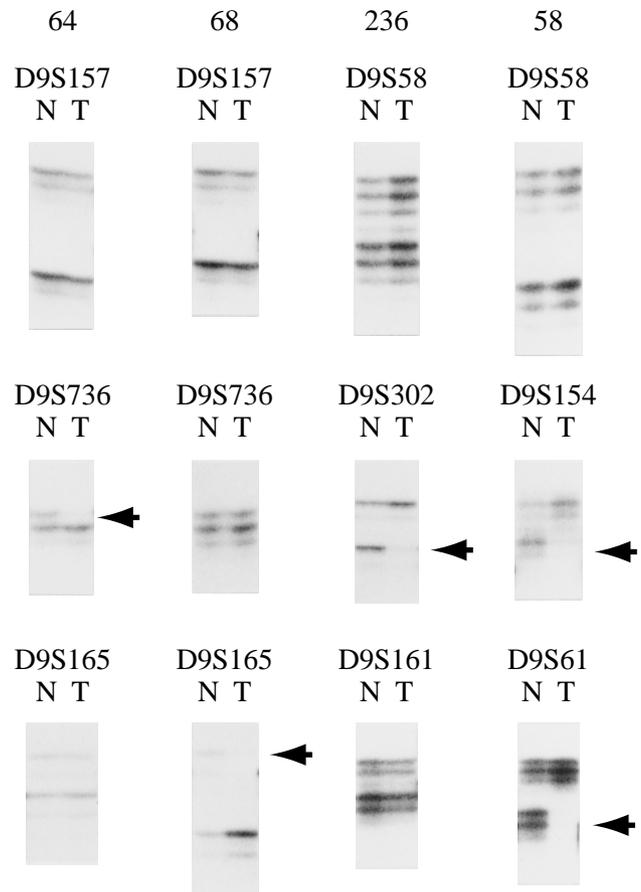


Fig. 1. Representative autoradiograms of normal and tumor genotypes that determined the commonly deleted regions at 9p21 and 9q31–34. The microsatellite markers yielding these results are indicated above their respective autoradiographic images. N and T, matched DNA samples isolated from normal and tumor tissues, respectively.

rized in Fig. 2. On the p arm, two tumors (tumors 68 and 256) retained heterozygosity at a distal marker, D9S736, but showed LOH at more proximal loci. Four other tumors (tumors 50, 54, 64 and 78) retained heterozygosity at a proximal marker, D9S165, but showed LOH at more distal loci. Hence, we defined the commonly deleted region within the 14-cM interval on 9p21 flanked by D9S736 and D9S165.

On the q arm, tumors 236 and 260 retained heterozygosity at a distal marker, D9S61, but showed LOH at more proximal loci. The proximal limit was defined by D9S58; two tumors (tumors 58 and 236) retained heterozygosity at this locus and showed LOH at a distal marker, D9S154. Hence, we defined this commonly deleted region within the 23-cM interval on 9q31–34 flanked by D9S58 and D9S61. Since the *PTCH* gene lies in this region we exam-

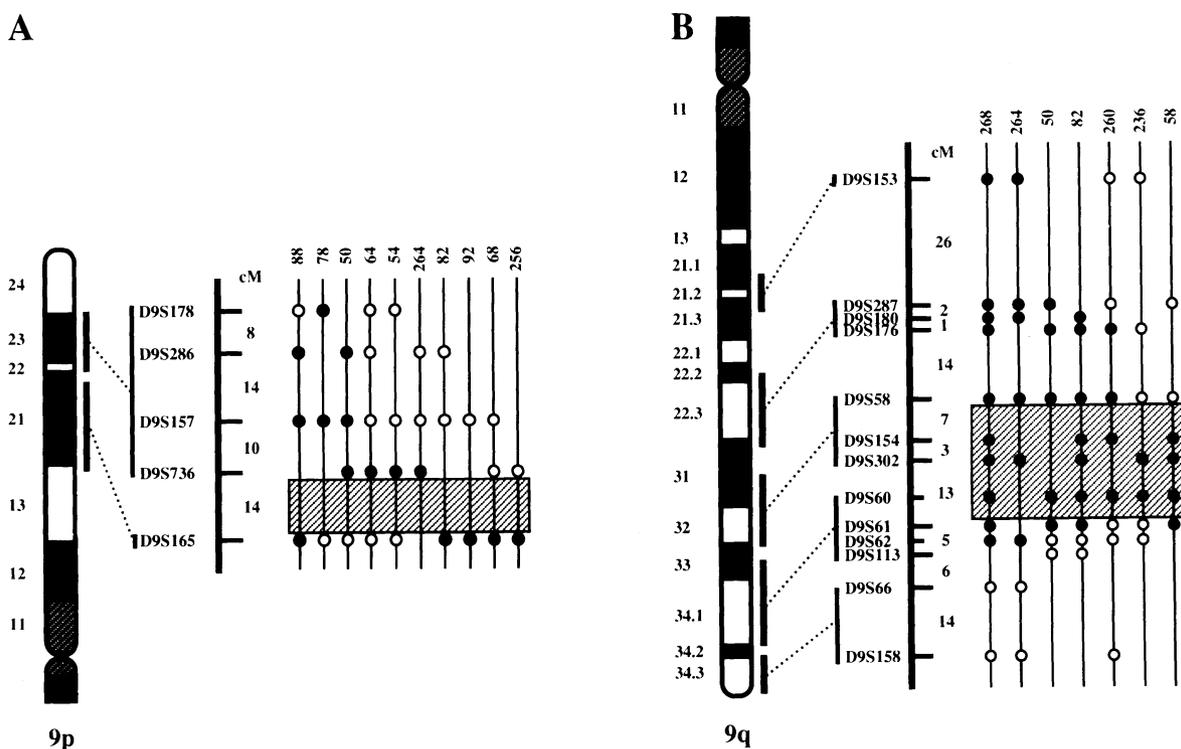


Fig. 2. Schematic representation of deletion mapping among 18 informative tumors that showed partial or interstitial deletions on 9p (A) or 9q (B) in urinary-bladder cancers. The karyograms show the location of each microsatellite marker used in the study. Numbers at the top of each column identify the tumors. Blank space indicates uninformative of the relevant marker in each case. The commonly deleted regions are indicated by shaded boxes superimposed on the map. Filled circles, LOH; open circles, retention of both alleles in the tumor; blank spaces, uninformative loci.

Table III. Correlation between LOH on Chromosome Arm 9p and 9q and Clinical Parameters^{a)}

		Grade				Staging				Lymph node metastasis			Distant metastasis	
		G1	G2	G3	pTa	pT1	pT2	pT3	pT4	N0	N1	N2	M0	M1
9p	LOH (+)	8	24	18	14	16	8	10	2	44	2	4	46	4
	LOH (-)	6	17	12	10	11	6	7	1	32	1	2	36	0
		NS ^{b)}				NS				NS			NS	
9q	LOH (+)	8	19	18	12	13	7	9	2	38	2	4	42	4
	LOH (-)	6	22	12	14	14	7	8	1	38	1	2	40	0
		NS				NS				NS			NS	

a) TNM classification of urinary bladder cancers according to criteria of the American Urological Association. Clinicopathological status and LOH status were available in all 85 tumors.

b) NS, not significant.

ined all its coding exons by PCR-SSCP in the 45 tumors that had shown LOH on the 9q arm, but detected no abnormalities (data not shown).

We attempted to correlate LOH on either 9p or 9q with clinicopathological parameters (tumor grade, staging, lymph node metastasis, local recurrence, distant metasta-

sis) in all tumors for which these data were available (Table III). Allelic loss on chromosome 9 was frequent in low grade and non-invasive tumors as well as in tumors of more advanced phenotype.

DISCUSSION

Loss of chromosome 9 is one of the most frequently observed cytogenetic abnormalities in urinary-bladder cancers. We present here the results of high-resolution deletion mapping of 85 primary bladder cancers using 18 microsatellite loci on chromosome 9. Allelic loss was observed in 64% of the tumors in our panel. We identified one target region of common deletion on 9p and another on 9q; the former was defined in the 14-cM interval between D9S736 and D9S165 at 9p21, and the 9q target region constituted the 23-cM interval between D9S58 and D9S61 at 9q31–34.

On deletion mapping of 9p in urinary-bladder cancer, Ruppert *et al.* located a commonly deleted region between D9S161 (9p21) and the telomere.²⁶⁾ Cairns *et al.* localized a commonly deleted region to a 10-cM region at 9p21–22 flanked by D9S162 and D9S171.²⁷⁾ Orlow *et al.* observed a high frequency of allelic losses with IFNA marker.²⁸⁾ Keen *et al.* described two commonly deleted regions on 9p²⁹⁾; a distal region at 9p21 between D9S126 and IFNA and a proximal region at a region centromeric to IFNA. The distal region of that study overlaps with the common regions defined in the former three studies. Simoneau *et al.*, on the hand, defined a commonly deleted region flanked by D9S970 at 9p12 and 9q21,³⁰⁾ which overlaps with the proximal region of Keen's study. Czerniak *et al.*³¹⁾ identified two deleted regions on 9p; a distal region at 9p11–13 between D9S268 and D9S285 and a proximal region at 9p21–23 between D9S52 and D9S165. The present study identified a commonly deleted region in a 14-cM interval at 9p21 flanked by D9S736 and D9S165 which overlaps with the proximal region of Czerniak's study, and provides evidence for the presence of a tumor suppressor gene in the region at 9p21. The commonly deleted region at 9p21 contains the *CDKN2* gene, whose mutant alleles, if inherited, predispose carriers to a syndrome of familial malignant melanoma. LOH at 9p21 has been observed in 57–73% of urinary-bladder cancers examined,^{5, 27–30, 32–34)} but this event also is frequent in malignant mesotheliomas,³⁵⁾ renal-cell cancers,³⁶⁾ non-small cell lung cancers,³⁷⁾ and cancers of the nasopharynx,³⁸⁾ head and neck,³⁹⁾ and breast.⁴⁰⁾ Homozygous deletions of *CDKN2* are frequently observed in cancers of the esophagus,¹³⁾ pancreas,¹⁴⁾ bladder,^{5, 32)} and breast,⁴⁰⁾ indicating inactivation of both alleles of the gene in those tumors. Baud *et al.*⁴¹⁾ suggested that simultaneous loss of both *CDKN2* alleles, by point mutation or homozygous deletion, was infrequent in primary bladder cancers. In a more

recent study, Benedict *et al.*⁴²⁾ indicated that loss of *CDKN2* protein function could be related to retinoblastoma (RB) protein overexpression by immunohistochemical analysis, since *CDKN2* could induce transcriptional down-regulation of RB, and its loss might lead to aberrant RB regulation. Another tumor-suppressor candidate on the short arm is Bcl2-associated gene 1 (*BAG1*), located at 9p12. The *BAG1* protein binds to *BCL2* and markedly increases the anti-apoptotic activity of *BCL2* induced by various stimuli.⁴⁴⁾

On deletion mapping of 9q in urinary-bladder cancer, Orlow *et al.* defined a commonly deleted region at 9q34.1–34.2 flanked by D9S10 and D9S7.²⁸⁾ Habuchi *et al.* described two commonly deleted regions on 9q in bladder cancer; the proximal region flanked by D9S153 and D9S109, and the distal region flanked by D9S61 and D9S66.⁶⁾ Simoneau *et al.* described a commonly deleted region at 9q21 around D9S283.³⁰⁾ Czerniak *et al.*³¹⁾ described three commonly deleted regions on 9q; the most proximal region flanked by D9S200 and D9S175 at 9q12–13, the middle region flanked by D9S152 and D9S176 at 9q21–22, and the distal region flanked by *ABL1* and D9S158 at 9q34. The commonly deleted regions described in these studies did not overlap with the critical region that we defined between D9S58 and D9S61 in the present study. On the other hand, Keen *et al.* located a large commonly deleted region that extended over 50 cM from D9S15 to D9S60.²⁹⁾ Recently, Simoneau *et al.*⁴³⁾ identified four unique deleted regions; the most proximal region flanked by D9S201 and D9S180 at 9q22.3, the next proximal region flanked by D9S53 and D9S58 at 9q31–32, the third region flanked by D9S103 and D9S260 at 9q33, and the most distal region flanked by *ASS* and *ABL-1* at 9q34. The critically deleted region on 9q in the present study partially overlaps with the region described by Keen *et al.*²⁹⁾ and the third region at q33 described by Simoneau *et al.*⁴³⁾

A candidate suppressor gene, named *DBCCR1*,⁴⁵⁾ was recently isolated from a 840 kb YAC present in the overlapping region at 9q32–33.¹⁸⁾ However the absence of mutation in bladder cancer excluded it from candidacy as a tumor suppressor. Therefore, the results of the present study suggest that our commonly deleted region flanked by D9S58 and D9S61 contains an as-yet unidentified tumor suppressor locus. The *PTCH* gene maps to the vicinity of the commonly deleted region we defined in a 23-cM interval on 9q31–34. *PTCH* was recently isolated as a predisposing gene for nevoid basal cell cancer (Gorlin syndrome).¹⁶⁾ Somatic mutations and loss of this gene have also been observed in some cutaneous basal-cell cancers, medulloblastomas, and meningiomas.¹⁷⁾ Suzuki *et al.*⁴⁶⁾ screened for mutations throughout the coding region of the *PTCH* gene using PCR-SSCP in esophageal and lung cancers. They detected two rare polymorphisms in exon 17

but no somatic mutations in these types of cancer. We did not detect the polymorphism, probably due to the difference in our electrophoretic conditions of SSCP procedures from their study which would have resulted in limited sensitivity in polymorphism detection. Simoneau *et al.*³⁰⁾ and Xie *et al.*¹⁷⁾ screened primary bladder cancers that have LOH on 9q for mutations in the gene but detected no mutations. We examined 45 tumors that showed LOH on 9q for mutations of this gene using PCR-SSCP but detected no mutation, in accord with the former studies. Recently, McGarvey *et al.*⁴⁷⁾ found mutations in two bladder tumors of highly advanced stage and grade (one tumor: pT3b, G3 and the other tumor: pT4, G3) in a panel of 54 primary bladder cancers. Although the latter study might suggest involvement of the *PTCH* gene in a late stage of tumorigenesis in a subset of invasive bladder cancers, from the combined results of these studies, it is unlikely that the *PTCH* gene plays an essential role in the majority of urinary bladder cancers.

There are a number of other known genes on the long arm whose inactivation could lead to uncontrolled cell growth: *MSSE*, a predisposing gene for multiple self-healing squamous epithelioma, lies at 9q22.2–22.3³⁰⁾; a growth arrest-specific gene (*GAS1*) is located at 9q22.3–31³⁰⁾; transforming growth factor β receptor-1 (*TGFBR1*) lies at 9q33–34⁴⁸⁾; the tuberous sclerosis-1 gene (*TSC1*) is at 9q34⁴⁹⁾; and the apoptosis-associated protein kinase-1 gene (*DAPK1*) is at 9q34.1.⁵⁰⁾ In the present study, the majority of the bladder cancers examined had lost one allele in a large region of 9q. Since deletions as large as 23-cM would cause haplo-insufficiency of most of the growth-controlling genes mentioned above, the deletions of 9q noted here may afford a selective advantage to some urinary bladder cells and render them prone to neoplastic transformation.

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Olumi *et al.*, Knowles *et al.*, and Brewsters *et al.* described association of LOH on 17p,²⁾ LOH on 8p,³⁾ and LOH on 18q,⁴⁾ respectively, with tumors of high grade and high stage in urinary-bladder cancer. These data suggested that inactivation of tumor suppressor genes on chromosomal arms 17p, 8p, and 18q plays a role in the progression of bladder cancer. Orlow *et al.* reported that LOH on 9p was associated with the development of superficial tumor with a more aggressive biological behavior,²⁸⁾ whereas Cairns *et al.* and Miyao *et al.* observed frequent LOH on chromosome 9 in low grade/stage tumors as well as high grade/stage tumors.^{51,52)} In the present study, LOH on chromosome 9 was observed frequently in low-grade tumors, tumors without lymph node metastasis, and tumors of non-invasive stage, as well as in tumors of more advanced phenotype, and provided evidence that loss of chromosome 9 is associated with tumorigenesis before progression to invasive cancer in urinary bladder. Our data are consistent with the notion that LOH on chromosome 9 is an early event in urinary bladder carcinogenesis, and suggest that inactivation of tumor suppressor genes on this chromosome is responsible. A further study on the correlation of patients' prognosis with LOH on 9p or 9q would be of interest.

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