

Allelic Deletions of Cell Growth Regulators during Progression of Bladder Cancer¹

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ABSTRACT

Cell growth regulators include proteins of the p53 pathway encoded by the genes *CDKN2A* (p16, p14arf), *MDM2*, *TP53*, and *CDKN1A* (p21) as well as proteins encoded by genes like *RBI*, *E2F*, and *MYCL*. In the present study we investigated allelic deletions of all these genes in each recurrent bladder tumor from well-defined clinical material with more than 3 years of follow-up. We followed three groups (22 or 23 patients/group) of patients with: (a) recurrent noninvasive tumors (Ta); (b) primary muscle-invasive tumors (T2–T4); and (c) progressing tumors (Ta/T1 ⇒ T2/T4). We found a significant difference in the numbers of gene loci hit by deletions in muscle-invasive versus noninvasive tumors ($P = 0.0000002$), with the genes most often hit by deletions in muscle-invasive tumors being *TP53*, *RBI*, and *MYCL*. A number of novel findings were made. Losses of *MYCL* and *RBI* alleles were more pronounced in patients having concomitant field disease because 11 of 14 informative cases showed losses compared with 3 of 8 cases without field disease. A more pronounced deletion of *TP53* ($P = 0.002$) and *RBI* ($P = 0.02$) was found in the progressing tumor group compared with the recurrent noninvasive group, and, finally, the combined loss of *TP53* and *RBI* was present only in the progressing tumor or muscle-invasive groups. Deletion of two or more loci in *TP53*, *MYCL*, *RBI*, and *CDKN2A* was found in 10 patients in the progressing tumor group and in only 1 patient in the recurrent noninvasive group ($P = 0.004$). The data demonstrate that a characteristic difference between recurrent noninvasive and recurrent progressing bladder tumors is loss of cell cycle-regulatory genes in the latter group.

INTRODUCTION

One of the most important features of urothelial cancers of the bladder and upper urinary tract is metachronous or synchronous multifocal occurrence with high frequency. Between 70% and 80% of patients with bladder cancer have only noninvasive disease (Ta) or tumors with invasion no deeper than the lamina propria (T1) on initial presentation, and the remainder have muscle-infiltrating or deeper cancers (T2–T4) (1). The risk of developing a muscle-invasive disease is only 10% in a patient with a noninvasive bladder tumor (Ta), whereas the majority of patients diagnosed with concomitant carcinoma *in situ* (flat lesion grade 3–4) will develop a muscle-invasive tumor. At present, one cannot predict which patient with a noninvasive tumor will experience progression to invasive disease and which one will not. The search for predictive markers has been the aim of many studies and has included, among others, studies of nuclear volume (2), blood group antigens (3, 4), adhesion potential (5, 6), epidermal growth factor receptors, (7) and matrix metalloproteinases (8). However, these markers have only shown a general relation to prognosis and provide no definitive information for the specific patient. In recent years, a large body of information has been accumulated on the growth-regulatory pathway through which the p53 and Rb

proteins are working, including proteins encoded by genes like *MDM2*, *CDKN1A* (p21), *CDKN2A*(p16, p14arf), *RBI*, *E2F*, and *MYCL* (Fig. 1). In the present study, we investigated allelic losses of these genes as predictors of disease course. Alterations of the *TP53* gene seem to be of importance in most cancers. It is known that LOH³ of the *TP53* gene is correlated to high grade and stage of bladder tumors (9, 10), and LOH of 17p (the *TP53* locus) is associated with an invasive phenotype (11). Furthermore, positive immunostaining for p53 protein correlates with disease progression (12). Other components of the p53 and Rb pathways have also been investigated in bladder cancers, including *MDM2* (13, 14), p21 (15, 16) *CDKN2A* (17), *E2F* (18), and *RBI* (19, 20). Despite the many studies of these components in bladder cancer (9, 10, 17, 21, 22), very few, if any, have analyzed the various components of this pathway in single individual tumors to verify the number of different alterations. This could be of importance because losses of some gene products may promote cancer, whereas others may inhibit it. Furthermore, alterations of specific genes should be interpreted in the context of the presence or absence of downstream effector proteins. Based on this, we examined all of the shown growth regulators (Fig. 1) related to the p53 and Rb pathways in each tumor from three groups of patients: (a) patients with primary muscle-invasive tumors; (b) patients with recurrent noninvasive tumors; and (c) patients with progressing tumors. A number of novel findings relating allelic deletions to clinicopathological data were made. We detected a significant difference in the numbers of cell growth-regulatory gene loci affected by allelic deletions in low-stage versus high-stage tumors, frequent deletions at the *MYCL* locus, a difference in the pattern of allelic deletions in high-stage tumors with and without field disease, a more pronounced deletion of *TP53* and *RBI* in the progressing tumor group, and the presence of combined deletions of *TP53* and *RBI* only in the progressing tumor or muscle-invasive groups.

MATERIALS AND METHODS

Patients. Three groups of patients were selected from a clinical data and tissue bank from approximately 1000 patients followed for more than 3 years on average. The noninvasive group consisted of 23 patients (18 males and 5 females; median age, 73 years; age range, 42–83 years) who had at least three metachronous stage Ta tumors and did not have a tumor of higher stage during a median follow-up of 205 weeks (range, 72–218 weeks). The muscle-invasive tumor group consisted of 22 patients (20 males and 2 females; median age, 67 years; age range, 46–84 years) who had a stage T2 or higher stage tumor as their first bladder tumor ever. The progressing group consisted of 23 patients (17 males and 6 females; median age, 71 years; age range, 50–83 years) who had a stage Ta or T1 tumor as their first tumor and whose disease later progressed to a higher stage. We included both Ta and T1 to get a reasonable number of tumors. From each patient, tumor tissue and blood were collected if informed consent was obtained.

Material. Tumors were obtained fresh from surgery, frozen immediately, and stored at -80°C . DNA was extracted from tumor tissue and blood by using a Puregene DNA extraction kit (Gentra Systems, Minneapolis, MN) following the manufacturer's instructions. Because of possible normal tissue contamination, all invasive tumors without evident allelic losses were reanalyzed using microdissected tumor tissue. Microdissection was performed using $\times 100$

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³ The abbreviations used are: LOH, loss of heterozygosity; MIN, microsatellite instability.

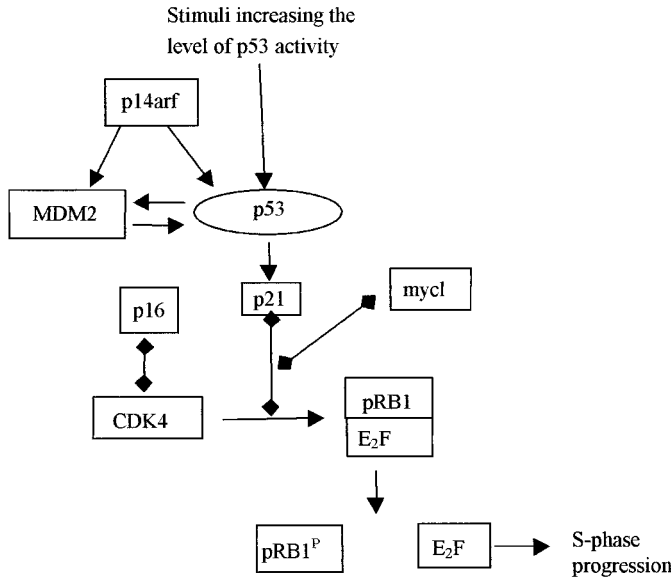


Fig. 1. Cell growth-regulatory pathways examined in this study. The action of the p53 protein is stimulated by any kind of genotoxic stress. An increased level of active p53 protein stimulates p21 to stop activation of cyclin-dependent kinases, phosphorylation of rb1, and release of e2f, which leads to S-phase progression. p16 is an inhibitor of cyclin-dependent kinases, leading to decreased release of e2f and cell cycle arrest. p14 binds to mdm2 and stabilizes p53. MYCL is a proto-oncogene encoding a protein that, among other actions, inhibits p21, leading to decreased inhibition of e2f release and thus S-phase progression. This model of cell growth regulation is simplified, and new knowledge of genes involved is still being acquired. Revised from Ref. 32.

magnification in a microscope and 4–10- μ m-thick serial sections of paraffin-embedded tumors. Microdissected tumor areas were deparaffinized by heating to 70°C, followed by rinsing in Xylo. DNA was extracted as described above.

LOH Detection. DNA from tumor and blood was analyzed for allelic deletions and MIN by using microsatellite markers. Microsatellites were chosen near growth-regulatory genes using information from the National Center for Biotechnology Information database Genemap 98 or based on published data (23, 24). Sequences of the primers used are listed in Table 1. Fluorescence-labeled primers were purchased from Hobolt DNA Synthesis (Hillerød, Denmark) and DNA Technology (Aarhus, Denmark).

PCR Amplification. PCR reactions were carried out in a 19- μ l volume containing 100–200 ng of purified genomic DNA; 2 pmol of each primer; 50 mM KCl; 10 mM Tris (pH 9.0); 1.5 mM MgCl₂; dATP, dTTP, dCTP, and dGTP (132 mM each); and 0.5 unit of Taq DNA polymerase. The reaction mixture was subjected to 5 min of denaturing at 95°C and 30–40 cycles of 95°C for 0.5–1.5 min, 50°C–63°C for 45 s to 1.5 min, and 72°C for 1.5 min. The final cycle was followed by a step at 72°C for 5 min. PCR conditions were optimized according to the sequence of the primers, and PCR was carried out in a MJ Research Thermocycler (PTC 200/PTC 225).

Electrophoresis. PCR products were analyzed by electrophoresis on 4.75% polyacrylamide/6 M urea denaturing gels using an ABI Prism 377 DNA sequencer and Genescan software (3 h, 51°C, 2.96 V).

Interpretation of Data and Definition of Terminology. MIN was defined as the presence of significant new bands, following PCR amplification of tumor DNA, that were not present in corresponding normal DNA. Tumors with MIN were excluded from allelic deletion examination. Allelic deletion was defined as loss of a significant part of one allele in tumor DNA compared with the corresponding normal DNA. This was determined by comparing the area under the curve for the two alleles from tumor and normal DNA in the curves obtained from the Genescan program. To determine the between-run variation, we repeated the entire procedure on 10 samples of corresponding tumor and normal DNA for the first six primer sets. We normalized the areas by dividing the ratio between tumor alleles with the ratio between normal alleles [(tumor allele 1:tumor allele 2)/(blood allele 1:blood allele 2)]. Based on these data, the variation of each amplicon was defined, and a significant loss was defined as a difference of more than 3 SDs between the allelic ratio in tumor and the allelic ratio in blood. Determinations of area under the curve in the first six primer sets were compared with a visual scoring by two independent observers

and showed a high degree of correlation (93%). Therefore, we decided to use visual scoring by two independent and experienced observers on the rest of the amplicons. All cases with allelic deletions or MIN in primary analysis were confirmed by repetition of the complete assay and rescored.

To analyze the importance of the number of genomic deletions in a single tumor, we divided the material into tumors having only one or no deletion, (low frequency) and tumors having two or more deletions (high frequency).

Statistics. Two different tests were used. For samples larger than 50 and figures expected to be larger than 5, we used the χ^2 test. For smaller samples and figures, we used the two-sided Fisher's exact test. When one locus was analyzed with more than one primer, the combined result was counted as one event.

RESULTS

The deletion of alleles was examined at the following loci in each tumor: TP53 (two markers); MDM2; CDKN1A (p21); MYCL; CDKN2A (p16 and p14arf); RB1; and E2F (Fig. 2). A pronounced difference in the frequency of allelic deletion was found between the noninvasive group of tumors and the muscle-invasive group of tumors. Seven of the 23 noninvasive tumors showed deletion of at least one locus compared with 19 of 22 muscle-invasive tumors (Table 2). In the muscle-invasive tumors, an average of 1.6 loci were deleted compared with 0.3 loci in the tumors of the noninvasive group ($P = 0.0000002$, χ^2 test). If a value of ≥ 2 deletions was used to define tumors with frequent deletions, then the number of tumors with frequent deletions amounted to 11 of 22 muscle-invasive tumors and only 1 of 23 noninvasive tumors ($P = 0.001$; two-sided Fisher's exact test; Table 2) because tumor 4 from patient 60 was the only one that lost both a TP53 and an E2F allele in the latter group. It was remarkable, however, that half of the muscle-invasive tumors had only one deletion (eight tumors) or no deletions (three tumors; Table 2).

In the muscle-invasive tumors, different combinations of deletions were detected from deletion of one RB1 allele to deletion of TP53, CDKN1A, CDKN2A, and RB1 alleles in a single tumor. The RB1 and MYCL loci showed significantly more deletions in muscle-invasive tumors than in noninvasive ones [RB1, $P = 0.001$; MYCL, $P = 0.003$ (two-sided Fisher's exact test)]. CDKN2A, which is located at chromosome 9p, was deleted with a similar frequency in noninvasive (3 of 13 tumors) and muscle-invasive (6 of 17 tumors) tumors, as expected.

The muscle-invasive tumors could be separated into two groups with or without concomitant carcinoma *in situ*. The patients with concomitant lesions have a field disease with grade 2 dysplasia or

Table 1 List of primers

Gene locus	Microsatellite locus	CHR*	Primer sequence
E2F	DIS482	1	S CGC TTG CCC AGG ATT TG AS AGG GGG ACA CTT GCC G
MYCL	DIS2743	1	S GAT GGG GTT TCA CTG TAG C AS TGA CCC AAA TCT TGA ACA GGA AT
MYCL	MYCL	1	S TGG CGA GAC TCC ATC AAA G AS CTT TTT AAG CTG CAA CAA TTT C
CDKN1A	D6S291	6	S CTC AGA GGA TGC CAT GTC TAA AAT A AS GGG GAT GAC GAA TTA TTC ACT AAC T
CDKN2A	IFNA	9	S GTA AGG TGG AAA CCC CCA CT AS TGC GCG TTA AGT TAA TTG GTT
MDM2	D12S80	12	S CCA GCC TGG AAT GAT ATG TA AS GAA TGT CAA TGG ACC AGA TG
CDK4	D12S1691	12	S GGT AAA CAC TGA GAC ACG CC AS TGA TGA CNC AGA AGT TGA GC
RB1	RB	13	S CTC CTC CCT ACT TAC TTG T AS AAT TAA CAA GGT GTG GTG G
P53	P53i1	17	S CTT GTA GTC CTA GCT ACT CAG CA AS CAA AAC ATC CCC TAC CAA AC
P53PCR	P53	17	S AGG GAT ACT ATT CAG CCC GAG GTG AS ACT GCC ACT CCT TGC CCC ATT C

* CHR, chromosome number; S, sense primer; AS, antisense primer.

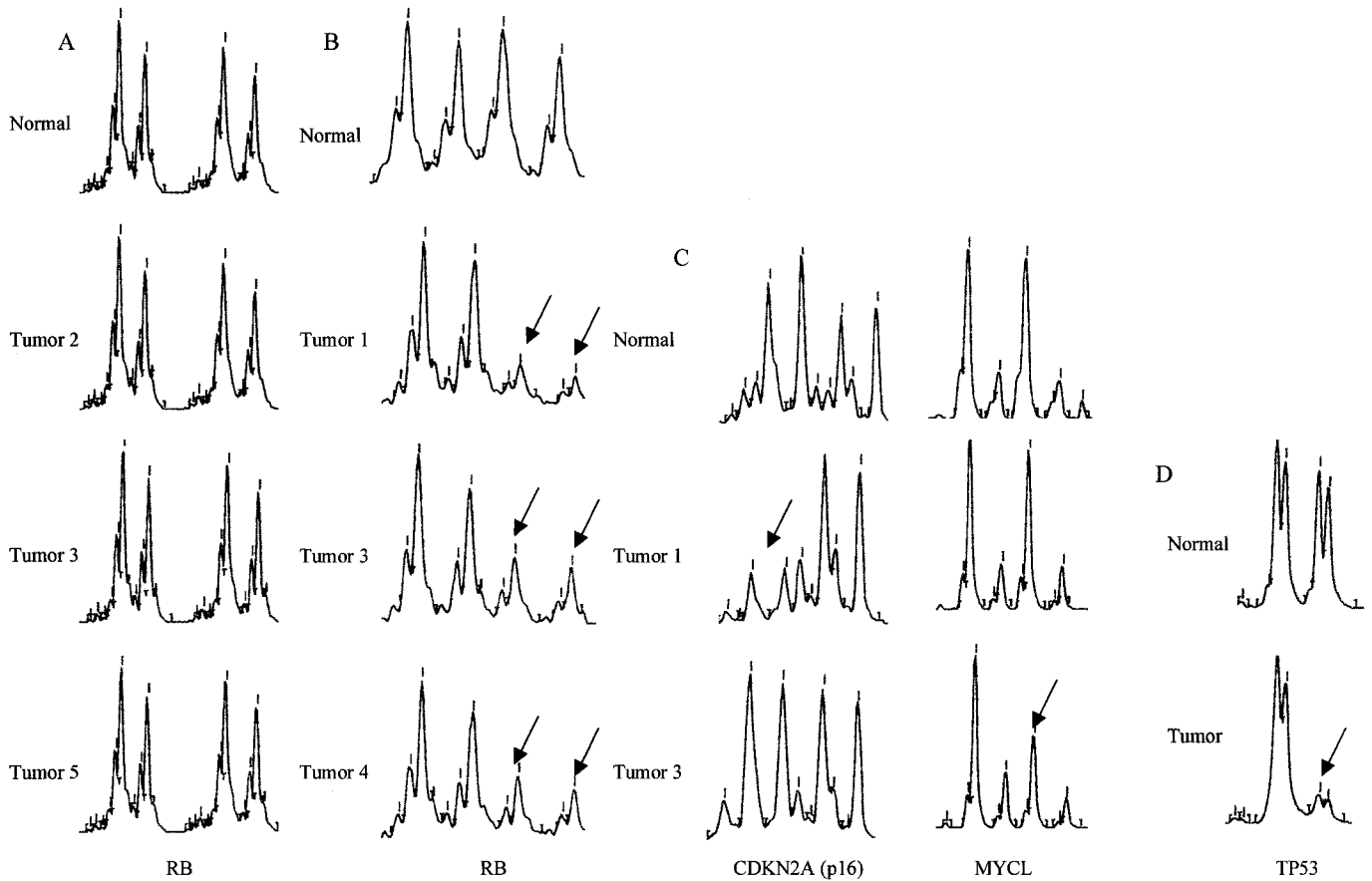


Fig. 2. Examples of electropherograms of microsatellites from tumors and corresponding blood. Each vertical row represents normal and tumor DNA from metachronous tumors in the same patient. A, gene locus *RB1* in patient 157; both alleles are retained in all three tumors. B, gene locus *RB1* in patient 172; allelic deletion occurs in all three tumors. C, patient 679; allelic deletion of *CDKN2A* (p16) is seen in tumor 1 (first row), and allelic deletion of *MYCL* is seen in tumor 3 (second row). D, gene locus *TP53* in patient 157; allelic deletion is seen in the tumor.

carcinoma *in situ*/invasive carcinoma in selected site biopsies. As a novel finding, we observed that the deletions of *MYCL* and *RB1* alleles were more pronounced in patients having field disease because 11 of 14 informative cases showed deletions compared with 2 of 8 cases without field disease ($P = 0.04$, two-sided Fischer's exact test). All tumors with more than two deletions had concomitant field disease (Table 2; Fig. 3).

Because of the significant difference between the noninvasive and muscle-invasive tumors, we decided to examine losses of *TP53*, *CDKN2A*, *MYCL*, and *RB1* in a group of patients with progressing tumors and in patients with recurrent noninvasive tumors (Table 3). As a novel finding, we demonstrate that the number of patients with allelic deletions of *RB1* in any tumor was significantly higher in the progressing group (7 out of 21 informative cases) than in the recurrent Ta group (0 out of 17 informative cases; $P = 0.02$, two-sided Fisher's exact test). For the *TP53* locus, 12 of 17 versus 3 of 19 informative patients had deletions ($P = 0.002$, two-sided Fisher's exact test). We compared all Ta tumors later developing into invasive tumors with tumors from patients with recurrent Ta tumors and found allelic deletion of *RB1* in 2 of 11 tumors in the progressing group versus 0 of 43 tumors in the recurrent noninvasive group ($P = 0.076$, two-sided Fisher's exact test). For *TP53*, the numbers were 4 of 9 versus 4 of 46 ($P = 0.037$, two-sided Fisher's exact test). The most striking difference was that *RB1* never showed deletions in the group with recurrent noninvasive tumors. The *CDKN2A* locus and *MYCL* were deleted to the same extent in both groups. The deletions of *RB1* were present from the beginning of the progressive disease course in all but

one case, as was *TP53* except in three cases. Deletions of alleles at these two loci could be important predictors for the disease course. Deletions were not detected at all visits in some cases (Table 3). This might indicate different tumor cell populations. As shown by case 679 in the recurrent noninvasive group, different populations of tumor cells seemed to be present because the *CDKN2A* locus showed deletion in tumor 1 and a normal pattern in tumor 3, whereas the *MYCL* gene locus showed the opposite pattern (Fig. 2C). Seven of the 23 patients that showed progression had no deletions at all of the examined loci. Intervals between recurrences were similar in the two groups. An average of 236 days passed between the recurrence of noninvasive tumors in the group that did not progress, and an average of 217 days passed from the last Ta or T1 tumor to the first tumor of a higher stage in the progressing group.

If we define the simultaneous deletion of two or more gene loci in *TP53*, *MYCL*, *RB1*, and *CDKN2A* as a high frequency of deletions, then 10 patients showed a high frequency of deletion in the progressing group, and only 1 patient showed such a high frequency in the recurrent noninvasive group. The number of tumors with a high frequency of deletion differed markedly between the groups examined (Fig. 3) but was always 50% or less of examined tumors.

DISCUSSION

We have studied the allelic deletion of genes involved in cell cycle regulation in a well-characterized clinical material consisting of bladder cancer patients with stable or progressing tumors. We found a

Table 2 Clinical and genetic profiles for noninvasive and muscle-invasive tumors

PT, patient number; VISIT, visit number in mob project; FIELD, whether or not the patient had corresponding field disease; CIS, carcinoma *in situ*; FD, frequency of deletions. High (H) if two or more loci showed deletions; Low (L) if zero or one loci showed deletion.

Group 1: Non invasive tumors with no known disease progression

STAGE	PT/VISIT	Gene locus ^a								FD
		TP53	MDM2	CDKN1A	MYCL	CDKN2A	Rb1	E2F		
pTa gr 2	3/4	○	○	○	○	○	○	○	○	L
pTa gr 2	32/3	○	○	○	○	○	○	○	○	L
pTa gr 2	38/4	○	○	○	○	○	○	○	○	L
pTa gr 1	58/2	○	○	○	○	○	○	○	○	L
pTa gr 2	60/4	○	○	○	○	○	○	○	○	H
pTa gr 2	73/3	○	○	○	○	○	○	○	○	L
pTa gr 3	74/1	○	○	○	○	○	○	○	○	L
pTa gr 0	92/6	○	○	○	○	○	○	○	○	L
pTa gr 2	115/2	○	○	○	○	○	○	○	○	L
pTa gr 2	154/5	○	○	○	○	○	○	○	○	L
pTa gr 1	157/2	○	○	○	○	○	○	○	○	L
pTa gr 2	166/5	○	○	○	○	○	○	○	○	L
pTa gr 2	252/4	○	○	○	○	○	○	○	○	L
pTa gr 2	269/2	○	○	○	○	○	○	○	○	L
pTa gr 2	272/2	○	○	○	○	○	○	○	○	L
pTa gr 2	327/6	○	○	○	○	○	○	○	○	L
pTa gr 2	335/3	○	○	○	○	○	○	○	○	L
pTa gr 2	424/3	○	○	○	○	○	○	○	○	L
pTa gr 3	576/1	○	○	○	○	○	○	○	○	L
pTa gr 2	597/1	○	○	○	○	○	○	○	○	L
pTa gr 2	662/1	○	○	○	○	○	○	○	○	L
pTa gr 2	679/1	○	○	○	○	○	○	○	○	L
pTa gr 2	716/1	○	○	○	○	○	○	○	○	L

Group 2: Primary muscle invasive tumors

STAGE	PATIENT	Gene locus ^a								FD	FIELD
		TP53	MDM2	CDKN1A	MYCL	CDKN2A	Rb1	E2F			
pT3 gr3	131	○	○	○	○	○	○	○	○	H	0
pT3b gr3	265	○	○	○	○	○	○	○	○	H	0
pT2 gr3	318	○	○	○	○	○	○	○	○	L	0
pT3 gr3	341	○	○	○	○	○	○	○	○	H	0
pT4b gr4	378	○	○	○	○	○	○	○	○	L	0
pT2 gr3	444	○	○	○	○	○	○	○	○	H	0
pT3b gr3	566	○	○	○	○	○	○	○	○	L	0
pT2-4 gr3	770	○	○	○	○	○	○	○	○	L	0
pT4b gr3	51	○	○	○	○	○	○	○	○	L	CIS
pT4b gr3	120	○	○	○	○	○	○	○	○	L	CIS
pT3b gr3	292	○	○	○	○	○	○	○	○	L	CIS
pT4 gr4	377	○	○	○	○	○	○	○	○	L	CIS
pT3 gr4	451	○	○	○	○	○	○	○	○	H	CIS
pT4b gr4	456	○	○	○	○	○	○	○	○	L	CIS
pT2 gr3	538	○	○	○	○	○	○	○	○	H	CIS
pT2 gr3/4	563	○	○	○	○	○	○	○	○	L	CIS
pT4a gr3	564	○	○	○	○	○	○	○	○	H	CIS
pT3 gr4	650	○	○	○	○	○	○	○	○	H	CIS
pT2-4 gr4	733	○	○	○	○	○	○	○	○	H	CIS
pT2-4 gr3	427	○	○	○	○	○	○	○	○	H	GRADE 2
pT2 gr3	697	○	○	○	○	○	○	○	○	H	GRADE 2
pT3b gr3	362	○	○	○	○	○	○	○	○	L	INV

○ No deletion/retention of heterozygosity
 ○ Homozygote or instable (non-informative)
 ● Allelic deletion
 ○ Not examined on microdissected tissue

^a Gene loci examined by the following microsatellites: TP53: P53i1 and p53PCR; MDM2: D12S80; CDKN1A (p21): D6S291; MYCL: MYCL and D1S2743; CDKN2A (p16): IFNA; RB1: RB; E2F: D1S482.

significantly higher frequency of allelic deletions in high-stage *versus* low-stage tumors and, as a novel finding, in progressing *versus* recurrent noninvasive disease. The genes most often affected by allelic deletions in muscle-invasive tumors were *TP53*, *RB1* and, as a novel finding, *MYCL*. Dichotomizing the patients into those with tumors having a high frequency of deletions (two or more deletions) and those with a low frequency of deletions showed, for the first time, a remarkable overrepresentation of high-frequency deletions in progressing tumors. Although allelic deletions were frequent in certain patient groups, some patients in each group showed a complete absence of deletions, indicating that the muscle-invasive or progressing tumors might consist of two different groups, one with allelic deletions and one with other characteristics.

The higher frequency of allelic deletions in muscle-invasive tumors

corresponds well with the findings by other authors that mutations, allelic loss, and abnormal expression of the p53 protein are more pronounced in high-stage tumors than in low-stage tumors (9, 10, 12). Allelic loss was found mainly in *RB1*, *MYCL*, *CDKN2A*, and *TP53* and was rare for the genes *MDM2* and *E2F*.

mdm2 up-regulation is expected to be unfavorable because the protein abrogates the growth suppression function of p53. In human breast cancer, *mdm2* overexpression was seen in 24 of 33 cases (25). In bladder cancer, a positive correlation between p53 accumulation and *mdm2* overexpression was shown, but *mdm2* overexpression alone had no prognostic significance (13). The *CDKN2A* gene encodes cell cycle-regulatory proteins p16 and p14arf, which share one exon with different reading frames. Both are tumor inhibitors; p14 binds to *mdm2* and inhibits p53 degradation, and p16 binds to cyclin-

Table 3 Clinical and genetic profiles for tumors from patients with progressing and recurrent noninvasive tumors

The table only shows selected tumors from each patient based on availability of tissue and the absence of urinary tract infection. Furthermore, a selection of last noninvasive, first invasive for progressing group was used. PT, patient number; VISIT, visit number; FD, frequency of deletions. High (H) if two or more out of four loci showed deletions. Low (L) if zero or one loci showed deletion.

STAGE	PT/VISIT	Gene locus ^a				FD	STAGE	PT/VISIT	Gene locus ^a				FD
		CDKN2A	MYCL	Rb1	TP53				CDKN2A	MYCL	Rb1	TP53	
pTa gr1	13/3	●	○	○	●	H	pTa gr2	3/2	▨	○	○	○	L
pT1 gr2	13/4	●	○	○	○	L	pTa gr2	3/3	▨	○	○	○	L
							pTa gr2	3/4	▨	○	○	○	L
pTa gr2	18/1	○	○	○	○	L	pTa gr1	32/1	○	○	○	○	L
pT2-4 gr3	18/7	○	○	●	○	L	pTa gr2	32/3	○	○	○	○	L
							pTa gr1	32/5	○	○	○	○	L
pTa gr2	31/2	▨	○	▨	○	L							
pT2-4 gr3	31/3	▨	○	▨	○	L	pTa gr2	38/4	○	▨	▨	▨	L
							pTa gr2	38/6	○	▨	▨	▨	L
pT1 gr3	112/3	●	○	○	●	H							
pT1 gr3	112/5	○	○	○	●	L	pTa gr1	58/2	○	○	○	○	L
							pTa gr2	58/5	○	○	○	○	L
pTa gr3	139/1	▨	▨	▨	●	L	pTa gr1	58/6	○	○	○	○	L
pT1 gr3	139/3	▨	○	○	●	L							
							pTa gr2	60/4	○	○	○	●	L
pTa gr3	172/1	○	○	●	○	L	pTa gr2	60/6	○	○	○	○	L
pT1 gr3	172/3	○	○	○	●	H							
pT3 gr3	172/4	○	○	●	●	H	pTa gr1	73/1	○	○	○	○	L
							pTa gr2	73/3	○	○	○	○	L
pT1 gr3	225/3	▨	○	▨	●	L							
pT1 gr3	225/4	▨	○	▨	●	L	pTa gr3	74/1	▨	○	○	○	L
pT3 gr3	225/5	▨	●	▨	●	H							
							pTa gr2	92/1	○	○	○	○	L
pTa gr3	237/4	▨	○	●	●	H	Ta gr0	92/6	○	○	○	○	L
pT2?	237/6	▨	○	●	●	H	pTa gr2	92/8	●	○	○	○	L
pT1 gr3	245/1	●	○	●	▨	H	pTa gr2	115/2	●	▨	○	○	L
pT1 gr3	245/3	●	○	●	▨	H	pTa gr2	115/4	●	▨	○	○	L
pT3 gr3	245/5	●	○	●	▨	H	pTa gr2	115/7	○	▨	○	○	L
pTa gr2	255/1	▨	○	○	▨	L	pTa gr2	154/1	○	○	○	○	L
pT1 gr3	255/5	▨	○	○	▨	L	pTa gr2	154/5	○	○	○	○	L
							pTa gr2	154/6	○	○	○	○	L
pT1 gr3	365/1	○	○	○	▨	L							
pT1 gr3	365/2	○	○	○	▨	L	pTa gr1	157/2	▨	▨	○	●	L
pT2-4 gr3	365/3	○	○	○	▨	L	pTa gr2	157/3	▨	▨	○	●	L
pTa gr3	365/4	○	○	○	▨	L	pTa gr2	157/5	▨	▨	○	○	L
pTa gr2	397/1	○	○	▨	▨	L	pTa gr2	166/5	▨	○	▨	○	L
pTa gr3	397/5	○	○	▨	▨	L							
pT1 gr3	397/6	○	○	▨	▨	L	pTa gr1	252/1	▨	○	▨	▨	L
pT1 gr2	397/7	▨	▨	▨	▨	L	pTa gr2	252/4	▨	○	▨	▨	L
pTa gr2	463/1	○	○	○	○	L	pTa gr2	269/2	○	○	○	▨	L
pT1 obs	463/6	●	○	○	○	L	pTa gr2	269/4	○	○	○	▨	L
pT1 gr3	463/9	●	○	○	○	L	pTa gr2	269/6	○	○	○	▨	L
							pTa gr2	269/7	○	○	○	▨	L
pT1 gr3	501/1	▨	▨	○	○	L							
pTa gr3	501/3	▨	▨	○	○	L	pTa gr2	272/1	○	○	○	○	L
pT2 gr3	501/5	▨	▨	○	●	L	pTa gr2	272/2	○	○	○	○	L
							pTa gr1	272/4	○	○	○	○	L
pT1 gr3	606/1	○	○	○	●	L	pTa gr1	272/5	○	○	○	○	L
pT3 gr?	606/2	○	○	○	●	L							
							pTa gr2	327/6	▨	▨	○	○	L
pT1 gr2	607/1	●	○	●	○	H	pTa gr2	327/8	▨	▨	○	○	L
pT2-4 gr3	607/2	●	○	●	○	H							
							pTa gr2	335/1	▨	○	▨	○	L
pT1 gr3	621/1	○	○	○	○	L	pTa gr2	335/3	▨	○	▨	○	L
pT1 gr?	621/3	○	○	○	○	L	pTa gr2	335/4	▨	○	▨	○	L
pT4 gr?	621/4	●	○	○	●	H							
							pTa gr2	424/3	○	○	○	○	L
pT1 gr3	638/1	○	▨	○	○	L							
pT1 gr3	638/3	○	▨	○	○	L	pTa gr3	576/1	●	○	○	▨	L
pT2 gr3	638/4	○	▨	○	○	L	pTa gr2	576/4	●	●	○	▨	H
pT1 gr3	704/1	○	●	●	●	H	pTa gr2	597/1	▨	○	○	○	L
pT2 gr3	704/2	○	●	○	●	H	pTa gr2	597/4	▨	○	○	○	L
pT1 gr3	744/1	▨	▨	●	●	H	pTa gr2	662/1	○	○	▨	●	L
pT1 gr3	744/2	▨	▨	●	○	L	pTa gr2	662/2	○	○	▨	○	L
pT2-4 gr3	744/3	▨	▨	○	●	H	pTa gr2	662/3	○	○	▨	○	L
pTa gr?	781/1	▨	▨	○	●	L	pTa gr2	679/1	●	○	○	○	L
pT1 gr3	781/2	▨	●	○	○	L	pTa gr2	679/3	○	●	○	○	L
pTa gr2	789/2	○	○	○	▨	L	pTa gr2	716/1	○	○	▨	○	L
pT1 gr2	789/3	○	○	○	▨	L	pTa gr2	716/3	○	○	▨	○	L
pTa gr2	846/1	○	○	○	▨	L							
pT2-4 gr3	846/2	○	○	○	▨	L							

○ No deletion/retention of heterozygosity
 ▨ Homozygote or unstable (non informative)
 ● Allelic deletion
 ○ Not examined on microdissected tissue

^a Gene loci examined by the following microsatellites: CDKN2A (p16): IFNA; MYCL: MYCL; RB1: RB; TP53: p53i1 and P53PCR.

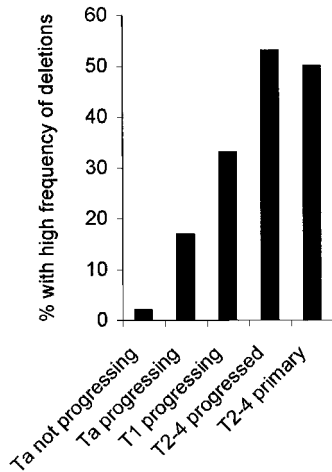


Fig. 3. Percentage of tumors within each patient group showing allelic deletion of two or more of four genes (*TP53*, *RBI*, *MYCL*, and *CDKN2A*). *Ta progressing* and *T1 progressing* represent the last tumor of that stage before progression.

dependent kinase 4 (Fig. 1; Ref. 26). A microsatellite at the *IFNA* locus is commonly used to investigate the *CDKN2A* locus (27, 28); however, deletions may occur that affect only the *CDKN2A* gene and not *IFNA*. Based on this, our findings concerning *CDKN2A* may underestimate the frequency of deletions of the *CDKN2A* gene. The tumor inhibitor p21, like p16, works through inhibition of cyclin-dependent kinases, abrogating the phosphorylation of *rb1* and arresting the cell cycle at the G_1 checkpoint. The *CDKN1A* gene is directly transcribed by p53 and is related to morphological indicators of cell proliferation. Its relation to prognosis in bladder cancer is ambiguous (15, 16). In a previous publication (17), the *CDKN2A* gene was deleted in 13 of 140 bladder tumors, all of which had small defined deletions at 9p21.

The *E2F* gene product is a transcription factor that promotes G_1 progression. Loss or inactivation of this protein could, in theory, arrest the cell cycle, but in a recent publication (18), lack of the *e2f* protein was related to disease progression, and *e2f* inactivation may be related to replication errors (29). The *rb1* protein binds to *e2f* and thus causes cell cycle arrest. Individuals with germ-line mutations in the *RBI* gene are at high risk of developing retinoblastoma and other cancers (30). Altered expression of *rb1* is associated with invasive bladder tumors (31) and bladder cancer recurrence (19), and the alteration of p53 and *rb1* in the same tumor is associated with tumor progression (20). The role of *MYCL* is difficult to interpret. The *MYCL* gene is a proto-oncogene. Among other mechanisms, it might work through inhibiting the p21-mediated inhibition of *rb1* phosphorylation (32). Another member of the *myc* family, *C-MYC*, plays a role in p53-dependent apoptosis (33). The role of *MYCL* is less clear because it accelerates apoptosis after interleukin 3 withdrawal, whereas overexpression produces resistance to cytotoxic drugs (34). We found one previous publication (35) on *MYCL* and bladder cancer in which the authors concluded that the *MYCL* genotype was not a prognostic factor in bladder cancer. In our material, allelic deletion of *MYCL* was associated with high-stage tumors, possibly indicating a growth advantage for cells with only one *MYCL* allele.

Lack of p53 protein is supposed to lead to increased cell division because the *CDKN1A* gene will not be encoding p21 protein, and there will be increased action of cyclin-dependent kinases and release of *e2f*, leading to S-phase progression and possible tumor growth. This will be enhanced by the lack of p16 protein, which is needed to stop the cyclin-dependent kinase-dependent phosphorylation of *rb1* and release of *e2f*. As a result, loss of *TP53*, *CDKN1A*, *CDKN2A*, and *RBI*

may give the cell a growth advantage, whereas loss of *E2F* and *MDM2* may stop S-phase progression and possible tumor growth (32). It was characteristic in our material that *E2F* and *MDM2* were rarely hit by deletions compared with the other genes, indicating that almost only unfavorable deletions leading to increased S-phase progression were present.

However, if the deletions we observed have any biological importance, they should influence the amount or quality of the gene products. This could be due to either a gene dose effect or inactivation of the remaining allele by a mutation or by methylation of CpG islands in the promoter region. The latter is a known mechanism that reduces the transcription of genes (36). There are some possibilities for underscoring deletions. We tried to rule out normal tissue contamination by microdissecting paraffin-embedded tissue in muscle-invasive tumors that showed no deletions when frozen tumor tissue was examined. Another possibility is too much template. Because almost all tumors showed some degree of deletion, it seems that saturation of the PCR process is no problem. Other authors have used 5–300 ng of template (37, 38). We conclude that allelic deletions of genes involved in cell growth regulation are of importance in the progression of bladder cancer. We detected a pronounced difference in the frequencies of deletions in noninvasive *versus* muscle-invasive tumors. If we define the simultaneous loss of two or more gene loci in *TP53*, *MYCL*, *RBI*, and *CDKN2A* as a high frequency of deletions, then 11 of 23 muscle-invasive tumors and only 1 of 23 noninvasive tumors showed this pattern. A striking new finding was that 10 patients in the progressing group and only 1 patient in the recurrent noninvasive group showed a high frequency of deletions. The differences were more pronounced for *RBI*, *MYCL*, and *TP53*. The number of tumors showing a high frequency of deletions was markedly different between the groups examined but was always 50% or less of examined tumors. Based on this, one might suggest that two different pathways may lead to bladder tumor progression, one in which deletion of alleles in cell cycle regulators is common and one in which such deletions do not occur.

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