REPORTS

Altered Patterns of MDM2 and TP53 Expression in Human Bladder Cancer

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Background: **The TP53 gene maps to the short arm of chromosome 17 (17p13.1) and encodes for a nuclear phosphoprotein of 53 kd (p53) involved in cell cycle control. The MDM2 gene is located on the long arm of chromosome 12 (12q13-14), and it encodes for a nuclear protein (Mdm2) of 90 kd of molecular mass. Genetic alterations in the TP53 gene have been reported as frequent events in bladder cancer and are associated with disease progression. The MDM2 gene has been shown to be amplified and overexpressed in sarcomas; however, these changes have not yet been analyzed in neoplastic lesions of the urinary bladder.** ***Purpose:*** **We undertook the present study in order to determine the frequency of MDM2 and TP53 abnormalities in bladder tumors, as well as to examine the clinical relevance of identifying their altered patterns of expression in patients affected with bladder cancer.** ***Methods:*** **We analyzed a cohort of 87 patients affected by bladder tumors. Altered patterns of expression of Mdm2 proteins were determined using an immunohistochemical assay with monoclonal antibody 2A10, and MDM2 gene amplifications were studied by Southern blotting. Mutant p53 proteins were identified using monoclonal antibody PAb1801. The presence of intragenic mutations in the TP53 gene were assessed utilizing single-strand conformation polymorphism and further characterized by sequencing. Associations were assessed statistically by the two-tailed Fisher's exact test.** ***Results:*** **Twenty-six of 87 cases had abnormally high levels of Mdm2 proteins; however, only one case showed an MDM2 amplification. Thirty-six of 87 cases displayed p53 nuclear overexpression. Sixteen cases had abnormally high levels of both Mdm2 and p53 proteins. There was a strong statistical association between Mdm2 and p53 overexpression (Fisher's exact test: $P = .018$). Moreover, there was a striking association between Mdm2 overexpression and low-stage, low-grade bladder tumors (Fisher's exact test: $P = .0005$).** ***Conclusions:*** **The results suggest that aberrant Mdm2 and p53 phenotypes are frequent events in bladder cancer and may be involved in tumorigenesis or tumor progression in urothelial neoplasias.** ***Implications:*** **This study is the first to report altered patterns of MDM2 expression in human bladder tumors and demonstrates that aberrant Mdm2 and p53 phenotypes may be important diagnostic and prognostic markers in patients affected by bladder cancer. [J Natl Cancer Inst 86:1325-1330, 1994]**

Bladder cancer is one of the most common malignancies occurring worldwide (*1*). The vast majority of these tumors are of epithelial origin (*2*). Based on morphological evaluation and natural history, urothelial neoplasia may be classified into two groups with distinct behavior and prognosis: 1) the low-grade papillary superficial tumors and 2) the high-grade papillary and nonpapillary lesions (*3*). Epidemiology and molecular studies (*4-6*) have revealed the involvement of chemical carcinogens and infectious agents as etiopathogenic factors in bladder cancer.

Molecular genetic and immunopathologic analyses of urothelial neoplasia have identified abnormalities in a number of chromosomes and genes that appear to be implicated in the development and progression of such tumors (*7-9*). The TP53 gene maps to the short arm of chromosome 17 (17p13.1) and encodes for a nuclear phosphoprotein of 53 kd (p53) involved in cell cycle control. Mutations of the TP53 gene are commonly found in bladder cancer and are associated with an aggressive clinical course (*10-13*). In addition to this molecular mechanism of TP53 inactivation, several viral oncoproteins may bind to p53 and eliminate its ability to function as a transcription factor and cell cycle checkpoint control (*14,15*). This is the case with the human papillomavirus (HPV) E6 oncoprotein (*16*). More recently, a cellular proto-oncogene product, the Mdm2 (*17,18*), has been shown to bind to p53 and act as a negative regulator, inhibiting its transcriptional transactivation activity. The MDM2 gene is located on the long arm of chromosome 12 (12q13-14), and it encodes for a nuclear protein (Mdm2) of 90 kd of molecular mass (*17,18*).

We undertook the present study in order to determine the frequency of MDM2 and TP53 abnormalities in bladder tumors, as well as to examine the clinical relevance of identifying their altered patterns of expression in patients affected with bladder cancer.

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See "Notes" section following "References."

Materials and Methods

Patient Characteristics and Tissues

A cohort of 90 patients with bladder tumors was initially selected for the present study. Three cases were excluded because of tissue characteristics or lack of clinical follow-up. Demographic data on this group may be summarized as follows: 67 patients were males and 23 were females, median age was 63.6 years, and median follow-up was 20 months (range 1-78). Tumor staging of the 87 evaluable cases was done according to the TNM (UICC) system (*19*): eight Ta, two Tis, 11 T1, and 66 T2+ (muscle invasive lesions). Eight tumors were classified as low grade (grade 1), 16 as intermediate grade (grade 2), and 63 as high grade (grade 3). Twenty-nine patients had received previous treatment prior to the time of surgery. This treatment consisted of chemotherapy for the majority.

We analyzed tumor tissues, which were obtained from the Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, N.Y., from all 87 patients. These tissues were embedded in cryopreservative solution (OCT compound; Miles Laboratories, Elkhart, Ind.), snap frozen in isopentane, and stored at –70 °C until used. Representative hematoxylin–eosin-stained sections of each block were examined microscopically to confirm the presence of tumor and to evaluate the percentage of tumor cells comprising these lesions (V. E. Reuter). DNA was extracted from 20-30 sections 30 µm thick from each of the blocks studied. Adjacent normal and tumor tissue specimens were also collected for molecular genetic assays in 40 of the 87 cases.

Monoclonal Antibodies and Immunohistochemistry

A panel of mouse monoclonal antibodies to Mdm2 and p53 proteins was used for the present study. The pattern of immunostaining of anti-Mdm2 antibody 2A10 was first assessed using 3T3-BALB/c and 3T3-DM cells. Antibody 2A10 identifies an epitope in the central portion of Mdm2 (*20,21*). Antibody PAb1801 (Ab-2; Oncogene Science, Manhasset, N.Y.) recognizes an epitope located between amino acids (aa) 32 to 79 of both wild-type and mutant human p53 proteins (*22*). MIgS-KpI, a mouse monoclonal antibody of the same subclass as the anti-Mdm2 and anti-p53 antibodies, was used as a negative control at similar working dilutions.

The avidin–biotin peroxidase method was performed on 5-µm-thick frozen tissue sections fixed with cold methanol–acetone (1:1 dilution). Briefly, sections were incubated for 15 minutes with 10% normal horse serum (Organon Tecknika Corp., Westchester, Penn.), followed by a 2-hour incubation with appropriately diluted primary antibodies (2A10 was used at 1:1000 dilution; PAb1801 or Ab-2 was used at 200 ng/mL). After the sections were washed extensively, they were subsequently incubated for 30 minutes with biotinylated horse anti-mouse IgG antibodies (1:200 dilution; Vector Laboratories, Burlingame, Calif.) and then incubated for 30 minutes with avidin–biotin–peroxidase complex (1:25 dilution; Vector Laboratories). Diaminobenzidine (0.06%) was used as the final chromogen and hematoxylin as the nuclear counterstain.

Immunohistochemical evaluation was done by at least two independent investigators who scored the estimated percentage of tumor cells that showed nuclear staining. Both Mdm2 and p53 nuclear immunoreactivities were considered positive when at least 20% of the tumor cells showed nuclear staining. The immunohistochemical analysis was done in a blinded fashion, without knowledge of the clinical information or molecular results.

Southern Blotting for MDM2 Amplification

DNA was extracted from paired normal and tumor samples by the nonorganic method developed by Oncor (Gaithersburg, Md.), digested with *Eco*RI enzyme, electrophoresed in 0.8% agarose gel, and blotted onto nylon membranes. The membranes were prehybridized with Hybrisol I (Oncor) at 42 °C for 1 hour, and hybridized with probes labeled to high specific activity with [^{32}P]deoxycytidine triphosphate overnight. A human MDM2 complementary DNA fragment probe (*21*) of 1.6 kilobase, pHDM (*Eco*RI), was used in Southern blots to assess gene amplification. A control probe (D12S2, *Eco*RI; American Type Culture Collection 57181) from the same chromosome as MDM2 was used to control for variations in sample loading and to control for the effect of nonspecific polysomies of chromosome 12 on the quantitation of gene copy number (*23*). Membranes were then washed and subjected to autoradiography using intensifying screens at –70 °C. Densitometry was performed using an Ultrascan XL Laser Densitometer (Pharmacia LKB Biotechnology, Piscataway, N.J.) to confirm the results. A case was considered to have an MDM2 amplification when it had at least five copies of the gene/cell.

SSCP Analysis and DNA Sequencing for TP53 Mutations

These studies were performed according to a slight modification of the method reported by Orita et al. (*24*). Amplifications were performed using 100 ng of genomic DNA extracted from the samples described above. The primers used were obtained from previously published (*25*) intronic sequences flanking exons 2 through 9 of the human TP53 gene. DNA was amplified following 30 cycles of polymerase chain reaction (PCR) (30 seconds at 94 °C, 30 seconds at 58 °C for exons 8 and 9, and 63 °C for exons 2-7, and finally 60 seconds for all samples at 72 °C) using a Thermal Cycler (Perkin Elmer Cetus, Foster City, Calif.). Amplified samples were then denatured and loaded onto a nondenaturing acrylamide gel containing 10% glycerol and run at room temperature for 12-16 hours at 10-12 watts. Gels were dried at 80 °C under vacuum and exposed to x-ray film at –70 °C for 4-16 hours.

Amplification of genomic DNA for sequencing assays was independent of that used for single-strand conformation polymorphism (SSCP) analysis, which used 35 cycles (60 seconds at 94 °C, 60 seconds at 58 °C and 63 °C as above, and 90 seconds at 72 °C). DNA fragments were isolated from 2% low melting point agarose gels, purified and sequenced by the dideoxy method (*26*). Both strands were sequenced for each DNA analyzed, and genomic DNA from control samples containing wild-type TP53 were sequenced in parallel to confirm the mutations.

Statistical Methods

Fisher's exact test (*27*) (two-tailed) was used to assess the association between clinicopathologic factors and altered patterns of MDM2- and TP53-encoded products.

Results

Overexpression of Mdm2 Proteins and MDM2 Amplification

A strong nuclear staining was seen in 3T3-DM cells, reported to have an amplified mdm2 gene and to overexpress the p90 protein. 3T3-BALB/c cells were unreactive and had very low levels of mdm2 proteins (data not shown).

Nuclear overexpression of Mdm2 proteins was observed in 26 of 87 (30%) evaluable cases (Fig. 1). Ten cases presented with Mdm2 nuclear immunoreactivities as the sole abnormality. Normal urothelium and mesenchymal elements, including endothelial cells and fibroblasts, were unreactive. An association was observed between tumors of lower stage and Mdm2-positive phenotype; while five of eight (62.5%) papillary superficial tumors (Ta) were Mdm2-positive, only 16 of 66 (24%) evaluable muscle invasive lesions (T2+) possessed that phenotype. Similarly, a significant association was found between low-grade tumors and Mdm2-positive phenotype: 50% of the low-grade (grade 1) tumors were positive, while only 17% of the high-grade (grade 3) tumors displayed Mdm2 immunoreactivities (Fisher's exact test: $P = .0005$).

Forty cases were analyzed for MDM2 amplifications, and in only one case was this molecular abnormality found (Fig. 2). This case corresponded to a patient affected with a pT1 transitional cell carcinoma and showed Mdm2 overexpression in all nuclei of tumor cells.

Overexpression of p53 and TP53 Mutations

p53 nuclear overexpression was observed in 36 of 87 (41%) cases (Fig. 1). Twenty cases had the p53-positive phenotype as the only abnormality observed. Molecular genetic analyses, using PCR–SSCP and sequencing, were per-

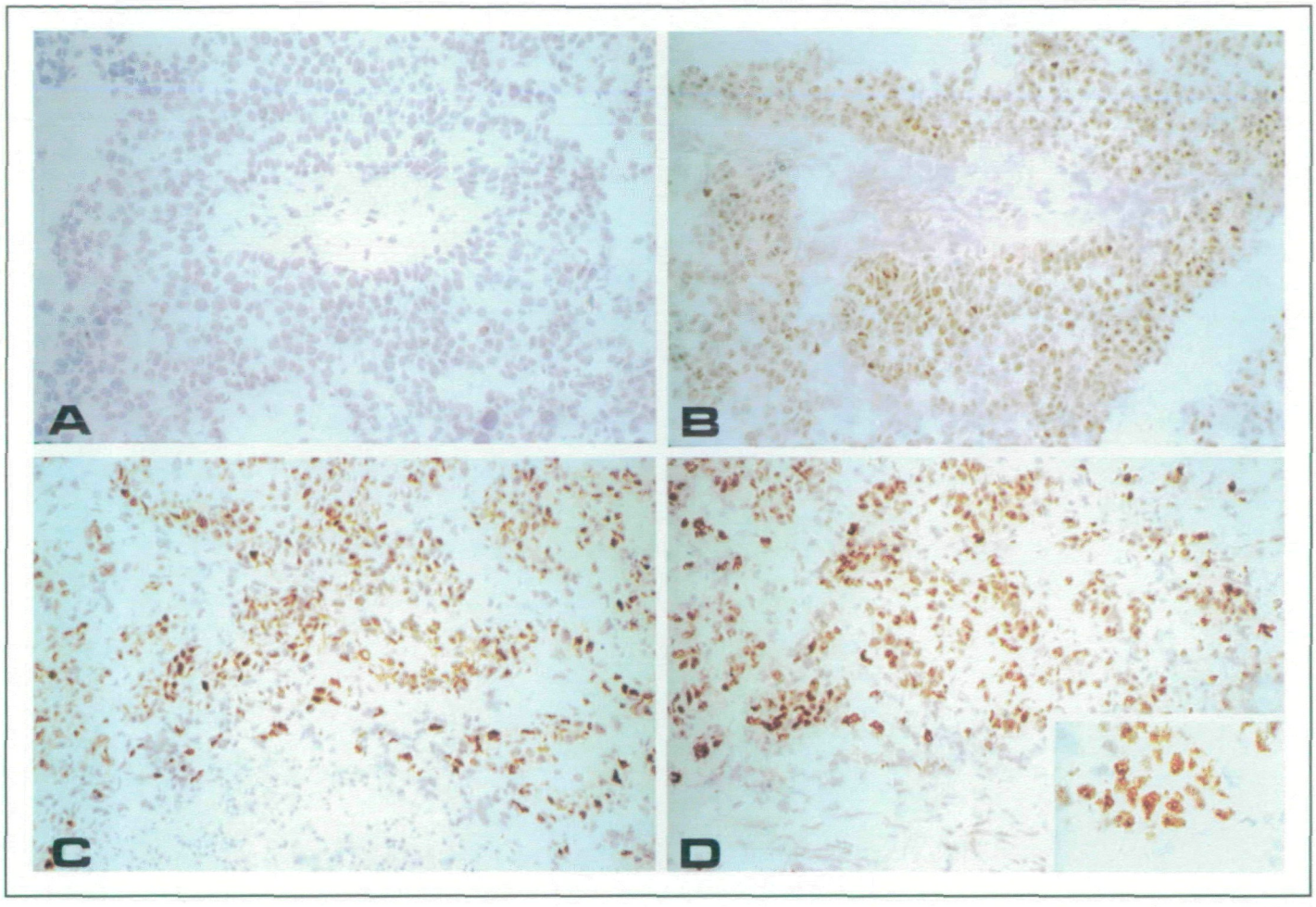

Fig. 1. Photomicrographs of two bladder tumors using immunohistochemical staining with antibodies PAb1801 (**A,C**) and 2A10 (**B,D**). Panel **A**: absence of staining with PAb1801 in a papillary superficial bladder tumor. Panel **B**: positive immunoreactivity with anti-Mdm2 antibody 2A10 in a consecutive section of the case illustrated in panel **A**. Panels **C** and **D**: an invasive bladder cancer displaying p53- and Mdm2-positive phenotypes, respectively. Note strong nuclear immunostaining with antibody 2A10 in insert of panel **D** (original magnification ×200).

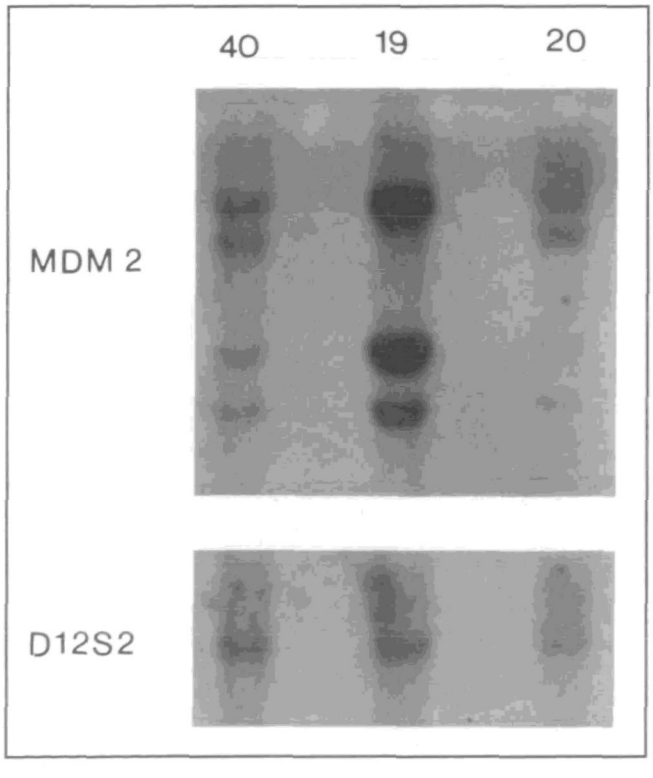

Fig. 2. Southern blot analysis of the MDM2 gene in bladder tumors. Southern blotting was performed using a complementary DNA fragment probe (pHDM, *Eco*RI), as described in the "Materials and Methods" section. A control probe on the same chromosomal arm (D12S2) was used in the analysis. The quantitative comparison of hybridization signal shows an MDM2 amplification in case 19, while cases 40 and 20 had no amplification.

formed in a subset of the tumors studied in order to correlate p53-positive phenotype and specific TP53 mutations (Fig. 3). Shifts in mobility and point mutations were identified and affected exon 5 (five cases), exon 7 (three cases), and exon 8 (six cases). We did not observe any abnormalities occurring in exons 2, 3, 4, and 9. All cases that displayed shifts in mobility by PCR–SSCP had a p53-positive phenotype.

Altered Genotype and Phenotype of MDM2 and TP53

Sixteen cases (18%) showed positive nuclear immunoreactivities for both Mdm2 and p53 proteins (Fig. 1). A strong statistical association was found when comparing the concomitant nuclear overexpression of Mdm2 proteins and p53 products (Fisher's exact test: $P = .018$). In order to better understand the possible correlation of TP53 mutations as they re-

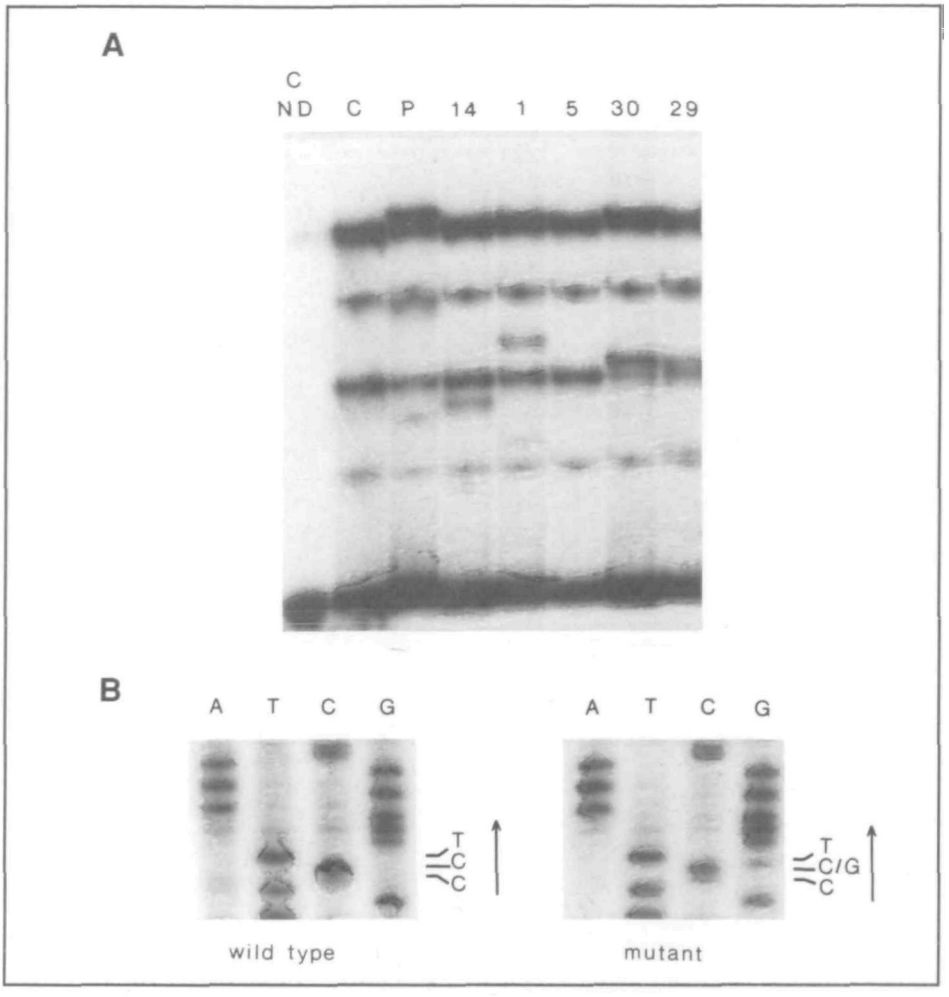

Fig. 3. Panel **A** illustrates intragenic TP53 mutations as detected by single-strand conformation polymorphism (PCR–SSCP) analysis of exon 8. Shifts in mobilities were observed for all cases, with the exception of case 5. CND = negative control (not denatured). C = negative control (single stranded). P = positive control. Panel **B** illustrates the direct sequencing of case 14, revealing a point mutation at codon 278 (proline to arginine).

late to p53 and Mdm2 phenotypes, we analyzed a group of 13 cases in which shifts in mobilities were observed for different exons of the TP53 gene. As stated above, all of these cases showed positive nuclear immunostaining for anti-p53 PAb1801 antibody. However, only those cases that possessed a mutation in exon 8 of the TP53 gene showed a positive Mdm2 phenotype. None of the seven cases that had mutations either on exon 5 or exon 7 rendered a positive nuclear staining using anti-Mdm2 2A10 antibody. In one case, we detected two mutations, affecting exons 7 and 8. This case was positive for p53 but unreactive for Mdm2. A strong association was found between TP53 mutations on exon 8 and an Mdm2-positive phenotype (Fisher's exact test: $P = .001$).

To evaluate the possible effect of chemotherapy in the subgroups described, we compared treated (n = 29) and untreated (n = 58) patients with tumor phenotypes. No statistically significant correlations between treated and untreated groups were seen, which may be due to the small sample size of the treated subgroup.

Discussion

Several etiopathogenic mechanisms have been postulated to be involved in the development of bladder tumors. These include primary molecular abnormalities of a candidate tumor suppressor gene in chromosome 9 (*9,28,29*), certain chemical carcinogens (*30*), and infection with high-risk HPV types 16 and 18 (*31,32*). On the other hand, TP53 mutations and altered patterns of p53 expression have been reported to occur in early stages of bladder carcinogenesis and to be associated with an aggressive biological behavior (*10,13*). In addition to the inactivation through germ-line or somatic mutations of the TP53 gene, several viral and cellular proteins have been shown to interact with p53 and alter its function (*14,16*). The p53 protein appears to operate as a transcription factor (*33*). The genes regulated by TP53 or the p53 protein itself appear to be involved in cell cycle progression (*34*), cell cycle checkpoint control after DNA damage (*35*), and the commitment of some cells to apoptosis (*36,37*). Very recently, the Mdm2 proto-oncogene product(s) has been shown to bind to p53 and inactivate its physiological role as a transcription factor and cell cycle regulator (*17,18*).

In the present study, we first analyzed the frequency of detecting altered patterns of Mdm2 and p53 expression as they occur in human bladder tumors. We observed that Mdm2 proteins were overexpressed in 32% of the evaluable cases. However, only one of these cases showed an MDM2 amplification. Moreover, a striking association was found between Mdm2-positive tumors and low-grade/low-stage lesions (62.5% of papillary superficial tumors versus 24% of muscle invasive lesions had a positive Mdm2-phenotype). On the other hand, only three of eight (37.5%) Ta tumors were positive for p53. Based on these data, one may postulate that different tumor phenotypes might be associated with different etiopathogenic mechanisms. Since p53 products are degraded via the ubiquitin-directed pathway by E6 oncoproteins encoded by HPV-16 (*38*), it might be expected that tumors with HPV infections would not accumulate wild-type p53 proteins. It follows from this assumption that Mdm2 proteins, which are transactivated by wild-type p53, would not be produced under these circumstances.

We then analyzed patterns of Mdm2 staining in tumors displaying a p53-positive phenotype that had a bona fide TP53 mutation. It was our working hypothesis that certain TP53 mutations would render inactive products, while others might maintain certain functions, mainly transactivation of the MDM2 gene. It has been reported that not only do some mutant p53 products conserve several functions, but they also acquire or "gain" specific properties, such as selective growth ad-

vantage and transactivation of certain genes that are not affected by the wild-type p53 (*39,40*).

We observed that all TP53 mutations affecting exons 5 and 7 rendered a Mdm2-negative phenotype. However, all exon 8 mutations showed an intense nuclear immunostaining with anti-Mdm2 2A10 antibody. Furthermore, a case that was characterized by the identification of two mutations, one in exon 7 and the other in exon 8, had a negative Mdm2-phenotype. These results suggest that certain mutants may be defective in certain functions while preserving transactivation properties of the wild-type p53 protein. It has been documented that the sequence-specific DNA binding of p53 that confers transactivation resides in the central region (*41*). Mutations affecting these binding sites, such as those occurring on exons 5 and 7, might render nonactive p53 products.

We were left with another unexpected phenotype: those tumors that were Mdm2-positive and p53-negative, without detectable TP53 mutations. Amplification of the MDM2 gene with overexpression of its encoded product would explain this phenotype (*42*). The alternative premise might be that chromosomal aberrations could cause sufficient DNA damage to alert the cellular machinery to overproduce wild-type p53. In turn, this elevation of wild-type p53, which is a transient event, could result in an increased expression of the p53-responsive gene MDM2. This phenomenon has been recently described in response to UV light (*43*) and irradiation (*44*), occurring in a p53-dependent manner. Since several of the tumors used for the present study were also screened for other molecular abnormalities in our laboratory (*9,45*), we were able to ascertain that all Ta cases with this phenotype had other chromosomal alterations, including loss of heterozygosity of chromosome 9 and/or instability at microsatellite loci.

Taken together, these observations have allowed us to postulate that specific Mdm2 and p53 phenotypes may reflect underlying biological mechanisms of tumorigenesis and tumor progression that occur in bladder cancer. Fig. 4 schematically illustrates possible carcinogenic mechanisms and expected phenotypes. Briefly, a double-negative phenotype might be associated with high-risk HPV infectious agents, such as viral types 16 and 18; nevertheless, it may be also encountered in cases with minimal chromosomal changes or genetic instability. In contrast, moderate-to-major chromosomal aberrations and microsatellite alterations would produce an increase of wild-type p53 that will result in an overexpression of Mdm2 proteins. Since wild-type p53 molecules have a very short half-life, these products would not be detectable; however, Mdm2 proteins appear to be chemically more stable and thus identifiable using immunohistochemically based assays. If these postulates are correct, detection of Mdm2 nuclear overexpression could be an important asset to the early diagnosis of bladder tumors. Finally, TP53 mutations may render either nonfunctional molecules or mutants that would maintain or even "gain" transactivation properties. It appears that mutations occurring in exon 8 do not affect the transcriptional activity of mutant p53 products upon the MDM2 gene.

This study is the first, to our knowledge, to report altered patterns of MDM2 expression in human bladder tumors. To date, MDM2 mutations and/or overexpression of its encoded product(s) have been documented in human sarcomas (*46-48*) and gliomas (*49*), but not in epithelial-derived tumors. This analysis also demonstrates that aberrant Mdm2 and p53 phenotypes are frequent events in bladder cancer. Furthermore, since concomitant alterations of TP53 and MDM2 in a given case may be synergistic and appear to be associated with tumor progression (*48*), they might become important prognostic adjuncts to our armamentarium of clinical variables and laboratory-based tumor markers. More studies are needed using well-characterized cohorts of patients, mainly those affected by superficial bladder tumors, in

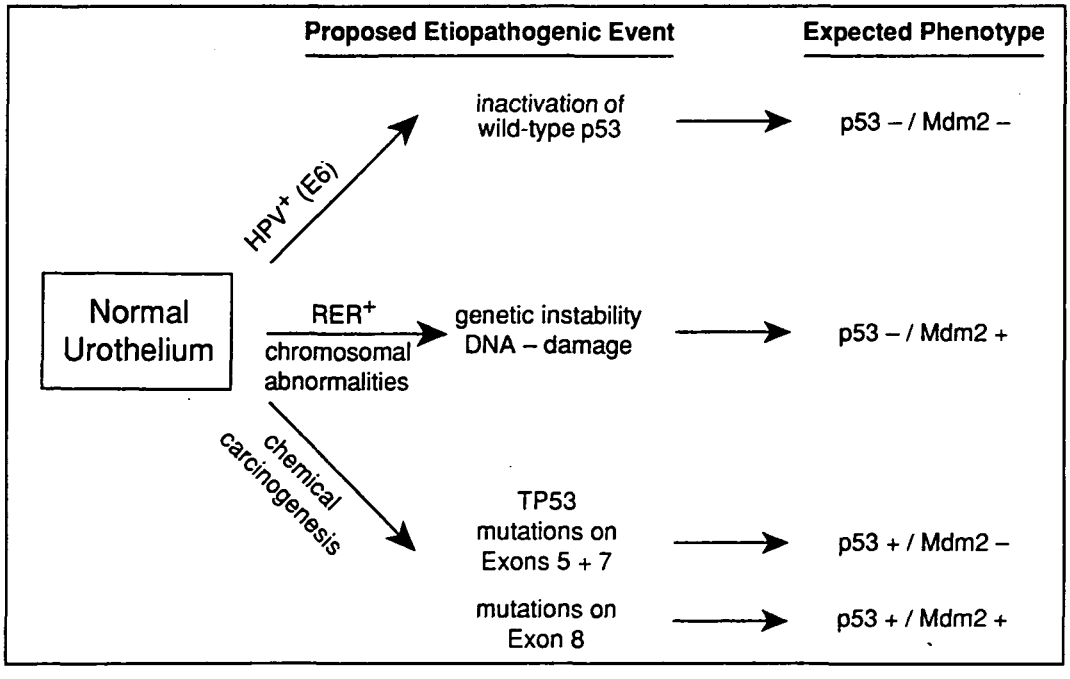

Fig. 4. Schematic illustration of observations that specific Mdm2 and p53 phenotypes may reflect underlying biologicial mechanisms of tumorigenesis and tumor progression that occur in bladder cancer. HPV = human papillomavirus. E6 = E6 oncoprotein positive. RER+ = replication error-positive phenotype.

order to confirm the working hypothesis and findings reported in this study.

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Notes

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