Genetic and Immunophenotype Analyses of TP53 in Bladder Cancer

TP53 Alterations Are Associated with Tumor Progression

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Abstract: Altered p53 status is a frequent event in bladder cancer and reported to have prognostic significance. We studied the *TP53* gene and its product in 76 patients affected with urinary bladder carcinomas by immunohistochemistry (mAb DO-7), polymerase chain reaction single-strand conformational polymorphism (exons 4-8) followed by direct sequencing of shifted bands, and loss of heterozygosity in 17p (p53CA). H-*RAS* mutations were also studied. The receiver operating characteristic curve and the logistic-regression analysis were used to evaluate the validity of immunohistochemistry in predicting *TP53* mutations. A p53-positive nuclear phenotype was defined by a cutoff of 20% tumor cells being immunoreactive and was found in 23 cases, while *TP53* mutations were detected in 22 cases, four of them with a negative p53 phenotype. *TP53* deletions were identified in 23 cases. No H-*RAS* gene mutations were observed. There was a significant association between phenotype and genotype results. Moreover, a significant association was observed between p53 status and tumor stage and grade, being alterations more common in high-stage and high-grade tumors (both χ^2 test; $P < .01$). Deletion of 17p significantly correlated with tumor stage ($P < .01$) and grade ($P = .01$), allelic losses being more common in advanced disease. Data from these studies suggest that genetic assays are necessary for the optimal determination of *TP53* alterations, mainly in tumors with a p53 negative phenotype, and especially in early stage tumors for which p53 status may assist in determining its progression to invasive disease. Since p53 alterations are significantly associated to clinicopathological features of poor prognosis, the inclusion of both p53 phenotype and TP53 mutation status into a predictive panel of tumor markers for bladder cancer is recommended.

Key Words: TP53 mutations, 17p allelic losses, immunohistochemistry, H-RAS, bladder cancer

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Transitional cell carcinoma is the most common histologic type of bladder cancer, superficial bladder tumors accounting for approximately 80% of all newly diagnosed bladder neoplasms.[1,2] Superficial bladder tumors have in general a good prognostic, with a 5-year survival rate of over 60%.[1] However, up to 10% to 15% of these cases will progress to muscle invasive or metastatic disease.

Genetic and immunopathological analyses of bladder cancer have identified abnormalities in a number of chromosomes and genes that appear to be implicated in the development and progression of such tumors. These include, among others, abnormalities of the chromosome 17p arm and the *TP53* suppressor gene, as well as activation of the H-*RAS* oncogene.[3]

The protein encoded by the *TP53* gene is a critical regulator of cell cycle progression, being also involved in apoptosis and preservation of genomic integrity. Loss of p53 function, most commonly through allelic deletion and point mutation of the contralateral allele within one of the evolutionary conserved domains, occurs in approximately half of most major cancers, including bladder tumors.[4] Different studies based on identification of p53 nuclear accumulation by immunohistochemical (IHC) assays, or *TP53* gene mutations by sequencing and gene-array analyses, have revealed the potential clinical implications of detecting p53 alterations and their association with bladder cancer progression and patient outcome.[5–8] Although this association is accepted, some discrepant studies have been reported.[9] These discrepancies might be due, at least in part, to differences in the methodology used to detect p53 alterations, as well as in the cohort of patients analyzed and the intrinsic nature of different carcinogenic exposures. Consequently, it seems desirable to standardize the methods to be used and to define the cutoff points of the distinct methodologies to establish a reliable system for the assessment of the p53 status. This issue is critical mainly when examining specific clinical populations in an attempt to conduct molecular epidemiology studies. In this context, the present analysis centers on a group of bladder cancer patients from the north-east part of

Spain. To the best of our knowledge, the database of p53 mutations maintained at the International Agency for Research on Cancer (IARC, Lyon, France), does not include TP53 genotypes in bladder cancer from that region or any other geographical location of Spain. Another aim of the study was to define the degree of concordance between different assays intended to detect *TP53* mutational spectrum and patterns of p53 expression. Associations between p53 alterations and standard histopathological variables were also analyzed as part of this study.

MATERIALS AND METHODS

Patient Characteristics and Tissues

A total of 76 consecutive patients (68 males and 8 females) were included in this prospective study, which dated from May 1999 to May 2002. All patients were diagnosed and treated in Barcelona, Spain. Median patient age was 69 ± 9.8 years (range 36-94). A total of 100 urothelial carcinomas were collected, corresponding to 58 lesions from patients with unique tumors, and 42 lesions from 18 patients with primary and recurrent tumors, but only one lesion selected at random from these patients was included in the present study. Five patients were treated by radical cystectomy, while 29 patients underwent endoscopic resection of their tumors, and in 42 patients tumors were obtained by transurethral biopsy. All specimens were staged following the TNM pathologic staging system.[10] Tumor stage was assigned as follows: pTa (n = 36), pTis (n = 1), pT1 (n = 23), and pT2-pT4 (n = 16). All tumors were also graded following the 1973 WHO classification, according to the Malstroms/Bergkvist system which includes grades 1, 2a, 2b, and 3. This approach is comparable to the 1999 WHO classification and allows cases to be grouped into a low-grade (grades 1 and 2a) and a high-grade (grades 2b and 3) categories, which are more suitable for statistical analyses. Tumors were classified as grade 1 (n = 10), grade 2 (n = 36; 2a n = 22, 2b n = 14) and grade 3 (n = 30) and, additionally for statistical evaluation, as low-grade (n = 32) and high-grade (n = 44) lesions. Specimens were processed for routine histopathology by formalin fixation and paraffin embedding. Normal and tumor tissue specimens from the same patient were always available and were investigated using immunohistochemistry and molecular assays.

Immunohistochemical Analysis

Immunohistochemistry (IHC) was performed using the primary mouse antihuman p53 monoclonal antibody DO-7 (DAKO, Glostrup, DK) at 1:2000 dilution (200 ng/mL). This antibody detects an epitope carried on the N-terminus of p53, mapping to amino acids 35 to 45, and identifies both wild-type and mutant p53 products. However, due to the short half-life and low levels of expression of wild-type p53, only mutant p53 proteins which accumulate in nuclei of tumor cells are visualized by IHC assays. The avidin-biotin immunoperoxidase method was used for this study. Briefly, 4-μm thick sections were treated with 0.1 mol/L citrate buffer (pH 6.0) as the antigen retrieval method. Primary anti-p53 DO-7 mAb (200 μg/mL) was incubated overnight at 4°C. Secondary reagents were biotinylated horse antimouse antibodies (Vector Laboratories Inc., Burlingame, CA) used at 1:100 final working dilution. Avidin-biotin complexes (Vectastain ABC PK 4000 ST, Vector Laboratories) were then applied at 1:100 dilution; 3,3′-diaminobenzidine was used as the final chromogen and Harris-modified hematoxylin as the nuclear counterstain. Positive controls, which were cases known to harbor *TP53* mutations displaying positive nuclear staining, and negative controls, in which the primary antibody was omitted, were included in each experiment.

Immunohistochemical evaluation was conducted by at least two independent observers that scored the estimated percentage of tumor cells showing nuclear staining, independently of the strength of the signal. This evaluation and data recording were conducted without previous knowledge of clinicopathological information or molecular results. p53 nuclear immunoreactivities were considered positive after statistical receiver operating characteristic (ROC) analysis (see below) when at least 20% of tumor cells showed nuclear immunoreactivities (see Discussion section).

Mutational Analysis

Tissue Microdissection, DNA Extraction, and Control Amplification

Ten 5-μm thick sections of both normal and tumor samples in all patients were used for each case. Tumor DNA was extracted, after microdissection to avoid normal tissue contamination, using a proteinase K-phenol/chloroform protocol. Two hundred ng of DNA were used for each polymerase chain reaction (PCR) amplification, using a GeneAmp PCR System 2400 thermal cycler (PE Biosystems, Foster City, CA). Efficiency of all PCR reactions was assessed in 2% agarose gels. A 268-bp fragment of the human β-globin gene was also amplified to test the quality of the genomic DNA extracted.

Characterization of *TP53* Gene Mutations

Exons 4 to 8 of the *TP53* gene were amplified in five independent PCR reactions. Mutational analysis was performed by single-strand conformational polymorphism (SSCP) in GeneGel Excel 12.5/24 polyacrylamide nondenaturing gels (Amersham Pharmacia Biotech, Uppsala, Sweden) at two different electrophoresis conditions, and gels were then developed by silver staining. Samples showing anomalous mobility were retested to verify the results. Shifted bands were excised from the gel, eluted in water, and used as a template for direct sequencing using the ABI PRISM BigDyeTM Termina-

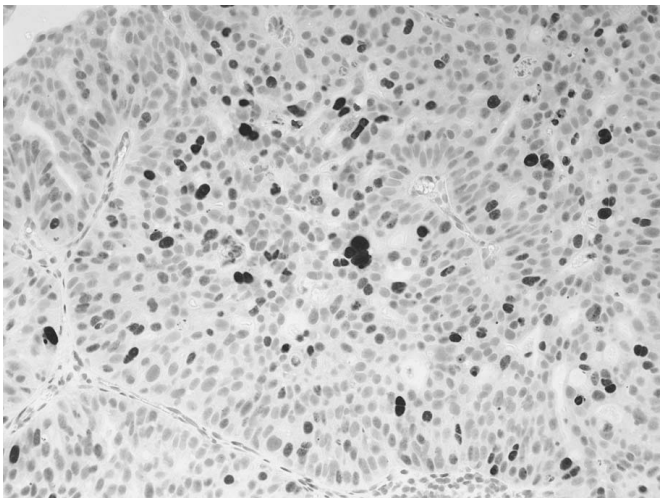

FIGURE 1. Immunohistochemical detection of p53 in bladder tumors using the anti-p53 monoclonal antibody DO-7. pT1/grade 3 transitional cell carcinoma in which the score of stained nuclei was 30%. In this particular case, when molecular analysis of the IHC positive areas was performed, no mutations were found (original magnification 200×).

tor Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Warrington, UK).

Detection of 17p Allelic Losses and Analysis of the H-*RAS* Gene

Amplification of the p53CA dinucleotide repeat[11] was performed for the analysis of allelic losses at 17p (TP53 locus). Products were also analyzed in Gene Gel Excel 12.5/24 polyacrylamide nondenaturing gels, developed by silver staining. Allelic losses were evaluated by two different observers and were considered when wild-type bands were reduced in at least 50% of the signal. Amplification and direct sequencing of H-*RAS* exon one (codons 12 and 13) was performed following the same protocol outlined above for the analysis of *TP53* gene mutations.

Statistical Analyses

Patients with primary and recurrent tumors were included in the study by randomly selecting one of the lesions to be studied using the Excel built-in random number generator. This approach allowed us to include only one specimen per patient, since the molecular analysis and technical aspects of the study did not reside on primary versus recurrent lesions. Both the ROC curve and the logistic regression analysis were used to evaluate the validity of IHC in predicting *TP53* gene mutations as measured by PCR-SSCP and sequencing.[12,13]

The χ^2 test was used to assess statistical association between p53 immunophenotype, *TP53* mutations, and TP53 deletion status with pathologic stage and histologic grade. *P* values quoted were two-tailed, and considered significant when they were less than or equal to .05. Statistical procedures were performed with SPSS 11.0.1 (Inc., Chicago, IL) and Statgrafics Plus 5.0 (Manugistics Inc., Rockville, MD).

RESULTS

Immunohistochemical Evaluation of p53 Nuclear Accumulation: Phenotype Studies

Definition of positive phenotype for p53 required nuclear immunoreactivity in at least 20% of tumor cells. Applying this criterion, we observed that 23 of 76 (30%) tumors displayed a positive phenotype (Fig. 1), while 20 (26%) lesions had undetectable p53 levels (score 0). The remaining 33 (44%) tumors displayed a percentage of immunostained nuclei ranging from 1% to 19%, values over which the cutoff point of positivity has been extensively discussed in the literature (see also Discussion section). We also observed that normal urothelium, inflammatory and mesenchymal cells in all tissues analyzed had nonreactive nuclei, serving as internal negative controls.

Incidence of *TP53* Mutations: Genotype Studies

Utilizing PCR-SSCP and direct sequencing, we identified and characterized a total of 24 *TP53* tumor specific mutations in 22 out of the 76 analyzed tumors (Fig. 2). Of interest,

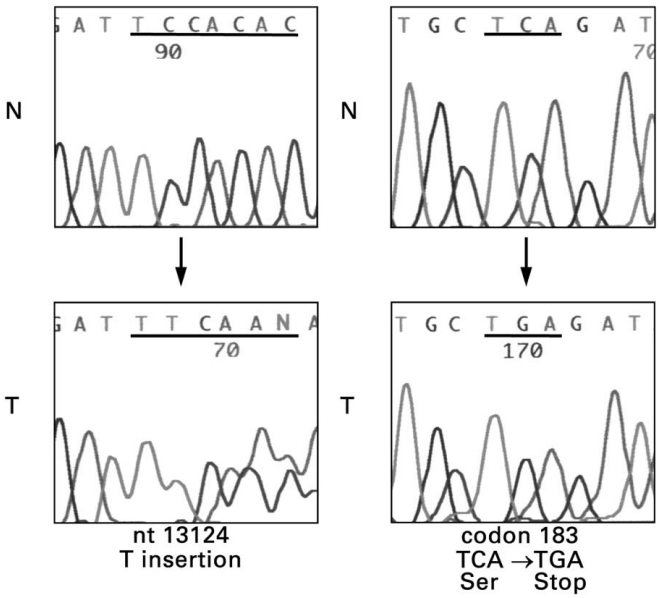

FIGURE 2. Molecular characterization of *TP53* mutations in urinary bladder tumors. This figure illustrates the detection of two different kinds of mutations by direct sequencing. (Left) An example of frameshift mutation is shown, corresponding to an insertion of a thymidine. (Right) A nonsense mutation that leads to a truncated protein is represented. Both mutations were detected in exon 5.

TABLE 1. Immunohistochemical and Mutational Analysis of p53 in Bladder Tumors

Number of Cases*	IHC Score (%)	Exon	Codon	Nucleotide	aa Change
1	20	—	—	—	—
4	30	—	—	—	—
1	40	4	96	TCT → TTT	Ser → Phe
1	65	5	135	TGC → TGG	Cys → Trp
1	60	5	139	AAG → GAG	Lys → Glu
1	70	5	149	Ins T	frameshift
		7	236	TAC → TGC	Tyr → Cys
1	75	5	158	CGC → TGC	Arg → Cys
		8	280	AGA → ACA	Arg → Thr
1	50	5	180	GAG → AAG	Glu → Lys
1	35	7	230	Ins GAC	
1	95	7	248	CGG → CCG	Arg → Pro
1	60	7	248	CGG → TGG	Arg → Trp
1	50	7	258	GAA → AAA	Glu → Lys
1	75	7	258	GAA → GTA	Glu → Val
1	60	8	271	GAG → AAG	Glu → Lys
1	85	8	273	CGT → CAT	Arg → His
1	40	8	274	GTT → CTT	Val → Leu
1	35	8	274	GTT → GAT	Val → Asp
1	80	8	285	GAG → AAG	Glu → Lys
1	25	8	291	AAG → AAC	Lys → Asn
1	90	Intron 4	—	C → T	frameshift
1	<20†	8	282	CGG → CCG	Arg → Pro
1	<20†	8	292	AAA → GAA	Lys → Glu
31	<20	—	—	—	—
1	0	4	86–89	Del 13 nt	frameshift
1	0	5	183	TCA → TGA	Ser → STOP
18	0	—	—	—	

*Bold numbers indicate discrepant cases between expression and detection of mutations.

†IHC scoring was 2% and 5%, respectively.

we observed two cases that displayed "double" mutations. Table 1 summarizes the mutational spectrum of this cohort. Nineteen of the 24 detected mutations were missense, while the remaining five mutations included one nonsense, one splicing, two frameshift (one deletion of 13 nucleotides, and one insertion of one nucleotide), and one in-frame insertion (3 bp).

Relationship Between p53 Phenotype and *TP53* Genotype

TP53 gene mutations were identified in 18 of 23 (78%) cases displaying a p53-positive phenotype. The two tumors harboring "double" mutations were found to display a p53 positive phenotype.

Only two cases displaying *TP53* mutations were found in the group scored as possessing less than 20% immunostained tumor cells, and thus considered as p53-negative phenotype, while the remaining two cases with *TP53* mutations were scored as having undetectable p53 immunoreactivities. Within the group of nonmutated tumors (n = 54), five showed positive immunoreactivities scoring between 20% to 30% (Table 1). Globally, there was a strong statistical correlation between p53 nuclear accumulation and detection of mutations by SSCP and sequencing ($P < .001$).

Figure 3 illustrates the results obtained by the ROC analysis. The area under the ROC curve may be considered as a measure of the diagnostic accuracy of detecting *TP53* mutations by the identification of a p53 positive phenotype using IHC. This accuracy was estimated to be 88% when the cutoff value for IHC was between 20% and 30%, values for which errors were mainly balanced (false positives and false negatives). The logistic regression analysis gave a cutoff value of 30%.

Detection of Allelic Losses and Analysis of the H-*RAS* Gene

LOH at 17p was evaluated in 74 tumors, 69 of which (93%) were informative for the marker. 46 (67%) tumors displayed normal patterns, while the remaining 23 (33%) showed allelic loss at the *TP53* locus (Fig. 4). Loss of heterozygosity (LOH) is distributed among tumor stage, grade, and p53 status as described in Table 2. A significant statistical association was observed between 17p LOH and p53 altered status (mutational spectrum and expression pattern) (χ^2 test; $P < .01$).

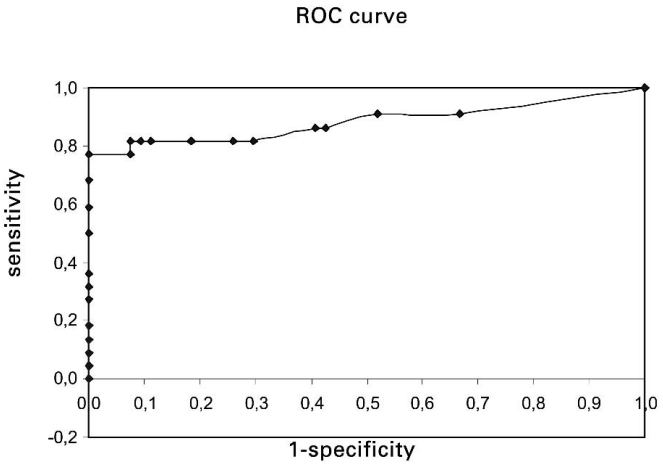

FIGURE 3. ROC analysis of the sensitivity and specificity of immunohistochemistry as it relates to PCR-SSCP and sequencing results. The area under the curve as a measure of diagnostic accuracy was estimated to be 88%. ROC analysis plots sensitivity versus specificity in a graphical display, allowing assessment of the validity in a fair manner, without the arbitrariness inherent in individual sensitivity/specificity pairs.

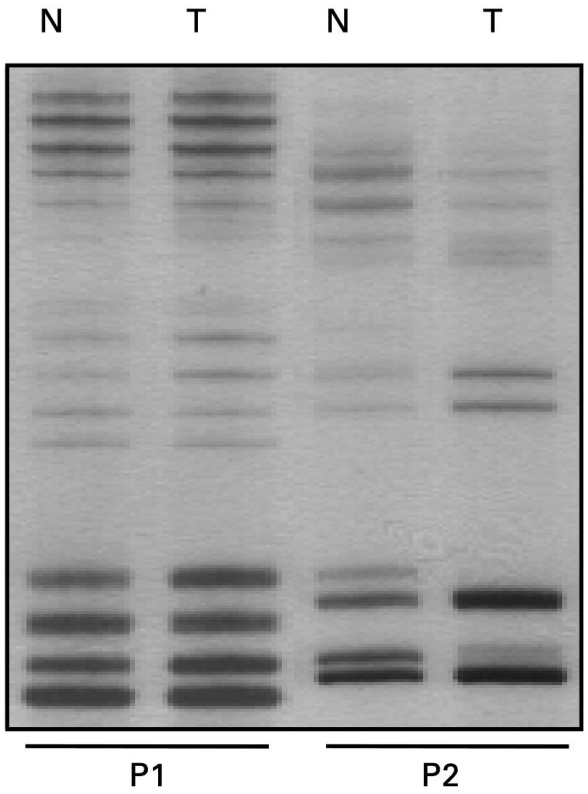

FIGURE 4. Representative cases to illustrate alterations of the p53CA microsatellite used to study 17p LOH (locus *TP53*). P1 lanes correspond to a tumor without *TP53* loss and P2 lanes exhibit 17p allelic loss.

No mutations were detected in any of the 73 tumors analyzed for the H-*RAS* gene.

Relationship Between Histopathological Features and p53 Alterations

The distribution according to histologic stage of tumors containing mutations was as follows: 3 of 36 pTa, 6 of 23 pT1, 12 of 16 pT2-4, and the only pTis tumor analyzed. Similarly, only one of 32 low-grade tumors displayed a *TP53* mutation, while 21 of 44 high-grade lesions had *TP53* mutations.

A cutoff of 20% of tumors cells with nuclear immunoreactivities was used to determine the p53 positive phenotype. This value was also used for statistical analysis of histopathological features and p53 alteration. Applying this criterion, p53 status as a combination of both strategies (IHC plus PCR-SSCP/sequencing) yielded a total of 28 cases with p53 alteration (37%). We found a significant association between p53 status and tumor stage and grade, being alterations more common in high-stage and high-grade tumors (both χ^2 test; $P < .01$). Statistical associations were also observed between histologic variables and 17p LOH (χ^2 test; $P = .01$), being allelic loss more common in advanced disease. Deletion of 17p significantly correlated with tumor stage ($P < .01$) and grade ($P = .01$) (Table 2).

DISCUSSION

The potential clinical value of p53 in bladder cancer as a predictive marker of tumor recurrence and treatment selection is still in debate.[7,8,14,15] Studies conducted in specific patient populations, using well-designed and -implemented technical approaches, are needed to further validate its clinical utility. In this context, molecular epidemiology studies aimed at defining rate of p53 alterations in diverse patient populations are of crucial importance. Defining methods to correctly categorize bladder cancer patients carrying altered p53 products is also critical, mainly in the framework of targeted therapeutic interventions.

Data from this study confirm that p53 nuclear accumulation, as detected by IHC with the antibody DO-7, is indicative of the presence of a TP53 mutation mainly affecting exons 4 to 8. This antibody has been reported to have high sensitivity and specificity for p53 detection,[16] especially accumulated p53 mutant products, which may explain the high accuracy of its association with TP53 mutations reported in this study.

The arbitrariness of the choice of cutoff values when scoring IHC results, together with the antibody used and the type of tissue analyzed, may affect the reliability of the assessment of the p53 status. Although the cutoff value of 10% tumor cells displaying p53 nuclear immunoreactivities has been used

TABLE 2. Distribution of TP53 Allelic Loss in Relation to Stage, Grade, and Mutational Status

	Normal	Loss	Total	*P* Value
Stage				
pTa	27	6	33	.008*
pT1	14	5	19	
pT2–4	4	12	16	
Grade				
Grade Low	23	5	28	.019
Grade High	22	18	40	
Mutational status				
TP53 wt	37	1	38	1.45E-07
TP53 mutation	8	14	22	
IHC accumulation				
IHC wt	38	8	46	3.45E-05
IHC accumulation	7	15	22	
Altered p53 (mutation + IHC)				
p53 wt	34	8	42	.001
p53 altered	11	15	26	

**P* value is referred to TP53 allelic loss versus dichotomized stage as pTa and pT1–4 (χ^2 test).

by other investigators, cutoff values up to 40% have been also used while evaluating the prognostic value of p53 phenotype in bladder cancer.[17] Methodological studies comparing p53 expression by IHC and TP53 mutations by genetic assays have revealed the superior accuracy of 20% tumor cells displaying p53 nuclear immunoreactivities as the cutoff value.[9,18] In our study, logistic regression analysis set the cutoff value at 30% positive tumor cells, on which all errors were mainly balanced. A cutoff between 20% and 30% tumor cell reactivities, as determined by the ROC analysis, is slightly superior to values previously described in studies on which similar accuracy rates were obtained.[9,18] In our series, the small number of tumors with these positive IHC values (20%–30%) could explain the wide cutoff range. We believe it is preferable to imbalance the error to false positives and set the cutoff to 20%, as previously reported.[18]

The biologic and clinical significance of identifying a p53 positive phenotype by IHC without detection of a *TP53* mutation is still uncertain. Some authors have suggested an association between poor prognosis and high p53 IHC values in the absence of detectable *TP53* mutations.[4] Alterations in genes others than p53 in its pathway, producing p53 accumulation, may account for this non–mutation-related positive phenotype, since DO-7 recognizes both mutant and wild-type p53 forms. A negative cooperative effect has been recently demonstrated for altered levels of gene products of the p53 and RB pathways in bladder cancer prognosis, being p53 positive phenotype identified by IHC the strongest predictor of disease outcome.[7,8] p53 stabilization could prevent its ubiquitination and subsequent proteosome degradation. Critical to this process is the phosphorylation of certain p53 sites, such as the recently reported TAF1 phosphorylation of p53 at Thr-55.[19] Significantly, the Thr-55 phosphorylation was reduced following DNA damage, suggesting that this phosphorylation contributes to the stabilization of p53 in response to DNA damage.

Allelic losses at the 17p locus have been previously reported in up to 55% of invasive bladder cancers,[19] being strongly correlated with the presence of *TP53* mutations. TP53-specific 17p deletions have been also correlated with tumor grade, stage, and the presence of vascular invasion.[20–22] We have found TP53 LOH in 33% of the informative cases analyzed, being the frequency in invasive tumors up to 50% of the cases. There was a statistically significant association between higher tumor grade and allelic deletion, as previously reported.[18,19] There were 18% deletions among pTa lesions (6/33), whereas 26% (5/19) pT1 and 75% (12/16) pT2-4 tumors exhibited LOH. There was also a statistical correlation between TP53 allelic loss and tumor grade, as well as with the presence of p53 alterations (positive phenotype and/or detection of gene mutations). These results further support early reported data revealing that in bladder cancer inactivation of the *TP53* gene involves both point mutation and loss of the concomitant allele, being allelic losses more common in advanced disease.

The H-*RAS* gene codes for a p21 membrane-bound protein involved in signal transduction. It was first shown to play a role in bladder cancer in the early 1980s, but the frequency on detecting H-*RAS* mutations in bladder cancer has been controversial. Rates of H-*RAS* mutations between 10% and 40% have been reported, being codon 12 G to T substitutions the predominant alterations identified.[23,24] H-*RAS* mutations were found in both noninvasive and invasive lesions, analyzing either tumor specimens or urine sediments.[25] Although H-*RAS* status may not be of great use in predicting progression, the ease with which H-*RAS* mutations can be detected in the urine has shown its potential use in the follow-up of those patients in whom the mutation is present. More recently, some studies have suggested that only minor cell populations harbor codon 12 H-*RAS* mutations,[26] independently of the methodology used for screening. In our series, no mutations have been found in codons 12 and 13 of the H-*RAS*, as analyzed by direct sequencing, suggesting that H-*RAS* mutations do not play a critical role in the development or progression of bladder cancer, at least in the population studied.

In conclusion, data from this study validate in the bladder cancer population examined the immunohistochemical approach of detecting p53-positive phenotype and that a 20% cutoff for nuclear immunoreactivity is indicative of *TP*53. Molecular assays are required for the optimal determination of p53 status. Nonsense mutations that lead to IHC false-negative results may also be determinant for progression to invasive disease, especially in early-stage tumors. Since p53 alterations are significantly associated to clinicopathological features of poor prognosis, we recommend the inclusion of both p53 phenotype and TP53 mutation status into a predictive panel of tumor markers for bladder cancer is recommended.

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