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Comparison of P53 Protein Overexpression
With P53 Mutation In Bladder Cancer:
Clinical and Biologic Aspects

Jacqueline A.M. Vet, Pierre Paul Bringuier, H. Ewout Schaalma, J. Alfred Witjes,
Frans M.J. Debruyne, and Jack A. Schalken

Department of Urology, University Hospital Nijmegen, and the Department of Pathology,
Canisius Wilhelmina Hospital, Nijmegen, The Netherlands

BACKGROUND: Alterations of the tumor suppressor gene p53 are known to occur in bladder cancer. Although p53 overexpression is associated with mutation of the p53 gene, a substantial discrepancy between molecular genetic alteration in p53 and overexpression of the protein has been found.

EXPERIMENTAL DESIGN: Tumor specimens of 39 bladder cancer patients were immunohistochemically analyzed for p53 overexpression, and the results were compared with the presence of a mutation as assessed by single strand conformation polymorphism (SSCP) and direct sequencing. Both clinical and biologic aspects were studied.

RESULTS: A significant correlation between p53 overexpression and poor survival in the whole group studied was found (p < 0.01). No association between p53 overexpression and decreased survival was found for invasive tumors in contrast with other studies. Differences in treatment of the patients and different Ab and scoring systems used might explain these differences. In our study, the Kaplan-Meier curves showed the same result for p53 overexpression and p53 mutation when the whole group and the invasive tumors were studied. However, in the group of superficial tumors, which was unfortunately too small for statistical analysis, we found p53 overexpression in three tumors, and no p53 mutations were found. A good concordance between p53 mutation and p53 overexpression was found (p < 0.02). However, two out of eight tumors with an SSCP-proven p53 mutation showed no p53 immunoreactivity, probably as a result of loss of the nuclear localization signal. Twenty three percent (7/31) of the tumors showed p53 overexpression without any sign of a mutation.

CONCLUSIONS: Our results indicate that, despite a good concordance between p53 mutation and p53 overexpression, there is no direct causal relationship between mutation and protein accumulation. Apparently, other events than mutation can trigger p53 stability.

Additional key words: Prognostic value, SSCP, Immunohistochemistry, TCC.

The tumor suppressor gene p53 is located on chromosome 17p13.1 and encodes a 53-kD nuclear phosphoprotein with specific DNA binding properties. Chromosomal losses of 17p13 occur during tumor progression in a variety of human tumors (1). In bladder cancer, loss of 17p13 is associated with high grade tumors and invasive disease (2, 3). In accordance with the classic tumor suppressor theory, the loss of heterozygosity (LOH) of 17p13 is often accompanied by a mutation of the remaining allele (4). In bladder cancer, p53 mutations correlate with grade and stage (5–7) and probably play a role in the progression of this disease.

Wild-type p53 acts as a cell cycle control protein at the level of G1-to S-phase transition (8). If DNA damage occurs, p53 levels rise and block cells in the G1-phase. The DNA damage can subsequently be eliminated either by DNA repair or by initiation of apoptosis (9). The up-regulation by p53 of p21, an inhibitor of G1 cyclin-dependent kinases, appears to be responsible for p53-mediated growth arrest (10). Recently, Smith et al. showed that Gadd45, which is up-regulated by p53, probably serves as a link between the p53-dependent cell cycle checkpoint and DNA repair (11). p53 seems to function through modulation of transcriptional activity, enhancing the expression of genes containing p53-binding sites and interacting with a variety of transcription factors to inhibit the expression of other genes (12, 13). Cells that lose this wild-type p53 function fail to show growth arrest if DNA damage occurs, which can lead to replication of incorrect DNA, resulting in genetic instability (14, 15). In addition to mutation, loss of wild-type p53 function can be the result of complexing with viral oncoproteins, e.g., simian virus 40 (SV 40), large T Ag (16, 17), adenovirus 5 E1b protein (18) and E6 protein of human Papillomavirus
16 and 18 (19). The cellular oncoprotein mdm2 also interacts directly with p53 and functionally inactivates it (20).

The occurrence of p53 mutations leads to conformational changes of the protein, resulting in a prolonged half-life and subsequently in accumulation of the protein (21). The extended half-life of the protein is the basis for immunohistochemical detection of p53. Although there is a good concordance between p53 overexpression and mutation of the p53 gene, several studies have shown a considerable discrepancy between molecular genetic alteration in p53 and overexpression of the protein (22-25). In this study, we compared the immunohistochemical detection of p53 overexpression with p53 mutation as assessed by single-strand conformation polymorphism (SSCP).

EXPERIMENTAL DESIGN

Tumor Specimens

Twenty-three snap frozen, superficial bladder carcinomas (pTa-pT1) and 24 muscle-invasive bladder carcinomas (pT ≥ 2) obtained from 45 patients were used for SSCP analysis, as described previously (7). The transitional cell carcinomas were classified according to the World Health Organization criteria (26). For immunohistochemistry, the same specimens were investigated, with the exception of two superficial and two invasive carcinomas that could not be analyzed because of poor quality of the frozen tissue. Moreover, the two squamous cell carcinomas were removed from this study because of their different pathologic background. The clinical and pathologic data are shown in Table 1. Genomic DNA was extracted from step-sectioned tumors (>70% tumor cells) using a method described by Miller and coworkers (27).

IMMUNOHISTOCHEMISTRY

Cryostat sections (5 μm) were dried overnight. Tissue sections were fixed in acetone for 10 minutes and then incubated overnight at 4°C with the mouse mAb DO-7 (Novocastra, Newcastle upon Tyne, UK) at a dilution of 1:100. This Ab recognizes both wild-type and mutant p53. Sections were subsequently incubated for 30 minutes with biotinylated sheep anti-mouse Ig Ab (1:200, Amersham, Buckinghamshire, UK) and then incubated for 30 minutes with streptavidin biotinylated-horseradish peroxidase complex (1:100, Amersham). After washing with PBS, 3,3'-diaminobenzidine (Sigma Chemical Company, St. Louis, MO) was used as a chromogen, and hematoxylin was used for counterstain.

Analysis of the immunohistochemical results was performed by two investigators (J.V., P.P.B.). The pattern of p53 nuclear overexpression was classified in four categories by estimating the percentage of stained tumor cells: −, no cells positive; +, 1 to 10% positive tumor cells; ++, 10 to 50% positive tumor cells; and ++++, > 50% positive tumor cells. Cytoplasmic staining was not scored.

PCR-SSCP

PCR-SSCP analysis (28) was performed to investigate p53 mutations in exons 5 to 8. The intron primers for amplification of exons 5 to 8 were: exon 5: S: 5′ tca ctt gtt ccc tga ctt 3′ and AS: 5′ gag gaa cag gcc tgg 3′; exon 6: S: 5′ gag gac aca aga gtt gtt 3′ and AS: 5′ gag acc cag tgg cca acc 3′; exon 7: S: 5′ cca agg cgc act ggc ctc 3′ and AS: 5′ gag gca aga aga gcc tgg 3′; and exon 8: S: 5′ cct tac tgc ctc tgg ctc 3′ and AS: 5′ tga atc tga ggc ata act 3′.

Genomic DNA (250 ng) was subjected to 35 cycles of PCR (95, 57, and 72°C for 0.5, 2, and 1.3 minutes, respectively). Exons 5, 6, and 8 were amplified in 50 μl containing: 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.75 mM MgCl₂, 250 μM deoxynucleotide triphosphates, 10 pmol of each 5’ end-labeled primer, and 1.5 U of Taq polymerase (Perkin Elmer/Cetus, Norwalk, CT). The PCR primers were used for sequencing in the dsDNA cycle sequencing system (Life Technologies, Inc). Electrophoresis was performed on 6% polyacrylamide gels with and without 10% (v/v) glycerol. Subsequently, autoradiography was performed at room temperature for 16 hours at 6 or 3 W, respectively.

SEQUENCE ANALYSIS

Direct sequencing of the double-stranded PCR products that showed a shift on the SSCP gels was performed as described previously (29). Amplified PCR products were purified using the magic PCR-preps DNA purification system (Promega, Madison, WI). The PCR primers were used for sequencing in the dsDNA cycle sequencing system (Life Technologies, Inc). Electrophoresis was performed on 6% polyacrylamide (19:1) gels containing 7 M urea.

STATISTICAL ANALYSIS

The Kaplan-Meier method was used to estimate survival probability as a function of time. Differences in survival were analyzed by a log-rank test. The χ² test (with Yates correction if relevant) was used for the other correlations.

RESULTS AND DISCUSSION

IMMUNOSTAINING PATTERNS

The DO-7 staining patterns of the four groups are shown in Figure 1. We defined a tumor as p53 positive if more than 10% of the tumor cells showed nuclear p53 expression. Of the 41 tumors studied, 13 showed p53 overexpression.

Correlation Between Tumor Grade/Stage and p53 Overexpression

p53 overexpression was found in 9% of the Grade 1, 23% of the Grade 2, and 60% of the Grade 3 tumors. Overexpression of p53 was found in 16% of the superficial tumors and in 50% of the invasive tumors (p < 0.05) (Tables 2, 3). p53 mutations found by SSCP analysis showed a high correlation with both increasing grade (p < 0.001) and stage (p < 0.001) (7).
CORRELATION BETWEEN p53 OVEREXPRESSION AND SURVIVAL

The survival according to p53 overexpression is shown in Figure 2. For the whole group, a shorter survival time of patients with p53 overexpression was observed ($\chi^2$, 8.00, $p < 0.01$, Fig. 2a). Although statistical analysis was not possible because of the small number studied, we observed a shorter survival time for patients with superficial bladder cancer that showed p53 overexpression (Fig. 2b). Within the group with invasive disease, no significant difference in survival time between the patients with and without p53 overexpression was seen (Fig. 2c).

COMPARISON BETWEEN p53 OVEREXPRESSION WITH p53 MUTATIONS FOUND BY PCR-SSCP

The overall comparison of p53 mutations analyzed by PCR-SSCP and p53 overexpression assessed by immunohistochemistry (IHC) is shown in Table 4. The sensitivity of IHC, defined as percentage of IHC-positive tumors among tumors with identified mutation, was 75%. The specificity of IHC, defined as percentage of IHC-negative tumors among neoplasms without a p53 mutation, was 77%. Despite the good concordance between p53 mutation and p53 protein overexpression ($p < 0.02$), 23% (7/31) of the tumors without a p53 mutation as assessed by SSCP analysis showed p53 overexpression. Table 5 shows the eight
We looked at both clinical and histologic aspects of p53 mutational analysis of SCCP G12p and a deletion of a G at codon 166, which showed no p53 immunopositivity at all. These showed p53 overexpression, except for cases 3 and 4, p53 mutational found by PCR-SSCP analysis. They all

**DISCUSSION**

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**Table 2. Relation Between p53 Overexpression and Stage**

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**Table 3. Correlation Between p53 Overexpression and Stage**

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The overexpression of p53 as assessed by IHC correlates with grade and stage ($p < 0.05$). However, correlation between mutations in the p53 gene as determined by SSCP analysis and grade and stage was higher ($p < 0.001$) (7). In concordance with other IHC studies (32, 33), we demonstrated that p53 overexpression is an unfavorable prognostic factor for bladder cancer patients ($p < 0.01$). No association between p53 overexpression and decreased survival was found for invasive tumors, in contrast with other studies. Differences in the treatment of the patients and the different Ab and scoring systems used might explain these differences. Esrig and coworkers (33) showed that p53 overexpression is a significant predictor of tumor progression if the disease is confined to the bladder. However, no association between p53 overexpression and tumor progression was found if the disease was not confined to the bladder ($pT3b$, $pT4$). Because of the relatively small number in our study, we cannot compare these two groups. The results of our study show that p53 overexpression has no additional prognostic value over stage, whereas for this same group of patients, we found that E-cadherin may have additional prognostic value over stage (34). Apparently, other mechanisms can lead to tumor progression, and they may override or bypass the function of p53.

The Kaplan-Meier curves showed the same result for p53 overexpression and p53 mutation (7) when the whole group and the invasive tumors are studied. For superficial tumors, however, three patients showed p53 overexpression without a mutation confirmed by SSCP analysis. Although this was not statistically significant, a trend was observed toward worse survival for the patients showing p53 overexpression. This was not observed when p53 mutations were analyzed, because we found no mutations at all in the group of superficial tumors. In high grade pT1 tumors, p53 overexpression had predictive value for progression of disease (35). These results, combined with the correlation of p53 overexpression with increasing grade and stage as shown here, imply that p53 overexpression plays a role in the progression of bladder cancer.

Analysis of p53 mutations at the molecular genetic level is rather difficult and time consuming and therefore is not suitable for routine use. IHC, however, is a standard technique in pathology laboratories. There is a good concordance between p53 mutation and p53 overexpression in relation to grade, stage and survival, so IHC detection of p53 alterations is preferable as a predictor of prognosis. Furthermore, IHC might reveal anomalies in the p53 pathway other than p53 mutations (see below).

A good concordance between p53 mutation by SSCP and p53 overexpression by IHC ($p < 0.02$) was observed. This strong correlation between high levels of p53 protein and mutation in the p53 gene has previously been described for a number of tumor types, and this led to the hypothesis that mutant p53 gene products are characterized by conformational changes of the protein, resulting in a higher stability and consequently in accumulation of the protein. However, we

alterations. We chose a cut-off of 10% p53 positive tumor cells for p53 overexpression. The appearance of occasionally stained cells in tumors, which we scored as negative, can be explained by the existence of clones of cells with a p53 mutation or by the occurrence of a genotoxic event that raises levels of wild-type p53 protein in normal cells, resulting in cell cycle arrest (30, 31). More detailed examination of microdissected focally stained areas by means of mutation analysis (SSCP) has to be performed to address this problem and to check the biologic importance of this phenomenon.

![Kaplan-Meier survival curves of bladder cancer patients](image)

**Fig. 2.** Kaplan-Meier survival curves of bladder cancer patients according to the presence or absence of overexpression of the p53 protein. A All patients ($p < 0.01$, log rank test). B Patients with superficial (pTn,pT1) tumors (not significant). C Patients with muscle invasive disease (not significant).
have observed discrepancies between p53 mutation and p53 overexpression.

Of the eight tumors with a p53 mutation as assessed by SSCP, two tumors showed no p53 immunoreactivity at all. In one tumor, sequence analysis showed a transversion in codon 166 of exon 5 generating a stop codon and thereby a truncated protein, which does not contain the nuclear localization domain at amino acids 316 to 325 (36). In the other tumor, a deletion of a guanine nucleotide found in codon 282 of exon 8 gave rise to a frameshift, which also affected the downstream nuclear localization sequence. In both cases, the loss of the nuclear localization signal most likely prevented nuclear accumulation, and therefore IHC detection was impossible.

In addition to these "false negatives," which can still be explained by the proposed theory of extended stabilization, we observed p53 overexpression in 23% of the tumors without any sign of p53 mutation as assessed by SSCP. This p53 overexpression without a concomitant mutation was also found in a considerable number of bladder tumors in two other studies (37, 38). Although we studied exons 5 to 8, which are known to contain the majority of the p53 mutations (1), mutations outside this region and intron mutations might explain this result. Moreover, although SSCP is a sensitive method for detecting mutations (39), not all mutations are detected by this technique. The presence of nonneoplastic tissue, although reduced to ≤30%, can lead to a negative SSCP result if the percentage of tumor cells overexpressing p53 is approximately 10%. This cannot be the explanation for p53 overexpression in the 23% of the tumors without a SSCP-proven p53 mutation because most of these tumors contained at least 20% of p53 overexpressing tumor cells.

All of the mechanisms leading to p53 overexpression are not yet fully understood. According to the already mentioned hypothesis, mutant p53 would always be stable, and wild-type p53 would be unstable. Thus the tight correlation between mutation and overexpression could indicate a causal relationship. However, this is not always true. In fibroblasts obtained from Li-Fraumeni patients who carried heterozygote germ-line p53 mutations, the mutant p53 protein did not accumulate to a high level and was unstable like the wild-type p53 (40). By contrast, in the tumors of these patients, when the wild-type allele was lost, the mutant p53 protein did accumulate. Clearly, p53 mutation per se does not cause p53 protein accumulation.

Alternatively, wild-type p53 can accumulate in some circumstances, e.g., as a result of complexing with viral oncoproteins (16–18) and with the cellular oncoprotein mdm2 (20). Accumulation of wild-type p53 in normal tissue was shown to occur in a novel cancer family syndrome (42). One can hypothesize that anomalies elsewhere in the p53 pathway can result in stabilization of p53 proteins as well as in the ability to ignore its growth-suppressive commands.

We found p53 overexpression in 23% of the cases without any sign of a mutation, which is in concordance with two other studies (37, 38), so we suspect that some other event(s) in addition to mutation plays a role in stabilization. The viral oncoproteins are not relevant for the human system. The mdm2 oncprotein has been implicated in the progression of bladder cancer (42). Whether mdm2 overexpression represents...
an alternative to p53 mutation in inactivating the p53 regulatory pathway is still unclear. Events other than mutation that lead to stability remain to be elucidated. It is noteworthy that p53 can accumulate in normal cells upon DNA damage. Thus some tumor cells behave as if they were in a permanent state of DNA damage (40). Additional research is necessary to characterize the signals that trigger this state and to understand the mechanisms through which this overexpression reflects alteration of p53 function and consequently gives rise to an altered phenotype in cancer cells.

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Address reprint requests to: J. Schalken, Urological Research Laboratory, University Hospital Nijmegen, P.O. Box 8101, 6500 HB Nijmegen, The Netherlands.

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