p73 Expression in Human Normal and Tumor Tissues: Loss of p73α Expression Is Associated with Tumor Progression in Bladder Cancer

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ABSTRACT

Purpose: To characterize the expression profile of p73 in human normal tissues by immunohistochemistry (IHC) and to analyze the correlation between p73 expression and bladder cancer progression.

Experimental Design: CJDp73 was characterized for p73α detection in Western blot and IHC through its application to isofrom-transfected 293 cells. Normal tissues were analyzed by IHC with the CJDp73 antiserum. Transitional cell carcinoma (TCC)–derived cell lines were subjected to reverse transcription-PCR and Western blot. TCC tissue microarrays were analyzed for p73α expression by IHC, and the results were statistically analyzed.

Results: p73 immunostaining was nuclear and restricted to epithelial cells of certain organs such as squamous epithelium of the epidermis and transitional epithelium of the bladder. The expression was also observed in certain specialized glandular epithelia such as acinar cells of breast and parotid gland. Four of seven TCC–derived cell lines had low to undetectable p73α protein levels. We found undetectable or low p73α expression in 104 of 154 (68%) TCC cases, this phenotype being more frequently observed in invasive tumors when compared with superficial lesions. This association was statistically significant (P < 0.0001). We also observed a significant association between p53, p63, and p73α alterations with bladder cancer progression (P < 0.0001).

Conclusions: p73α plays a tumor suppressor role in bladder cancer, and its inactivation occurs through an epigenetic mechanism, most probably involving protein degradation.

INTRODUCTION

p73 was identified as the first homologue of p53 and along with p63 have added an additional level of complexity to the analysis of p53 function (1, 2). The p73 gene maps to 1p36.33, a region reported to harbor frequent deletions in certain human tumors, including neuroblastoma and colon cancer (3, 4). Three different promoters (P1, P2, and P3) have been described for p73 at the RNA level, giving rise to either the TA (transcriptionally active) or ΔN (dominant-negative) variants (5). Additionally, p73 is subject to extensive COOH-terminal alternative splicing of exons 11 through 14, yielding at least six isoforms (6–8).

p73 shares significant amino acid identity to p53 in the transactivation, DNA binding, and oligomerization domains (6). Not surprisingly, TAp73 can transactivate many p53 target genes such as p21 and BAX, leading to cell cycle arrest or apoptosis, respectively (6–11). In opposition, ΔNp73 acts as dominant-negative inhibitor, not only toward TAp73, but also toward other members of the p53 family (2).

Initially, because of its similarities to TP53 and the chromosomal location in a region frequently deleted, p73 was hypothesized to be a suppressor gene. This observation has been challenged by the low frequency of p73 mutations found in primary tumors, suggesting that the tumor suppression activity of p73 could be lost epigenetically and is not conforming to Knudson’s two-hit hypothesis (4). A recent study demonstrated that the simultaneous absence of p73 and p63 affected the induction of p53-dependent apoptosis in response to DNA damage in E1A-expressing cells and in the developing mouse central nervous system (12). However, p73-deficient mice were not tumor prone or display an increase in tumor incidence (13).

Previous studies have shown that p73α and p73β transcripts are ubiquitously expressed at very low levels in some normal human organs, including brain, heart, kidney, liver, and spleen (14). Bladder cancer is the fifth most common cancer in the United States (15). Alterations in proto-oncogenes such as Ras (16) and alterations of certain tumor suppressors such as TP53 and RB (17) have been associated with bladder cancer progression. Approximately 50% of bladder tumors harbor TP53 mutations or an altered p53 expression (18). A previous study from our group, centered in a closely related set of patients to the here described, found p53 nuclear overexpression and TP53 mutations in ~50% of the cases. p53 overexpression, associated with loss of p21 and mdm2 nuclear overexpression, was found to be a significant prognostic factor associated with patient survival (19). More recently, we reported the significant
association between loss of p63 expression and tumor progression in bladder cancer, analyzing the same cohort of patients studied here. p63 expression is lost in most invasive bladder TCC,\(^3\) whereas papillary superficial tumors maintain p63 expression (20).

In this study, we analyzed the expression of p73 in human normal tissues at the protein level. We also examined p73 expression patterns in a cohort of patients with bladder tumors previously examined for p53 and p63 and studied the relationship between bladder cancer progression and the loss of p73 expression, both individually and related to p63 expression and p53 status. A role for p73 as a tumor suppressor is hypothesized for bladder cancer.

### PATIENTS AND METHODS

**Human Tissue and Patient Characteristics.** A broad spectrum of paraffin-embedded normal human tissues were studied for the expression of p73 at the protein level (Table 1). Normal tissues used for RNA extraction were embedded in cryopreservative solution OCT (Miles Laboratories, Elkhart, IN), snap frozen, and stored at \(-80^\circ C\). Representative H&E-stained sections of each frozen and paraffin blocks were examined microscopically to confirm the presence of normal tissue in the samples. Tumors from 154 patients with TCC of the bladder were also analyzed. Tumor specimens included 49 papillary superficial tumors (T\(_a\) and T\(_1\)) and 105 invasive lesions (T\(_2\)–T\(_4\)). These cases were coded to ensure patient confidentiality.

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\(^3\) The abbreviations used are: TCC, transitional cell carcinoma; RT-PCR, reverse transcription-PCR; IHC, immunohistochemistry; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
**CJDp73 Antibody Production.** A 518-bp DNA fragment, spanning amino acids 428–599 and mapping to the COOH terminus of simian p73α, was inserted downstream in frame of the polyhistidine (6xHIS) tag of the pRSET-C-T7 expression vector (Invitrogen Life Technologies, Inc., Carsbad, CA). The p73α fusion protein was expressed in BL21(DE3) Escherichia coli and purified on an 8% SDS-polyacrylamide gel. Crushed gel slices containing the fusion protein were used to raise polyclonal antibodies in rabbits (Covance Research Products, Inc., Denver, PA). The resultant hyperimmune serum was designated CJDp73 and an aliquot was affinity purified using recombinant 6xHIS-p73α protein attached to a Nickel-NTA column (21).

**Cell Lines.** Nine bladder cancer cell lines were studied, including RT4, which is derived from a superficial TCC; T24, J82, 5637, HT-1197, HT-1376, UM-UC-3, and TCC-SUP derived from invasive TCC; and SCaBER derived from an invasive squamous cell carcinoma of the bladder. For transfection experiments, transformed human primary embryonal kidney 293 cells were used. Bladder tumor-derived cell lines and 293 cells were obtained from and maintained as recommended from the American Type Culture Collection (Manassas, VA). H224 cell lines expressing tetracycline-repressible simian HA-p73α and murine myc-ΔNp63α (for use in Western blots and RT-PCR) were a gift from Xinbin Chen (22). These stable cell lines do not express p63 or p73 when grown in the presence of 1 μg/ml tetracycline.

**Plasmids, Cell culture, and Transfection.** For CJDp73 characterization by Western blotting, transformed human primary embryonal kidney 293 cells were transfected in 35-mm dishes with human HA-tagged p23, TAp73α, TAp73β, TAp73γ, TAp73ε, ΔNp73α, ΔNp73β, ΔNp73γ, and vector control (pcDNA3) by the Fugene method (Roche Diagnostics, Indianapolis, IN). After 48 h, the cells were washed with a PBS solution and pelleted by centrifugation. For the CJDp73 characterization by IHC, transformed human primary embryonal kidney 293 cells were transfected in 2-well chambered slides (Nalge Nunc International, Naperville, IL) with TAp73α, TAp73β, TAp73γ, TAp73ε, ΔNp73α, ΔNp73β, ΔNp73γ, and vector control (pcDNA3). After 48 h, the slides were washed twice with PBS, fixed for 10 min in formalin, washed again with PBS, and subjected to the IHC protocol described below.

**Tissue Array Construction.** Normal and tumor tissues were fixed with formalin and embedded in paraffin. Five-μm sections stained with H&E were obtained to identify viable, representative areas of the specimen. From the defined areas core biopsies were taken with a precision instrument (Beecher Instruments, Silver Spring, MD), as described previously (23). Tissue cores with a diameter of 0.6 mm from each specimen were punched and arrayed in triplicate on a recipient paraffin block (24). Five-μm sections of these tissue array blocks were cut and placed on charged poly-lysine-coated slides. These sections were used for immunohistochemical analysis (25). Cell lines known to express p73 were used as positive controls (see above).

**IHC.** Five-μm tissue sections were deparaffinized, rehydrated in graded alcohols, and processed using the avidin-biotin immunoperoxidase method. Briefly, sections were submitted to antigen retrieval by microwave oven treatment for 15 min in 10 mM citrate buffer (pH 6.0). Slides were subsequently incubated in 10% normal serum for 30 min followed by the overnight incubation at 4°C with the appropriately diluted primary antibody. The rabbit antismimian CJDp73 polyclonal antibody (~50 ng/μl) was used at a 1:400 dilution to a final concentration of 0.125 ng/μl. The mouse monoclonal antihuman p53 antibody (PAb1801 clone) recognizing an epitope located between amino acids 46 and 55 was used at 0.2 μg/ml (Oncogene Research Products, Boston, MA). p63 was analyzed with the monoclonal 4A4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (20). After the primary antibody, samples were incubated with the biotinylated antirabbit or antimouse immunoglobulins for 30 min, followed by avidin-biotin peroxidase complexes for 30 min (Vector Laboratories, Inc., Burlingame, CA). 3,3′-Diaminobenzidine was used as the chromogen and hematoxylin as the nuclear counterstain. Slides were reviewed by several investigators (P. P., P. C., M. D., C. C-C., and C. J. D.). Some normal tissues were only available as frozen samples. These sections were cut, fixed on formalin for 10 min at 4°C, and analyzed as the paraffin embedded samples, except for the deparaffinization step.

Results were scored in TCC lesions by estimating the percentage of tumor cells showing characteristic nuclear staining. An arbitrarily defined 10% cutoff was taken to classify the TCC data into two categorical groups (positive versus negative). TCC samples were considered to have a negative phenotype for the p73α expression when <10% of nuclei showed staining. The cutoff point for p53, p53 ≥ 20% immunoreactive tumor cells, was selected based on previous publications (19). The cutoff point for p63 was chosen at 30% of tumor cells displaying nuclear immunostaining (20).

**Western Blotting.** For Western blotting, total cell extracts of cultured cells were prepared as described previously (26). The rabbit antismimian CJDp73 polyclonal antibody was used at 1:500 dilution. The anti-HA monoclonal antibody (HA.11) was obtained from Covance (Princeton, NJ) and used at 1:1000 dilution. The anti-myc monoclonal antibody (S1826) was obtained from Clontech (Palo Alto, CA) and used at 1:500 dilution. The goat anti-human Ran polyclonal antibody (C-20, sc-1156) was purchased from Santa Cruz Biotechnology and used at a dilution of 1:1000. Horseradish peroxidase-conjugated antibodies (Amersham, Arlington Heights, IL, and Sigma, St. Louis, MO) were used as secondary antibodies at a 1:1000 dilution. Proteins were visualized with an enhanced chemiluminescence plus detection system (Amersham).

**RNA Isolation and RT-PCR.** Total RNA was extracted from the nine bladder carcinoma cell lines using TRizol (Invitrogen Life Technologies, Inc.) according to manufacturer’s instructions. Total RNA (250 ng) was then amplified using p73 isof orm-specific primers using the Superscript One-Step RT-PCR kit with Platinum Taq (Invitrogen Life Technologies, Inc.) using the manufacturer’s protocol (50 μl of reaction volume). All reverse transcription reactions were carried out for 30 min at 50°C, then 4 min at 94°C, followed by isof orm-specific PCR conditions for each primer set: A, TAp73 isoforms (nucleotides 61–366 of TAp73α): 40 cycles at 94°C (30 s), 56°C (40 s), and 72°C (30 s) using SK053 (sense, 5′-TTCTGGAAACAGACACAGCACAC3′) and SK054 (antisense, 5′-GGGTTAGCTCGTGTTGGAG3′); B, ΔNp73 isoforms (nucleotides 5–218 of...
Characterization of the CJDp73 Antibody. A p73α fusion protein was used for immunization of rabbits by standard methods and an aliquot of the resultant hyperimmune serum, designated CJDp73, was affinity purified (see “Materials and Methods”). Western blot analysis of 293 cell extracts ectopically expressing HA-TAp73α, was affinity purified (see “Materials and Methods”). Isoform-specific RT-PCR (including GAPDH and a no-RNA control) was performed in triplicate. Forty-five μl of RT-PCR products were resolved in 2% agarose gels. Total RNA from H24 cells expressing HA-TAp73α was used as positive control.

Statistical Analyses. In this study, the association between p73α expression levels and histopathological variables of the cases analyzed was evaluated. p73α expression was treated as both a continuous variable and a categorical variable (positive versus negative taking 10% of positive cells as the cutoff point). p63 (positive versus negative) and p53 (wild-type versus mutant) were treated as categorical variables. These determinations were made as a final call at the tumor, as opposed to the core, level. The difference on tumor stages between groups split according to data from p53, p63, and p73α staining was analyzed as categorical variables. p73α expression as a categorical variable was also individually analyzed versus tumor stage and grade. A Fisher’s exact test was used to test the hypothesis of no difference between the categorical groups analyzed. When treating the p73α expression as continuous variable, each tumor was treated as a random effect to account for the inherent but unknown correlation between the different cores of an individual tumor. The data were then fit using the method of restricted maximum likelihood, and an F test was used to test the hypothesis of no difference between groups.

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expressing HA-p73, HA-p53, myc-p63, and vector control constructs demonstrated that CJDp73 recognized both TAp73 and ΔNp73, as well as ΔNp73β but to a lesser extent (Fig. 1). Interestingly, although CJDp73 reacted weakly with ΔNp73, this antibody does not react with TAp73 (Fig. 1). As expected, CJDp73 does not react with p73 and ΔNp63α (Fig. 1). More importantly, CJDp73 does not recognize p53 and ΔNp73 (Fig. 1).

Immunoreactivity of CJDp73 was also examined in tissues and cell lines by IHC. We first titrated the CJDp73 antibody on human normal skin sections and obtained an optimal concentration for IHC of 0.125 ng/μl. At this concentration, the skin displayed intense p73 nuclear immunoreactivity in cells of the basal and parabasal layers of the epidermis, hair follicles, and sweat glands (Table 1). We next examined 293 cells ectopically expressing the seven p73 constructs used in the above Western analysis. Only those cells expressing TAp73 and ΔNp73α displayed intense p73 nuclear immunoreactivity with CJDp73 (Fig. 2). The other p73 isoforms and vector control showed a lack of immunoreactivity (Fig. 2). CJDp73 was raised against p73α and p73β isoforms, the antiserum being mainly directed to the α-derived isoforms, as revealed by both Western blot and IHC. The affinity for the β isoforms is much lower, and it appears to be only over the detection limit of immunoblotting for the ΔNp73β isoform. Transient transfection experiments for IHC and Western blot were conducted in triplicate and in parallel. In all cases, the ΔNp73β isoform was detected by Western blot but not by IHC. Thus, we conclude that CJDp73 is an appropriate reagent to specifically discriminate between p73α and other p73 isoforms by IHC assays.

Expression of p73α in Stratified, Transitional, and Simple Epithelia. In contrast to wild-type p53, where protein expression is rarely detectable by immunohistochemical methods but is present at low levels in all cell types (27), we found a relatively restricted tissue-specific distribution of TA- and ΔN-p73α in normal tissues using the anti-p73 polyclonal antibody CJDp73. Table 1 summarizes the patterns of p73α expression in normal tissues.

We observed that the stratified squamous epithelia studied, which included the epidermis of the skin and tonsil mucosa, displayed intense p73 nuclear immunoreactivity in cells of the basal and parabasal layers of the epidermis, hair follicles, and sweat glands (Table 1). We next examined 293 cells ectopically expressing the seven p73 constructs used in the above Western analysis. Only those cells expressing TAp73α and ΔNp73α displayed intense p73 nuclear immunoreactivity with CJDp73 (Fig. 2). The other p73 isoforms and vector control showed a lack of immunoreactivity (Fig. 2). CJDp73 was raised against p73α and p73β isoforms, the antiserum being mainly directed to the α-derived isoforms, as revealed by both Western blot and IHC. The affinity for the β isoforms is much lower, and it appears to be only over the detection limit of immunoblotting for the ΔNp73β isoform. Transient transfection experiments for IHC and Western blot were conducted in triplicate and in parallel. In all cases, the ΔNp73β isoform was detected by Western blot but not by IHC. Thus, we conclude that CJDp73 is an appropriate reagent to specifically discriminate between p73α and other p73 isoforms by IHC assays.
Expression of p73α in Other Normal Tissues. We observed intense p73α expression in spermatogonia cells of the testis (Fig. 3). p73α was generally undetectable in mesenchymal elements, including smooth and striated muscle. Additionally, p73α was undetectable in lymphocytes. Other organs such as liver, spleen, thyroid, and placenta had undetectable levels of p73α expression (Table 1).

Expression of p73 in Human Transitional Cell Carcinoma Cell Lines. We applied the CJDp73 antibody in immunoblotting and the RT-PCR conditions previously described to the characterization of p73α in a group of nine bladder tumor derived cell lines. Western blot analysis of the seven invasive bladder cell lines revealed a heterogeneous pattern of p73 expression (Fig. 4). Interestingly, only three of the invasive TCC derivatives expressed p73α. They showed two prominent species migrating at Mr 65,000 (T24, 5637, and H-1376). The upper band is likely to be TA.p73α isoform because the HA-tagged TA.p73α ran slightly higher in the same gel (Fig. 4). We further examined the samples with other antibodies to check for the identity of the lower bands (Mr ~65,000 and Mr ~55,000). We could not find any other suggestion to match these bands with ΔN.p73α or ΔN.p73β (data not shown).

In summary, the expression of TAp73α is lost in four of seven invasive tumor-derived cell lines (Fig. 4).

In an attempt to further clarify the identity of the p73 isoforms seen by Western blot, we performed RT-PCR for TA, ΔN, and the various COOH-terminal splice variants of p73 on total RNA isolated from the nine bladder cancer cell lines (Fig. 5). We found that most of the nine cell lines expressed the TA, ΔN, and COOH-terminal splice variants tested (Fig. 5). TAp73 RNA was found in all of the bladder cancer cell lines but in SCaBER and UM-UC-3. We observed a correlation between high expression of TAp73 RNA (Fig. 5) and TAp73α protein accumulation by Western blotting (Fig. 4). Additionally, whereas we detected ΔN.p73 in all of the lines, we did not detect the p73γ splice variant and any TA RNA in the UM-UC-3 cell line, suggesting that these cells do not express any transcriptionally active isoform and only expresses some ΔN.p73 isoform different from gamma. SCaBER cells only expressed the ΔN.p73 isoforms.

Expression of p73α in Bladder Cancer: Relation to Tumor Stage and Grade. We examined 154 well-characterized primary bladder tumors compiled onto three tissue arrays. TCC arrays were analyzed by IHC using CJDp73 antibody (Fig. 6). p73α expression was reported both as continuous and categorical data (positive versus negative). Cores with no tumor or insufficient tumor cells were not used in the final analysis. Similar to what we observed in the invasive TCC-derived cell lines, most invasive tumors lost p73α expression. Although most superficial tumors (28 of 49; 57%) express p73α, only 22 of 105 (21%) invasive TCC lesions retain p73α expression (Fig. 6). p73α expression, as a continuous variable, was found to be statistically associated with tumor stage (P < 0.0001; Table 2). Using Fisher’s exact test, p73α as a categorical variable was also found to be associated tumor stage (P < 0.0001; Table 3).

Analysis of p73 Expression and p53 Status in Bladder Cancer. p53 status was previously analyzed in these 154 tumor samples. We examined the correlation between tumor stage (superficial versus invasive) and the combined data referring to p53 status and p73α expression. Tumors were classified according to phenotypes into four categories (Table 4). The first category included those tumors with wild-type p53 and p73α-

![Fig. 4](image-url)  
Fig. 4 p73 expression levels in bladder cancer cell lines. Protein extracts from seven different bladder tumor-derived cell lines were analyzed by Western blotting using CJDp73 and anti-Ran antibodies. Lanes 1–7 depict levels of p73 on the following TCC lines: J82, T24, HT-1197, 5637, UM-UC-3, TCC-SUP, and HT-1376. A protein extract obtained from 293 cells transiently expressing HA-tagged p73α was loaded onto Lane 10 and was used as a positive control for CJDp73. The bottom panel shows the anti-Ran antibody used in blots from protein extracts at an equivalent loading through all lanes.

![Fig. 5](image-url)  
Fig. 5 Analyses of p73 transcript levels in bladder cancer cell lines. Isoform-specific sets of primers were applied by RT-PCR to a panel of nine bladder cancer cell lines. The first lane shows the water control. Lanes 2–10 show the bladder cell lines in the following order: SCaBER, J82, T24, HT-1197, 5637, UM-UC-3, TCC-SUP, HT-1376, and RT4. The positive control is RNA obtained from a TAp73α-overexpressing cell line (last lane). GAPDH amplification is used as a control. Most bladder cancer cells, with the exception of UM-UC-3, displayed all p73 transcripts.

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positive phenotype. We observed that 22 of 31 (71%) tumors in this group were papillary superficial lesions. The second category included those tumors that had wild-type p53 and a p73-negative phenotype (n = 62). We observed that only 17 of 62 (27%) tumors in this group were superficial lesions. There was a significant difference between these two categories and distribution of tumor stage (P < 0.0001). The third category included those tumors that had a TP53 mutation and a p73-positive phenotype (n = 19). We found that 6 of 19 (32%) cases were superficial lesions in this group. The final category included those tumors with TP53 mutation and a negative p73 phenotype (n = 42). Only 4 of 42 (10%) tumors were found to be superficial lesions. There was a significant difference between these two last groups and tumor stage (P = 0.06). Furthermore, we observed a significant association between p53 status and tumor stage, independent of p73 phenotype. We observed that 39 of 93 (42%) p53 wild-type tumors were non-invasive superficial lesions, whereas only 10 of 61 (16%) p53 mutant tumors were superficial lesions (P < 0.0001). Interestingly, the inhibitory function of p53 over tumor progression decreases when the TCC samples have lost the expression of p73. The group with functional p53 and a negative p73 phenotype is statistically closer to the group with mutant p53 and a negative p73 phenotype than the comparison between the groups segregated only according to p53 status (P = 0.03 versus P < 0.0001). p53 mutations and p73-negative phenotype were associated with bladder cancer progression, as revealed by the comparison of this category with the groups that had a wild-type p53 and a positive p73 phenotype (P < 0.0001; Table 4).

Table 2  Loss of p73α expression, as a continuous variable, is associated with tumor stage

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>p73α expression</th>
<th>Range</th>
<th>Cores</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invasive</td>
<td>4.88 ± 10.27</td>
<td>0–45</td>
<td>243</td>
<td>&lt;0.0001</td>
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<tr>
<td>Superficial</td>
<td>17.60 ± 20.06</td>
<td>0–70</td>
<td>109</td>
<td></td>
</tr>
<tr>
<td>Low grade</td>
<td>22.32 ± 22.11</td>
<td>0–70</td>
<td>44</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>High grade</td>
<td>14.99 ± 17.71</td>
<td>0–55</td>
<td>65</td>
<td></td>
</tr>
</tbody>
</table>

* Mean percentage of tumor cells ± SD.

* F test via method of restricted maximum likelihood.

Table 3  Loss of p73α expression, as a categorical variable is associated with tumor stage

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>p73α expression</th>
<th>Tumors</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invasive</td>
<td>Positive</td>
<td>22</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>83</td>
<td>0.0001</td>
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<tr>
<td>Superficial</td>
<td>Positive</td>
<td>28</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>21</td>
<td>1.00</td>
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<tr>
<td>Low grade</td>
<td>Positive</td>
<td>10</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>7</td>
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<td>32</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>14</td>
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</tbody>
</table>

* Fisher’s exact test.
ground of wild-type p53. More specifically, tumors that had a p53 wild-type and p63/p73α-positive phenotype \((n = 25)\) comprised 21 (84%) superficial lesions, whereas wild-type p53 and p63/p73α-negative phenotype tumors \((n = 31)\) included only 4 (13%) superficial lesions \((P < 0.0001)\). Similarly, the cooperative effect of these three genes is revealed through the significant association to tumor stage. Tumors with a wild-type p53 and positive p63/p73α phenotype \((n = 25)\) included 21 (84%) superficial lesions, whereas tumors carrying p53 mutations and negative p63/p73α phenotype \((n = 32)\) included only 2 superficial lesions \((P < 0.0001)\). There is no statistical difference according to tumor stage between the group with wild-type p53 and negative p63/p73α expression and the samples with mutant p53 and a p63/p73α negative phenotype \((P = 0.43)\).

### DISCUSSION

Using the affinity-purified anti-p73 polyclonal antiserum CJDp73 in immunohistochemical assays, we found that it detects p73α isoforms specifically, not identifying any other p73 isoforms tested. When applied to human normal adult tissues, we observed p73α immunoreactivities in basal cells lining glandular epithelia such as breast and prostate but not in the luminal cells of these organs. p73α was also expressed in basal cell layers of stratified epithelia such as the skin epidermis and in all cells of the transitional epithelium of the ureter and urinary bladder. Expression of p73α in proliferating cells such as spermatogonia and basal epidermal cells suggests a potential role for p73α in mediating tumor suppression activity, as suggested by Flores et al. (12). Thus, accumulation of p73α might have a

### Table 4

Altered expression of p53 and p73α is associated with tumor stage

<table>
<thead>
<tr>
<th>p53 status</th>
<th>p73α expression</th>
<th>Histology</th>
<th>Tumors</th>
<th>(P^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Positive</td>
<td>Superficial</td>
<td>22</td>
<td>9</td>
</tr>
<tr>
<td>Wild type</td>
<td>Negative</td>
<td>Invasive</td>
<td>17</td>
<td>45</td>
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<tr>
<td>Mutant</td>
<td>Positive</td>
<td>Superficial</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>Mutant</td>
<td>Negative</td>
<td>Invasive</td>
<td>4</td>
<td>38</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>39</td>
<td>54</td>
</tr>
</tbody>
</table>

\(P^a\) (p53–p73)

<table>
<thead>
<tr>
<th>Wild type-negative</th>
<th>Mutant-positive</th>
<th>Mutant-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>(&lt;0.0001)</td>
<td>0.01</td>
<td>(&lt;0.0001)</td>
</tr>
</tbody>
</table>

\(P^a\) (p53–p73)

<table>
<thead>
<tr>
<th>Wild type-negative</th>
<th>Mutant-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>

\(a\) Fisher’s exact test.

### Table 5

Altered expression of the p53 family members is associated with tumor stage

<table>
<thead>
<tr>
<th>p53 status</th>
<th>p63 expression</th>
<th>p73α expression</th>
<th>Histology</th>
<th>Tumors</th>
<th>(P^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Positive</td>
<td>Positive</td>
<td>Superficial</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>Wild type</td>
<td>Negative</td>
<td>Negative</td>
<td>Invasive</td>
<td>4</td>
<td>27</td>
</tr>
<tr>
<td>Wild type</td>
<td>Positive</td>
<td>Positive</td>
<td></td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Mutant</td>
<td>Positive</td>
<td>Positive</td>
<td></td>
<td>11</td>
<td>18</td>
</tr>
<tr>
<td>Mutant</td>
<td>Negative</td>
<td>Negative</td>
<td></td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Mutant</td>
<td>Negative</td>
<td>Positive</td>
<td></td>
<td>2</td>
<td>30</td>
</tr>
<tr>
<td>Mutant</td>
<td>Positive</td>
<td>Negative</td>
<td></td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>46</td>
<td>104</td>
</tr>
</tbody>
</table>

\(P^a\) (p53-p63-p73)

<table>
<thead>
<tr>
<th>Mutant-positive-positive</th>
<th>Mutant-negative-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.08</td>
<td>(&lt;0.0001)</td>
</tr>
</tbody>
</table>

\(P^a\) (p53-p63-p73)

<table>
<thead>
<tr>
<th>Wild type-negative-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.43</td>
</tr>
</tbody>
</table>

\(a\) Used Fisher’s exact test.
preventive function in the event of cellular stress, which could menace the integrity and viability of the cell. The accumulation of p73α, as well as that reported for p63 (20), may prepare the cell to react to genotoxic insults.

The expression pattern of p73α in normal tissue is similar to that reported for p63 (28). However, there are certain differences, including the more predominant expression of p63 on the suprabasal layer of stratified epithelia when compared with the intense basal expression of p73. In addition, p63 was not detected in umbrella cells of the bladder, whereas p73α is expressed in these cells (Fig. 3). Similarly, p63 was not detected in the pancreas, whereas we report herein the expression of p73α in exocrine pancreas.

Our previous observation that normal bladder urothelium expresses high levels of p63 and that the loss of p63 expression is associated with tumor progression in bladder cancer (20) prompted us to investigate the role of p73 in transitional cell carcinoma. TAp73α expression was lost in four of seven invasive derived cell lines (Fig. 4). We also observed that p73α was more frequently lost in invasive TCC cases with superfi cial invasive TCC lesions. Statistical analyses revealed that loss of p73α expression was a frequent event associated with advanced tumor stage in bladder cancer. Our findings are in agreement with a study recently reported showing a decline in p73 expression in esophageal cancer (29).

In our study, we also compared p73α, p63, and p53 expression patterns in the same cohort of bladder cancer patients (19, 20). Although the three genes have some shared functions their expression patterns in normal tissue are different. Although p53 levels are very low, as a matter of fact undetectable by IHC, p63 and p73 are usually expressed in the nuclei of certain normal cells, including urothelium. Interestingly, it appears that the loss of p63 and p73 expression, as assessed by IHC, represents an epigenetic tumor suppressor event, because tumor-specific mutations have been reported to be very rare (see below).

p73α expression and p53 status were both significantly associated with tumor stage. We observed that the impact of p53 status in samples displaying the p73α-positive phenotype was less significant than p73α expression in samples with wild-type p53. Thus, p73α activity appears to have a critical tumor suppression function. Moreover, p73α expression and p53 status were found to have a negative cooperative effect and to be significantly associated with tumor stage.

We also observed a negative cooperative effect for the three p53 family members. Tumors with wild-type p53 and positive p63 and p73α phenotype had a significantly better prognosis than those with wild-type p53 and undetectable p63 and p73α. The dependence of p53 on p63 and p73α expression is in concordance with the results of Flores et al. (12). These investigators have reported that p63 and p73 expression is needed to induce a p53-dependent response to DNA damage in E1A-expressing cells and in the developing mouse central nervous system. There is a strong association between p53, p63, and p73 alterations with bladder cancer progression, as revealed by comparing the group with wild-type p53 and positive p63 and p73α phenotype versus the p53 mutant group lacking p63 and p73α expression with tumor stage (Table 5). The collaborative effect of p63 and p73α on the p53 function is also revealed by comparing the TCC samples with wild-type p53 versus mutant p53 when both groups share a negative phenotype for p63 and p73α. The dependence of the wild-type p53 function on p63 and p73α to block bladder tumor progression can be hypothesized because both groups have a highly similar fraction of invasive tumors, and the only difference is the p53 status. On the basis of data presented here, it can be postulated that assessment of p53 status alone might not be as robust as if incorporating the status of p63 and p73α.

In conclusion, this study reports the characterization of the CIDp73 antibody by Western blot and IHC on normal and tumor tissue, as well as the RT-PCR study of p73 expression in nine bladder tumor-derived cell lines. It also reports the significant association between p73α loss and advanced tumor stage in bladder cancer. In addition, it describes for the first time the negative collaborative effect produced by alterations of the three p53 family members in bladder cancer progression using a large and well-characterized cohort of patients. Results from this study support a tumor suppressor role for p73α, where inactivation appears to be through epigenetic events in bladder cancer.

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Clinical Cancer Research

p73 Expression in Human Normal and Tumor Tissues: Loss of p73 α Expression Is Associated with Tumor Progression in Bladder Cancer

Pere Puig, Paola Capodieci, Marija Drobnjak, et al.


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