Impaired p63 Expression Associates with Poor Prognosis and Uroplakin III Expression in Invasive Urothelial Carcinoma of the Bladder

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ABSTRACT

Purpose: p63 is proposed to play roles in normal development and differentiation of stratified epithelia including urothelium. We recently reported that impaired p63 expression is a common feature of high-grade invasive urothelial carcinomas and associates with reduced β-catenin. On the basis of these facts, we propose that impaired p63 expression contributes to biological aggressiveness of urothelial neoplasms. Uroplakin (UP) III expression was also evaluated to investigate a possible association between loss of p63 expression and terminal urothelial differentiation.

Experimental Design: Expression of p63, β-catenin, and UP III was immunohistochemically analyzed in 75 cystectomy specimens of high-grade invasive bladder carcinoma. p63 expression was semiquantified and compared with pathological parameters, expression of β-catenin and UP III, and cancer-specific survival.

Results: Lower p63 expression was significantly associated with higher Tumor-Node-Metastasis (TNM) stage (P = 0.0004), lymph-node metastasis (P = 0.013), and reduced β-catenin expression (P = 0.003). By univariate analysis, lower p63 expression, along with TNM stage and lymph-node status, were significantly associated with a poor prognosis (P = 0.0005), whereas reduced β-catenin was not. By multivariate analysis, the prognostic effect of p63 expression was independent of TNM stage and lymph-node status with marginal statistical significance (P = 0.074). UP III expression was restricted to a subset of p63-negative carcinoma cells, including even anaplastic carcinoma cells.

Conclusions: Impaired p63 expression characterizes biological aggressiveness of high-grade invasive urothelial carcinomas. Moreover, loss of p63 expression is a prerequisite for UP III expression. Our data suggest that p63 plays critical roles in tumor progression and biochemical terminal differentiation of urothelial neoplasms.

INTRODUCTION

p63, also known as p51, p40, p73L, or KET (1–5), is a homologue of the p53 tumor suppressor gene located at 3q27–29. This gene encodes for two major classes of protein: those containing an acidic amino terminus analogous to the transactivation domain of p53 (TAp63) and those with a truncated amino terminus that lacks this region (ΔNp63). TAp63 isoforms can transactivate p53 target genes and induce apoptosis when overproduced (1, 5), whereas ΔNp63 isoforms potentially suppress transactivation by both p53 and TAp63 isoforms in a dominant-negative manner (5). In addition, alternative splicing yields at least three p63 carboxyl termini (α, β, and γ) that could modify the transcriptional activity of p63 protein (1, 5). Therefore, at least six p63 isoforms are possible.

Unlike the conditionally expressed p53, p63 is constitutively expressed in the basal cell/progenitor cell compartment of many epithelial tissues, in which the ΔNp63 products account for virtually all p63 protein expressed (5). The p63-null mice show lack of limbs and severe epithelial defects of the skin, prostate, breast, and urogenital tract (6–9). In the skin of the p63-null mice, epidermal cells could not sustain stem cell populations and eventually undergo apoptosis via terminal differentiation (7). These facts indicate that ΔNp63 plays a key role in normal development, in maintaining progenitor cell populations, and in regulating differentiation of these epithelial cells. The urothelium of the p63-null mice comprises a single-layered cuboidal epithelium lacking normal urothelial structure (9). On the basis of these data, ΔNp63 appears to play critical roles in maintaining the normal urothelial structure and is possibly associated with urothelial differentiation.

Despite the initial enthusiasm surrounding the cloning of p63 as a homologue of the p53 gene, a rare incidence of p63 mutations in human tumors indicates that p63 is unlikely to be a tumor suppressor gene that conforms to the two-hit model of carcinogenesis (1, 10–12). Instead, ΔNp63 is considered to have oncogenic properties based on the following observations: first, ΔNp63 overexpression is often observed in and enhances oncogenic growth of squamous cell carcinomas (13, 14); second, ΔNp63 could function as dominant-negatives against the p53 tumor suppressor activities (5); third, ΔNp63 overexpression induces nuclear accumulation of β-catenin and activates β-catenin signaling that promotes cell proliferation (15).
With regard to clinical management, urothelial neoplasms are divided into two major phenotypic variants, low-grade papillary noninvasive (T<sub>a</sub>) tumor and high-grade invasive (T<sub>1</sub>-T<sub>4</sub>) carcinoma (16, 17). The former has limited potential to progress to invasive disease and eventually provides excellent prognosis (16, 17). The latter originates in carcinoma in situ and results in a high risk of the development of incurable distant metastases (18). Thus far, three studies have investigated the role of p63 in urothelial neoplasms. The first group of investigators proposed that overexpression of /<sub>H9004</sub>Np63 mRNA or down-regulation of TAp63 mRNA relates to carcinogenesis and tumor progression (12). In contrast, we demonstrated that high-grade invasive urothelial carcinomas frequently diminish /<sub>H9004</sub>Np63 expression, whereas low-grade papillary noninvasive tumors highly preserve the normal p63 expression pattern characterized by the abundant and well-organized /<sub>H9004</sub>Np63 expression (19), which is consistent with another study (9). These conflicting findings prompted us to investigate the prognostic value of /<sub>H9004</sub>Np63 expression in urothelial neoplasms. In the previous study, we also found that the impaired ΔNp63 expression associates with reduced expression of β-catenin (19), which plays a crucial role in cell-cell adhesion (20). On the basis of these facts, we propose that impaired ΔNp63 expression contributes to biological aggressiveness of urothelial neoplasms. In the current study, we focused on high-grade invasive bladder carcinoma, the life-threatening phenotypic variant of urothelial neoplasms, and investigated the prognostic value of ΔNp63 expression, along with that of β-catenin expression. We further assessed expression of UP<sup>3</sup> III, a component of the plaques of the asymmetric unit membrane lining apical surface of umbrella cells and a putative terminal differentiation marker of urothelial derivatives, in the same tumor cohort to evaluate a possible association between p63 expression and terminal urothelial differentiation.

**MATERIALS AND METHODS**

**Patients.** Included in this study was a cohort of 75 bladder urothelial carcinoma patients (17 females and 58 males) undergoing radical cystectomy between January 1983 and December 1997. The median age was 67 years (range, 35–80 years). None of the patients had received cytotoxic therapy before cystectomy. None of the patients had evidence of distant metastasis at cystectomy. Histological grade of the tumor was determined according to the 1973 WHO grading system (21). The stage of the tumor was determined based on the 1997 TNM system (22). Informed consent was obtained from each patient before commencing this study. End point for survival analysis was bladder cancer death.

**Immunohistochemistry.** The deepest invasive portion of bladder cancer was selected to evaluate its biological aggressiveness. Immunostaining was performed on 5-μm paraffin-embedded tissue sections using a mouse antihuman p63 monoclonal antibody 4A4 (Santa Cruz Biotechnology, Santa Cruz, CA).
CA) and a mouse antihuman β-catenin monoclonal antibody clone 14 (Transduction Laboratories, Lexington, KY), as described previously (19). UP III expression was examined using ready-to-use solution of a mouse antihuman UP III monoclonal antibody AU1 (Progen, Heidelberg, Germany; Refs. 23–25) on the serial tissue sections immunostained for p63. Briefly, the sections were rehydrated, treated in 0.3% hydrogen peroxide in methanol, pretreated in a 550 W microwave oven for 15 min in 10 mM citrate buffer (pH 6.0), and incubated with 10% goat serum. The sections were incubated with the 4A4 at a 1:200 dilution for 60 min at room temperature, with the clone 14 at 1:250 dilution overnight at 4°C or with the AU1 for 60 min at room temperature. After incubation with a horseradish peroxidase-labeled secondary antibody [HISTOFINE Simple Stain MAX PO(M), Nichirei, Tokyo, Japan] for 30 min at room temperature, color was developed with 3,3-diaminobenzidine (Nichirei). Finally, the sections were counterstained slightly with hematoxylin. Negative controls were included by replacement of the primary antibody with PBS.

Evaluation of Immunoreactivity. The slides were independently reviewed by two of the authors (K. F. and O. Y.) who were blinded to the clinicopathological data. Because the tumor samples showed various degrees of staining intensity and different numbers of positive cells, p63 immunoreactivity was semiquantified using a combined intensity and percentage of positive scoring method (26, 27). Intense nuclear staining was scored as 2, weak as 1, and negative as 0. Normal urothelial cells of the basal to intermediate layer showed intense nuclear staining, which was used as an internal positive control of intensity 2 (19). The percentage of cells with each intensity score was estimated. A p63 staining score was defined as the sum of the percentage of positive cells with each intensity level multiplied by the intensity score [e.g., a case with 60% intense staining and 20% weak staining would be scored as 140 (60 × 2 + 20 × 1)].

We have shown previously that Np63 isoforms account for p63 protein expressed in normal and neoplastic urothelial tissues (19). To validate this scoring system, we assessed a correlation between p63 staining scores and Np63 mRNA levels in another set of 32 urothelial tumor samples. The p63 staining scores correlated significantly with Np63 mRNA levels (the Spearman’s rank correlation test, \( r_s = 0.61; P = 0.0004 \)), confirming the validity of this scoring system.

As described previously, nuclear β-catenin expression is rare in urothelial carcinomas (19). Therefore, we evaluated membranous β-catenin expression exclusively in this study. The normal urothelium shows homogeneously membranous β-catenin immunoreactivity at virtually all intercellular borders (19, 28). If the β-catenin staining pattern in carcinoma tissues was similar to this normal pattern, it was evaluated as normal. Negative and heterogeneous β-catenin staining was evaluated as reduced (28).

Statistical Analysis. The independence of variables was assessed using the Fisher’s exact test. The difference in non-parametric data between two or more groups was assessed using the Mann-Whitney U test. Survival curves were calculated by the Kaplan-Meier method and the difference among survival curves was analyzed by the log-rank test. Associations of variables with CSS were tested using univariate and multivariate analyses by the Cox proportional hazard model. Differences were considered significant at \( P < 0.05 \).

RESULTS

Patient Characteristics. A total of 75 tumors consisted of 27 T1, 20 T2, 23 T3, and 5 T4 urothelial carcinoma of the bladder. All of carcinoma samples contained grade 3 urothelial carcinoma component. Pelvic lymph-node metastasis was pathologically confirmed in 13 patients (17%). Follow-up periods after radical cystectomy ranged from 2 to 196 months.

![Box plot of p63 staining scores according to TNM stage (A), lymph-node status (B), and β-catenin expression status (C) in 75 high-grade invasive bladder carcinomas. The horizontal line, ends of each box, and lines across the middle of each box identify the mean value of the all samples, the 25th and 75th quartiles and median values, respectively. Whiskers indicate 1.5 times the interquartile range.](clincancerres.aacrjournals.org)
During these periods, 19 patients died of bladder cancer. None of the 27 patients with T1 carcinoma died of bladder cancer. A 5-year CSS rate of this cohort was 72%.

**Impaired p63 Expression Associates with Advanced Disease and Reduced β-catenin.** p63 expression was restricted to the nuclei of normal and neoplastic urothelial cells in bladder tissues (Fig. 1, A and B). In normal urothelium, almost entire basal to intermediate cells showed p63 immunoreactivity of intensity 2, but umbrella cells showed immunoreactivity of intensity 0. In carcinoma tissues, p63 immunoreactivity was heterogeneous and its distribution was chaotic particularly in invasive portions. The p63 staining scores ranged from 0 to 180 (median, 70; mean ± SD, 74.6 ± 44.9). Lower p63 staining scores were significantly associated with higher TNM stage (P = 0.0004) and positive lymph-node metastasis (P = 0.013; Fig. 2, A and B). p63 staining scores of T1 carcinomas were significantly higher than those of T2–4 (data not shown; P = 0.0013).

β-catenin expression was normal in 38 carcinomas (51%, Fig. 1C), whereas it was reduced in 37 (49%, Fig. 1D) including 11 with negative expression. β-catenin expression did not associate with pathological parameters (Table 1). As shown in Fig. 2C, the reduced β-catenin group showed a significantly lower p63 staining score than the normal β-catenin group (P = 0.003). When cases were divided into high and low p63 expression groups at the mean p63 staining score of 74.6, low p63 expression was significantly associated with reduced β-catenin (P = 0.011, Table 1).

**Impaired p63 Expression Associates with Unfavorable Clinical Outcome.** Next, we evaluated prognostic value of p63 and β-catenin expression in the 75 patients. As shown in Fig. 3A, the low p63 expression group showed significantly shorter CSS than the high p63 expression group with respective 5-year CSS rates of 53% versus 91% (P = 0.0008). β-catenin expression was not associated with CSS (Fig. 3B). Table 2 summarized results of univariate and multivariate analyses by the Cox proportional hazard model. By univariate analysis, both higher TNM stage and positive lymph-node status were identified as significant and independent prognostic indicators. In a multivariate analysis including p63 expression, TNM stage, lymph-node status, and β-catenin expression, the prognostic power of p63 expression was marginally significant (P = 0.074). TNM stage and lymph-node status were identified as significant and independent prognostic indicators.

**UP III Expression Restricted to p63-Negative Carcinoma Cells.** Finally, we evaluated UP III expression to reveal the possible association between p63 expression and terminal urothelial differentiation. UP III is not only highly specific for urothelial neoplasms (23, 29), but also proposed as a marker of their terminal differentiation (23, 29, 30). Expectedly, UP III expression was restricted to the apical surface of umbrella cells.

### Table 1

<table>
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<tr>
<th>Pathological parameters</th>
<th>β-Catenin expression</th>
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<td>Normal</td>
<td>Reduced</td>
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<tr>
<td>TNM stage</td>
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<tr>
<td>T1</td>
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</tr>
<tr>
<td>T2–4</td>
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<td>27</td>
</tr>
<tr>
<td>Lymph-node metastasis</td>
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</tr>
<tr>
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<td>29</td>
</tr>
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<td>Low</td>
<td>14</td>
<td>25</td>
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*a* Fisher’s exact test.

b Cases were divided into high and low groups at the mean p63 staining score of 74.6.
in normal urothelium (Fig. 4A), whereas p63 expression was absent from these cells (Fig. 4B). In the current cohort, 23 carcinomas (31%) expressed UP III. Of the 23 carcinomas, 14 expressed UP III in invasive portions, whereas the others expressed UP III in only superficial portions. In carcinoma tissues, UP III expression pattern took the form of heterogeneously linear staining of the cell membrane of carcinoma cells bordering the stroma (Fig. 4C). Less frequently, sporadic cytoplasmic UP III staining was observed even in morphologically anaplastic carcinoma cells (Fig. 4E). UP III expression in carcinoma cells, if present, was virtually restricted to a subset of p63-negative cells (Fig. 4, C and D). UP III expression did not associate with TNM stage, lymph-node metastasis (Table 3), or CSS (Fig. 3C).

**DISCUSSION**

This is the first study to demonstrate not only the prognostic value of p63 expression, but also its association with UP III expression in urothelial neoplasms. The current study investigated the roles of p63 in high-grade invasive urothelial carcinoma of the bladder. First, we showed that impaired p63 expression is associated with more advanced disease and reduced β-catenin. This is consistent with our previous study showing that impaired ΔNp63 expression associates with aggressive pathological phenotypes and reduced β-catenin in urothelial neoplasms including low-grade papillary noninvasive tumors (19). Second, impaired p63 expression, but not reduced β-catenin, was correlated with an unfavorable clinical outcome. Third, a subset of p63-negative carcinoma cells expressed UP III. In addition, we demonstrated a limited effect of UP III expression status on tumor progression and prognosis.

Low-grade papillary noninvasive urothelial tumors of the bladder provide excellent prognosis, whereas high-grade invasive carcinomas are life-threatening (16–18). Therefore, we investigated the prognostic value of p63 expression in high-grade invasive bladder carcinomas. The current study demonstrated that impaired p63 expression is another prognostic marker along with the established prognostic factors, such as TNM stage and lymph-node status, indicating that impaired ΔNp63 characterizes biological aggressiveness of urothelial neoplasms. Thus, the roles of ΔNp63 in urothelial neoplasms differ from those in squamous cell carcinomas where overexpression of ΔNp63 associates with oncogenic growth (13).

β-Catenin plays a role in cell-cell adherent junctions (20). Cancer invasion and metastasis could be promoted by reduced β-catenin, reflecting possible defective cell-cell adherent junctions (20). Our current study and a previous study (19) have shown that impaired ΔNp63 expression associates strongly with reduced β-catenin in urothelial neoplasms. Although impaired ΔNp63 expression had a prognostic impact in high-grade invasive bladder carcinoma, reduced β-catenin did not. Because a recent in vitro study has clearly shown that ΔNp63 regulates β-catenin levels (15), reduced β-catenin might be ascribed to impaired ΔNp63 expression in urothelial neoplasms.

The mechanisms underlying the impaired p63 expression remain unrevealed in urothelial carcinomas. Genetic mutations of p63 or p53, at least, do not seem to be involved (9, 12, 19). We investigated the possible association of p63 expression with expression of UP III, a putative terminal differentiation marker of urothelial cell derivatives (23, 29, 30). In the normal axis of urothelial differentiation, UP III is expressed only after extinction of ΔNp63. Interestingly, carcinoma cells of urothelial origin still retain this nature. These findings suggest that loss of p63 is a prerequisite for terminal differentiation. In the keratinocyte-squamous cell carcinoma lineage, down-regulation of ΔNp63 is clearly correlated with terminal differentiation (14). In addition, the p63-null mouse study strongly indicates that ΔNp63 prevents p63-bearing cells from differentiating toward a terminal stage (7). Our data imply that p63 plays a role in regulating terminal differentiation even in aggressive urothelial carcinomas.

Morphologically anaplastic urothelial carcinomas are described as “undifferentiated” carcinomas (31) and anticipated not to have a feature of terminal differentiation. Surprisingly, the current study demonstrated that a subset of anaplastic urothelial carcinoma cells express UP III in an aberrant manner. UP III expression status was not related to tumor progression or

### Table 2  Associations between variables and CSS by the Cox proportional hazard model in high-grade invasive bladder cancer patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate Hazard ratio (95% CI)*</th>
<th>P</th>
<th>Multivariate Hazard ratio (95% CI)</th>
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<tr>
<td>p63 expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>Reference</td>
<td></td>
<td>Reference</td>
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<tr>
<td>Low</td>
<td>2.51 (1.45–5.21)</td>
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<td>1.74 (0.96–3.77)</td>
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<tr>
<td>β-Catenin expression</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>Reference</td>
<td></td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>Reduced</td>
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<td>0.92 (0.58–1.49)</td>
<td>0.73</td>
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<tr>
<td>T1</td>
<td>Reference</td>
<td></td>
<td>Reference</td>
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<tr>
<td>T2,T3,T4</td>
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<td>ND</td>
<td>0.0008</td>
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<tr>
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<td>Reference</td>
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<tr>
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<td>2.78 (1.76–4.42)</td>
<td>&lt;0.0001</td>
<td>1.66 (1.03–2.71)</td>
<td>0.039</td>
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*CI, confidence interval.
†Cases were divided into high and low groups at the mean p63 staining score of 74.6.
ND, not determined, because none of 27 cases with T1 disease died of cancer.
prognosis, unlike the degree of morphological differentiation or histological grade that reflects well biological aggressiveness of urothelial neoplasms (23, 29). Previous studies on urothelial neoplasms demonstrated that UP III expression is independent of histological grade (23, 29). These facts depict a discrepancy between biochemical and morphological differentiation of urothelial neoplasms. Considering that p63 possibly regulates differentiation of urothelial cells, it is conceivable that highly aggressive urothelial carcinomas that have already lost ΔNp63 expression can undergo biochemical urothelial differentiation. In the normal urothelial lineage, integrated molecular pairing of UP Ia/II and UP Ib/III forms the plaques of the asymmetric unit membrane that stabilize urothelial structure and contribute to permeability barrier function of the urothelium (24), which represents the true and functional terminal urothelial differentiation. In urothelial neoplasms, however, UPs do not appear to be assembled into the functional urothelial plaques (25, 29, 30), indicating that UP III expression reflects biochemical, but not functional, terminal differentiation. Taken together, aggressive urothelial carcinomas might opportunistically express UP III after they have lost ΔNp63 expression during their progression.

In conclusion, we have demonstrated clearly that impaired

<table>
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<th>Variables</th>
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* Fisher’s exact test.
p63 expression characterizes biological aggressiveness in high-grade invasive urothelial carcinomas of the bladder. Moreover, loss of p63 expression is a prerequisite for UP III expression. Our data suggest that p63 plays critical roles in tumor progression and biochemical terminal differentiation of urothelial neoplasms.

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