Expressions of p16 and p27 in urothelial carcinoma and their prognostic value

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KEYWORDS
Immunohistochemistry; p16; p27; Urothelial carcinoma

Abstract
Expressions of human p16 and p27 were tested for correlations with clinicopathologic features of urothelial carcinoma (UC). Tissue microarrays (TMA) constructed from paraffin-embedded specimens from 78 patients with UC were analyzed by immunohistochemical staining. In 49 of the 78 tumors (63%), high p16 expression was associated with absence of tumor invasiveness and low-grade carcinoma (p = 0.003 and p = 0.046, respectively). The p27 expression was high in 33 of the 78 tumors (42%) and showed a significant negative association with invasiveness, carcinoma grade, and tumor size (p = 0.016, p = 0.046, and p = 0.014, respectively). Kaplan–Meier analysis indicated that patients with high p27 levels had longer than average overall survival (p = 0.021). This study demonstrates that p16 and p27 are prognostic indicators of tumor stage and grade in UC and that they provide clinicians with the ancillary information needed for selecting suitable therapeutic strategies.

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Introduction
Urothelial carcinoma (UC), the most common histological type of malignant urinary tumor, can arise anywhere in the urothelial lining of the urinary tract, from the urethra to the renal pelvis. Based on its morphology and invasiveness, this malignancy can be classified as low grade or high grade, and noninvasive or infiltrating, respectively [1]. In Taiwan, an unusually high incidence of upper urinary tract UC has...
been reported [2,3]. A recent study showed that one question remaining to be answered is whether the biological characteristics of UC differ by anatomical location [4]. Stage, lymph node metastasis, and grade are well-documented conventional prognostic factors for UC, but these factors are inadequate to successfully predict which patients will experience recurrence and/or metastasis [5,6].

Cell cycle progression is controlled by the complex interactions of cyclins, cyclin-dependent kinases (CDKs), and their inhibitors. The best prognostic markers for survival, recurrence, and progression are those involved in the G1/S phase transition, such as cyclin D1 and E, and cyclin-dependent kinase inhibitors (CDKI), such as p27 and p16 [7,8]. Two families of CDKI, INK4 (p16, p15, p18, and p19) and CIP/KIP (p21, p27, and p57), regulate cell proliferation and are essential for preventing neoplastic transformation. Most of these cell cycle proteins have been analyzed in immunohistochemical (IHC) studies of various carcinomas. The expressions of some cell cycle proteins reportedly predict recurrence and disease progression in patients with bladder UC [9–11].

At chromosome 9p21, a region that is often altered in various tumor types [12–15], the p16 gene acts as a tumor suppressor by negatively regulating the G1/S cell cycle. Point mutation, promoter hypermethylation, and p16 deletion are common in various human malignancies [16–23]. Mutation of p16 reportedly has a major role in early carcinogenesis and progression of many tumors [15,24–26]. Additionally, p27, a member of the CIP/KIP family of proteins, regulates the CDKs and, as a tumor suppressor, it is a major negative regulator of the G1–S cell cycle. Reduced p27 protein expression may result in tumor development and/or progression [27].

This study investigated the relevance of p16 and p27 immunoeexpression and evaluated its prognostic value in UC, particularly in terms of clinicopathologic parameters.

Materials and methods

Patients and follow-up

This study analyzed 78 UC specimens archived between 1995 and 2000 by the Department of Pathology, Kaohsiung Medical University Hospital, Kaohsiung City, Taiwan. All 78 patients had undergone nephrectomy, ureterectomy, ureteroscopic tumor excision, or cystectomy with complete tumor resection. Histologic data collection included invasiveness and tumor grade according to the latest World Health Organization (WHO) classification [1]. The analysis included 14 urinary bladder, 37 ureter, and 27 renal pelvis specimens obtained from 27 males and 51 females with UC (age range, 21–83 years; median age, 68 years). Fifty-four tumors were classified as invasive, and 24 were classified as noninvasive. Eleven tumors were classified as low grade, and 67 were classified as high grade. The duration of patient follow-up was calculated as the number of months from the date of the positive diagnostic surgical procedure to the date of the most recent cystoscopy, the most recent visit, or death. Of the 78 treated patients, 68 had survived until the median follow-up period (59.2 months). When calculating disease-free survival, patients with any recurrence (local, regional, or distant) or patients who had died from any cause were classified as failures at the time of recurrence or death; all other patients were surveyed at the last follow-up. When calculating overall survival, UC-related deaths were counted as failures at the time of death; all other patients were surveyed at the last follow-up.

Tissue microarray

The tissue microarray (TMA) was constructed using formalin-fixed paraffin-embedded UC tissue samples. All original slides were classified by two pathologists (C.C.W. and C.Y.C) according to WHO criteria [1]. Slides containing the representative area of the tumor were circled with a color pen. In each case, one core of the tumor (diameter, 2.0 mm) was carefully transferred with forceps from the selected areas to the recipient metal paraffin block box. Four-μm sections of the TMA block were cut and stained with hematoxylin-eosin to verify that the cores adequately represented diagnostic areas.

IHC staining

The IHC staining to detect p16 and p27 was performed by the streptavidin-biotin method. Briefly, sections were deparaffinized and autoclaved at 121°C for 10 minutes in 0.1M of a pH 6.0 citrate buffer (for p16) or in 0.1M of a pH 9.0 Target Retrieval Solution (for p27). Endogenous peroxidase in the section was blocked by incubation in 3% hydrogen peroxide for 5 minutes at room temperature. After washing with Tris buffer solution (TBS) and incubation with goat serum for 1 hour, the sections were incubated with primary antibodies p16 (Clone F12, 1:50 dilution; Santa Cruz Biotechnology, Dallas, CA, USA) and p27 (Clone F8, 1:50 dilution; Santa Cruz Biotechnology) at room temperature for 30 minutes. Biotinylated second antibody and peroxidase-conjugated streptavidin from the DAKO Universal LSAB kit (DAKO, Glostrup, Denmark) were applied for 20 minutes each. Finally, sections were incubated in 3’3’-diaminobenzidine (DAB) for 5 minutes, counterstained with hematoxylin, and mounted on a slide. Negative controls were obtained by replacing the primary antibody with nonimmune serum. Known tonsil and breast carcinoma cases positive for p16 and p27 were used as positive controls. Strong staining of lymphocytes was also used as an alternative internal control for p27.

Immunostaining evaluation

Immunostaining intensity was evaluated by light microscopy as described elsewhere [11]. Protein expressions were evaluated by two independent observers with no knowledge of the clinicopathologic data. The immunohistochemical results were evaluated using semiquantitative analysis. The samples were stratified into the following three groups according to percentage of positive cell nuclei: 1–10%, 11–50%, and >50%. After the first statistical analysis showed no significant difference between these three predefined cutoff values, ≤10% was defined as negative, and >10% was defined as positive. For p16, cells...
with cytoplasmic staining but no nuclear staining were considered negative [28,29].

Statistical analysis

Statistical analysis was performed by Chi-square test. Survival curves were calculated by the Kaplan–Meier method, and log-rank tests were performed. All statistical analyses were performed using the SPSS version 14 (SPSS Inc., Chicago, IL, USA). A p value < 0.05 was considered statistically significant.

Results

The analysis of p16 expression showed a combination of nuclear and cytoplasmic staining in several tumors, but only cells with clear nuclear staining were considered positive. High and low p16 expressions were observed in 49 (63%) and 29 (37%) cases, respectively. For p27, high and low protein expression was detected in 33 (42%) and 45 (58%) tumors, respectively (Fig. 1). Table 1 shows the IHC expression of p16 and p27 in relation to clinicopathologic variables. The p16 expression levels showed significant negative correlations with tumor invasiveness and grade (p = 0.003 and p = 0.046, respectively) whereas p27 expression showed significant negative correlations with tumor invasiveness, grade, and size 3.0 cm (p = 0.016, p = 0.046, and p = 0.014, respectively). Other parameters, including sex, age, and tumor location, were unassociated with p16 and p27 expression. In addition, significant correlation was found between expression levels of p16 and p27 (p = 0.043). When using the Kaplan-Meier method to test actuarial disease-related survival according to overall survival and disease-free survival for correlations with p16 and

<table>
<thead>
<tr>
<th>Factors</th>
<th>p16 expression</th>
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<th>p27 expression</th>
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<tbody>
<tr>
<td></td>
<td>Low (%)</td>
<td>High (%)</td>
<td>p value</td>
<td>Low (%)</td>
</tr>
<tr>
<td>Sex</td>
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<tr>
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<td>Female</td>
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<td>Age (y)</td>
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<tr>
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<td>24 (62)</td>
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<tr>
<td>≥ 68</td>
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<tr>
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<td>24 (65)</td>
<td>0.723</td>
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<tr>
<td>≥ 3</td>
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<td>25 (61)</td>
<td></td>
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<td>Location</td>
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<td>Urinary bladder</td>
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<td>11 (79)</td>
<td>0.237</td>
<td>5 (36)</td>
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<td>Kidney</td>
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<td>20 (54)</td>
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<td>18 (67)</td>
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<td>Tumor invasiveness</td>
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<tr>
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<td>26 (48)</td>
<td>28 (52)</td>
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<tr>
<td>Absent</td>
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<td>21 (87)</td>
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<tr>
<td>Low</td>
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<td>10 (91)</td>
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<td>28 (42)</td>
<td>39 (58)</td>
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* p < 0.05 was considered statistically significant.

a The p value was determined by Chi-square test.

b The p value was determined by Fisher’s exact test.

Figure 1. Immunohistochemical staining for p16 and p27. Non-invasive low-grade UC showing strong nuclear and cytoplasmic staining for p16 (A). Non-invasive low-grade UC showing strong nuclear staining for p27 (B) (A, B: original magnification, 200×).
p27 expression, the overall survival curves showed statistically significant differences between the groups with high and low p27 expression (log-rank test, $p = 0.021$) (Fig. 2). The patients who expressed low-level p27 in UC were associated with poor prognoses. However, p16 expression was unrelated to overall survival (log-rank test, $p = 0.489$). Nuclear p16 and p27 expression also showed no significant associations with disease-free survival (log-rank test, $p = 0.661$ and $p = 0.108$, respectively).

Discussion

Studies of cell cycle markers of disease-free, overall, and disease-specific survival in UC have obtained mixed results [30,31]. Possible reasons include the heterogeneity of cases evaluated in different studies [9]. Cell cycle progression is controlled by a system of CDKs and CDKIs. Hence, reduced CDKI production causes uncontrolled cell cycling. Low expression of p16 or p27 is a known marker of adverse prognosis in many human cancers, particularly cancer of the pancreas, esophagus, and head and neck (p16 expression) and cancer of the bowel, breast, and prostate (p27 expression) [25,32,33]. Because the CDKIs p16 is a tumor suppressor protein that binds to cyclin/CDK4 or cyclin/CDK6 complexes, it blocks their kinase activity and inhibits progression to the S phase of the cell cycle [25]. In human cancers, p16 is known to be an early marker for malignant transformation [34]. A polymerase chain reaction study of heterozygosity also showed a correlation with p16 expression but not with clinical outcome [35]. Therefore, in assessing the value of p16 as a prognostic marker, IHC may be better than analyzing point mutations or methylations [36]. The current study examined both nuclear and cytoplasmic p16 expression because an abnormal p16 accumulation in the nucleus can reportedly result in its penetration into the cytoplasmic region [37]. This study showed increased p16 expression in noninvasive and low-grade UCs.

Nevertheless, the correlation between p16 loss and tumor recurrence in superficial UC remains controversial. Some studies have shown that reduced p16 expression predicts recurrence of UC [38–40], which is consistent with the observation in the current study that p16 expression correlates negatively with invasiveness and grade in UC and that it probably correlates positively with prognosis.

The p27 in the Cip/Kip family regulates progression from G1 into S phase by binding and inhibiting the cyclin E/CDK2 complex needed for entry into S phase [32,41,42]. In combination with other cell cycle protein abnormalities, loss of p27 has been associated with an aggressive tumor course and an unfavorable prognosis in patients with bladder carcinoma [43,44]. The current study revealed high p27 expression in low-grade UC and low expression in muscle-invasive UC, which is consistent with other studies [43,45–47]. In patients with high p27 expression, tumors tended to be smaller and in an earlier stage. Those with high p27 staining had a better overall survival rate compared to those with low staining, which suggests that p27 inhibited their tumor proliferation and progression. Low p27 levels are associated with poor survival in breast, lung, prostate, and bladder cancer [48–51], which suggests that p27 acts as a tumor suppressor gene in various human tumors. In this study, however, p16 and p27 expressions revealed no significant associations with anatomic location of UC or with disease-free survival of UC. Therefore, further studies are needed to test these associations in a larger sample size or over a longer follow-up period.

In conclusion, the finding that p16 and p27 are negatively associated with prognostic indicators (tumor stage and grade) of UC provides clinicians with useful ancillary data for selecting therapeutic strategies in UC.

References


