p16INK4a Expression Analysis as an Ancillary Tool for Cytologic Diagnosis of Urothelial Carcinoma

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Key Words: Urinary bladder; Urothelial carcinoma; p16INK4a; Immunohistochemistry; Immunocytochemistry; Urine cytology

Abstract

We immunochemically studied p16INK4a expression in 116 urine cytologic samples and compared results with 190 histologic samples. The cytologic samples were classified into 4 groups: 1, mild cellular atypia; 2, moderate cellular atypia; 3, severe cellular atypia; and 4, malignancy. Overexpression of p16INK4a was detected in none of 32 cases in group 1, 8 (16%) of 50 cases in group 2, 5 (42%) of 12 cases in group 3, and 11 (50%) of 22 cases in group 4. In addition, by histologic analysis, p16INK4a overexpression was not detected in nonneoplastic urothelium, except for a few cases of reactive atypia, but it was detected in about 50% of urothelial carcinomas. In particular, a high incidence (16/20 [80%]) of p16INK4a overexpression in high-grade carcinomas was noted in cytologic samples. Immunocytologic analysis of p16INK4a expression in cytologic samples is a useful ancillary tool for detection of urothelial carcinoma with infiltrating potential.

Urothelial carcinoma of the urinary bladder is one of the most frequent malignancies in industrialized countries.1 Although the incidence of urothelial carcinoma in Japan is lower than in other industrialized countries, it is the 12th and 13th most frequently diagnosed malignancy in men and women, respectively, and more than 6,000 people die of the disease per year.2 Based on the 2004 World Health Organization classification of tumors of the urinary system, urothelial carcinoma has 2 subtypes based on cellular and structural atypia, low- and high-grade types, and 2 subtypes based on invasion, noninfiltrating and infiltrating types.3 Low-grade urothelial carcinoma, which has an incidence of 70% to 80%, is usually noninfiltrating and has an excellent prognosis but shows frequent relapse.3 About 30% of these recurrent tumors may show progression to higher grade with stromal invasion.3,4 High-grade noninfiltrating urothelial carcinoma, including papillary and flat types, namely carcinoma in situ (CIS), often progresses to infiltrating carcinoma. High-grade infiltrating carcinoma usually has a poor prognosis.

Treatment options for urothelial carcinoma vary depending on factors such as subtype, stage, and tumor size. Usually, low-grade noninfiltrating carcinoma is treated by transurethral resection (TUR); high-grade noninfiltrating or early infiltrating carcinoma is treated by TUR followed by bacillus Calmette-Guérin (BCG) therapy and chemotherapy; and total cystectomy with or without chemotherapy is used to treat advanced-stage infiltrating carcinoma.5 From the therapeutic point of view, it is important to detect high-grade malignancy and invasion potential.

Urine cytology is the primary mode of detecting and monitoring urothelial carcinoma. Cytologic specificity is relatively high for the detection of urothelial carcinoma, but
sensitivity is low, particularly in low-grade urothelial carcinoma. Diagnosis of urothelial carcinoma by fluorescence in situ hybridization (FISH) has been popular because of its comparable specificity and higher sensitivity than urine cytology.6-13 The Vysis UroVysion (Quest Diagnostics, Madison, NJ) FISH method can detect deletion of locus 9p21 and polymorphism of chromosomes 3, 7, and 17. The locus 9p21 contains the CDK2/p16(INK4a) gene, which encodes cyclin-dependent kinase (CDK) inhibitor and regulates cell proliferation.

Loss and overexpression of p16(INK4a) protein can affect carcinogenesis and progression of malignancy from various organs, such as the uterine cervix.14-20 Most previous studies that have analyzed p16(INK4a) in urothelial carcinoma have described loss of expression of p16(INK4a) in urothelial carcinoma, and only a few have reported overexpression of p16(INK4a) protein.21-31 Previous studies that have analyzed p16(INK4a) expression in urothelial cancer have used tissue sections, and none has evaluated the validity of p16(INK4a) expression analysis for urinary cytologic diagnosis. In the present study, we analyzed immunocytochemically p16(INK4a) expression in atypical cells in urine cytology and compared it with histologic analysis of p16(INK4a).

Materials and Methods

Cases

First, we examined 190 histologic lesions from 77 bladder biopsies, 99 TURs, and 14 surgical resections obtained from 149 patients (123 males, 26 females; mean age, 69 years). Multiple lesions from 1 patient and recurrent lesions were included. Then, we analyzed 116 urine cytologic samples from 112 patients (89 males and 23 females; mean age, 69 years). These samples consisted of 107 voided urine, 4 bladder washing, and 5 catheterized urine samples. Samples obtained from patients with recurrent cancer were also included. In 38 cases, p16(INK4a) expression was immunohistochemically studied in histologic and cytologic samples. The patients had urogenital symptoms, including hematuria and ureteral calculus. Of the patients, 23 had a history of urothelial carcinoma.

Histologic Diagnosis

Histologic classification was according to the 2004 World Health Organization classification.3 Histologic diagnoses from 190 histologic sections included the following: 20 normal urothelium, 12 reactive atypia after BCG therapy, 11 atypia of unknown significance, 19 dysplasia, 23 urothelial CIS, 37 noninfiltrating low-grade papillary urothelial carcinoma, 20 noninfiltrating high-grade papillary urothelial carcinoma, 29 early infiltrating high-grade urothelial carcinoma (pT1), and 19 advanced infiltrating urothelial carcinoma (pT2-4). The stage of most carcinomas was determined by TUR or from surgical material, while that of some noninfiltrating carcinomas was evaluated by biopsy and clinical findings.

Cytologic Diagnosis

Cytologic samples were classified into 4 groups: 1, mild cellular atypia (probably benign); 2, moderate cellular atypia (indeterminate for malignancy); 3, severe cellular atypia (“suspicious” of malignancy); and 4, malignancy. The cytologic diagnosis of 116 samples comprised 32 in group 1, 50 in group 2, 12 in group 3, and 22 in group 4.

Immunohistochemical and Immunocytochemical Studies

For immunohistochemical analysis, 4-μm-thick tissue sections on silane-coated glass slides were prepared. Urine cytologic samples were stained with Papanicolaou stain after centrifugation (1,500 rpm for 5 minutes) and fixed in YM’s Fluid Fixative (Muto Pure Chemicals, Tokyo, Japan) on silane-coated glass slides. After cytologic diagnosis, the samples were immunostained. p16(INK4a) immunostaining of the histologic and cytologic samples was performed using a CINtec p16(INK4a) Research Kit (clone E6H4; DakoCytomation, Glostrup, Denmark) with a DAKO Autostainer Plus (DakoCytomation Colorado, Fort Collins), after a high-temperature antigen unmasking technique using Epitope Retrieval Solution (DakoCytomation, Glostrup) (10 minutes, 95°C). The intensity of the immunoreactivity detected in the nuclei and/or cytoplasm of atypical cells was scored by the number of positive cells, as follows: negative (0), no discernible staining or fewer than 5% positive cells; 1+, 5% to 20% positive cells; 2+, 21% to 70% positive cells; 3+, more than 71% positive cells with strong intensity, usually in nuclei and the cytoplasm. Histologic and cytologic samples showing 2+ or 3+ immunoreactivity were considered to show overexpression of p16(INK4a).

Statistical Analysis

Statistical analysis was performed using the χ2 test for independence. A P value of less than .05 was considered as statistically significant.

Results

Expression of p16(INK4a) in Histologic Sections

Comparison Between Nonneoplastic and Neoplastic Lesions

Table II summarizes expression of p16(INK4a) in urothelial lesions. Among 43 nonneoplastic lesions, including 20 normal urothelium, 12 reactive atypia, and 11 atypia of unknown significance, 21 (49%) cases were negative for p16(INK4a) and 19 (44%) showed focal immunoreactivity (1+) Image 1. Of

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Expression of p16INK4a in Urothelial Carcinoma

The 12 samples of reactive atypia after BCG therapy, 3 (25%) showed 2+ positive overexpression of p16INK4a. No nonneoplastic cases had 3+ positive overexpression of p16INK4a. On the other hand, 105 (70.9%) of 148 neoplastic lesions showed positive immunoreactivity for p16INK4a. Moreover, overexpression (2+ or 3+) of p16INK4a was detected in 8 (42%) of 19 dysplasia cases, 16 (70%) of 23 CISs, 12 (32%) of 37 noninfiltrating low-grade papillary urothelial carcinomas, 9 (45%) of 20 noninfiltrating high-grade papillary urothelial carcinomas, 17 (59%) of 29 early infiltrating high-grade urothelial carcinomas, and 8 (42%) of 19 advanced infiltrating urothelial carcinomas. Overexpression (3+) of p16INK4a was detected only in definite malignant lesions.

Comparison Between Low- and High-Grade Neoplastic Lesions

In low-grade urothelial carcinoma, 18 (49%) of 37 cases indicated focal and weak immunoreactivity (1+) of p16INK4a. In the 12 (32%) of 37 cases of low-grade urothelial carcinoma that showed p16INK4a overexpression, scoring was 2+ for 7 cases (19%) and 3+ for 5 cases (14%). In high-grade noninfiltrating urothelial carcinoma, including 23 CISs and 20 noninfiltrating high-grade papillary urothelial carcinomas, 25 (58%) of 43 cases moderately to strongly overexpressed p16INK4a in both nuclei and cytoplasm (2+ and 3+). Fewer than half of the cases of high-grade advanced infiltrating urothelial carcinoma showed overexpression of p16INK4a (8/19 [42%]). There was a significant difference (P = .02) in incidence of cases with p16INK4a overexpression between high-grade (50/91 [55%]) and low-grade (12/37 [32%]) urothelial carcinoma.

Expression of p16INK4a in Cytologic Samples

Table 2 shows p16INK4a expression in urine cytologic samples. Atypical cells in group 1 revealed mild immunoreactivity but no overexpression of p16INK4a. Overexpression of p16INK4a was detected in 8 (16%) of 50 cases in group 2 with moderate atypia (indeterminate for malignancy). There was a significant difference (P = .02) in incidence of cases with p16INK4a overexpression between high-grade (50/91 [55%]) and low-grade (12/37 [32%]) urothelial carcinoma.

Table 2

<table>
<thead>
<tr>
<th>Score</th>
<th>Incidence of p16INK4a Overexpression (%)</th>
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<td>19</td>
<td>11</td>
</tr>
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<td>190</td>
<td>63</td>
</tr>
</tbody>
</table>

NM, normal urothelium; RA, reactive atypia.

* Scoring data are given as number of cases. Scoring was as follows: negative (0), no discernible staining or fewer than 5% positive cells; 1+, 5%-20% positive cells; 2+, 21%-70% positive cells; 3+, more than 71% positive cells with strong intensity in the nuclei and cytoplasm.

Image 1

Normal epithelium (A, H&E, ×200; B, p16INK4a immunohistologic stain, ×200). Expression of p16INK4a was detected in a few nuclei (score, 1+).
(P = .002; χ² test). Also, the incidence of p16INK4a overexpression between 2 groups was significantly different, except between groups 3 and 4 (groups 1 and 2, P = .02; 1 and 3, P = .0001; 1 and 4, P = .00004; 2 and 3, P = .05; 2 and 4, P = .01; and 3 and 4, P = .69; χ² test).

Comparison of p16INK4a Overexpression Between Cytologic and Histologic Diagnoses

Diagnosis in most cases in groups 3 and 4 was confirmed by histologic examination, while only 7 cases in group 2 in which malignancy was suspected clinically were diagnosed histologically. Thirty-eight cases were studied immunochemically for p16INK4a expression using histologic and cytologic samples. Comparison of the cytologic and histologic diagnoses with the immunohistochemical results for the 38 cases is summarized in Table 3. Of the 38 cases, 29 (76%), namely 16 (80%) of 20 cases with p16INK4a overexpression and 13 (72%) of 18 cases without overexpression by cytology, had consistent results in histologic sections and cytologic samples Image 1. All 20 cytologic samples with p16INK4a overexpression were confirmed to be neoplastic by histologic diagnosis. Of 7 cytologic cases with negative p16INK4a expression in group 4, 4 (57%) had infiltrating carcinoma.

![Image 2](https://academic.oup.com/ajcp/article-abstract/132/5/776/1766496/20177667861?from=1)

**Image 2** Reactive atypia after bacille Calmette-Guérin therapy (A, H&E, ×200; B, p16INK4a immunohistologic stain, ×200). Expression of p16INK4a was detected with moderate intensity in some nuclei (score, 2+).


**Image 3** Low-grade papillary urothelial carcinoma (A, H&E, ×200; B, p16INK4a immunohistologic stain, ×200). Expression of p16INK4a was detected with mild intensity (score, 1+).
Discussion

Immunohistochemical and cytogenetic studies have shown aberrant expression of various cell-cycle–related proteins based on losses and gains of chromosomal regions in bladder cancer.4,27-29 Human p16INK4a, the gene of which is located on the 9p21 locus, is one of the CDK inhibitors that regulates the cell cycle and prevents abnormal cell proliferation. Aberration of p16INK4a expression has also been considered as a cause of carcinomaogenesis and tumor progression in urothelial carcinoma.4,32-36 In most previous studies, aberration of p16INK4a expression in urothelial carcinoma has been seen as a loss of p16INK4a expression. Homozygote deletions, null mutations, and overexpression of p16INK4a are known cancer-related events.26-29 Overexpression of p16INK4a can replace the function of a deleted allele.4,29,32-36

Table 2I

<table>
<thead>
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<th>Expression of p16INK4a in Atypical Cells in Cytologic Studies*</th>
<th>Group</th>
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<th>2+</th>
<th>3+</th>
<th>Overexpression (%)</th>
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<td></td>
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<tr>
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<td>5</td>
<td>3</td>
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<td></td>
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<td>3 (n = 12)</td>
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<td>1</td>
<td>42</td>
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<td>4 (n = 22)</td>
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<td>5</td>
<td>4</td>
<td>3</td>
<td>50</td>
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</table>

* Group 1, mild cellular atypia (probably benign); group 2, moderate cellular atypia (indeterminate for malignancy); group 3, severe cellular atypia (suggestion of malignancy); group 4, malignancy. Scoring data are given as number of cases. Scoring was as follows: negative (0), no discernible staining or fewer than 5% positive cells; 1+, 5%-20% positive cells; 2+, 21%-70% positive cells; 3+, more than 71% positive cells with strong intensity in the nuclei and cytoplasm. Group 1 vs 2 vs 3 vs 4, P = .002.
Voided urine cytologic sample in group 2 (moderate cellular atypia; indeterminate for malignancy) (A, Papanicolaou, x600; B, p16[INK4a] immunocytologic stain, x600). Histologic diagnosis was urothelial dysplasia. Expression of p16[INK4a] was detected in many moderately atypical cells (score, 2+).

Table 3

Comparison of p16[INK4a] Overexpression Between Cytology and Histology

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<th>Case No./ Sex/Age (y)</th>
<th>Cytologic Diagnosis*</th>
<th>p16[INK4a] Expression in Cytologic Specimen†</th>
<th>Histologic Diagnosis</th>
<th>p16[INK4a] Expression in Histologic Specimen†</th>
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<td>NILGpUC</td>
<td>1+</td>
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<td>CIS</td>
<td>3+</td>
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<td>3+</td>
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</tr>
<tr>
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<tr>
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<td>0+</td>
<td>RA</td>
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<td>7/F/51</td>
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<td>1+</td>
<td>AUS</td>
<td>1+</td>
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**AIHGUC, advanced infiltrating urothelial carcinoma (pT2-4); AUS, atypia of unknown significance; CIS, urothelial carcinoma in situ; EIHGUC, early infiltrating high-grade urothelial carcinoma (pT1); NIHGpUC, noninfiltrating high-grade papillary urothelial carcinoma; NILGpUC, noninfiltrating low-grade papillary urothelial carcinoma; RA, reactive atypia.**

* 1, Mild cellular atypia (probably benign); 2, moderate cellular atypia (indeterminate for malignancy); 3, severe cellular atypia (suggestive of malignancy); 4, malignancy.

† Scoring was as follows: negative (0), no discernible staining or fewer than 5% positive cells; 1+, 5%-20% positive cells; 2+, 21%-70% positive cells; 3+, more than 71% positive cells with strong intensity in the nuclei and cytoplasm.
methylation of 5′ CpG islands, and mutations of the p16INK4a
gene have been reported in 0% to 66%, 15% to 67%, and 0%
to 7% of urothelial carcinomas, respectively, as causes of loss
of p16INK4a expression.21-23,37-42 On the other hand, expression
of p16INK4a protein has been reported in 11% to 100% of
urothelial carcinomas.21-31

In the present study, nonneoplastic lesions such as nor-
mal urothelium and atypia of unknown significance showed
only weak expression of p16INK4a, whereas 3 (25%) of 12
cases of reactive atypia after BCG therapy and 70 (47.6%) of
147 neoplastic lesions such as dysplasia and urothelial carci-
noma revealed overexpression of p16INK4a. In particular, CIS
showed a high incidence (70%) of p16INK4a overexpression.
It is interesting that advanced infiltrating carcinomas, even
high-grade tumors, showed a lower incidence of p16INK4a
overexpression than noninfiltrating high-grade tumors.

The mechanism of p16INK4a overexpression can be
explained in 2 ways. One is that increased p16INK4a gene
expression caused by polyploidy of chromosome 9 or amplifi-
cation of the 9p21 locus can augment directly protein expres-
sion. Another explanation is the self-regulation that accom-
panies abnormally high levels of cell proliferation, namely,
p16INK4a protein with a very long half-life accumulates in cells
and down-regulates proliferation.43 Self-regulation of p16INK4a
overexpression is best known in uterine cervical tumors with
human papillomavirus infection. The binding of E6 and E7
oncoproteins of the human papillomavirus to p53 and Rb pro-
teins inhibits the suppressor function of cell proliferation and,
consequently, induces overexpression of p16INK4a for self-
regulation of cell proliferation.14-20,44 In urothelial carcinoma,
one study reported polysomy of chromosome 9 as the cause
of p16INK4a overexpression.21 However, another study using
FISH analysis showed a very low incidence of polysomy of
chromosome 9 and a high incidence of deletion of the 9p21
locus in infiltrating carcinomas (data not shown). Based on
the preceding discussion, we conclude that the main cause of
p16INK4a overexpression in reactive atypia after BCG therapy
and urothelial carcinoma is self-regulation of abnormally
high cell proliferation, and the lower incidence of p16INK4a
overexpression in infiltrating cases results from deletion of
the 9p21 locus.

No previous study has evaluated the validity of p16INK4a
expression analysis for urinary cytologic diagnosis. In the pre-
tent study, the sensitivity of detection of p16INK4a expression
in cytologic samples was similar to that in histologic sections.
Weak expression but not overexpression of p16INK4a was found
in cells in group 1 (mild cellular atypia), while p16INK4a over-
expression was found in 16%, 42%, and 50% of cases in groups 2
(indeterminate for malignancy), 3 (suspicious of malignancy),
and 4 (malignancy), respectively. Unfortunately, we could not
compare histologic diagnosis and p16INK4a expression in most
cases in group 2; however, 5 cases with p16INK4a overex-
pression in group 2, which were histologically examined, were all
malignant. All cytologic samples with p16INK4a overexpression
were confirmed to be neoplastic by histologic diagnosis. We
consider that overexpression of p16INK4a in indeterminate or
suspicious cases is a useful finding for confirming malignancy
in cytology. Also, cytologic samples with p16INK4a overex-
pression had a high incidence (80%) of high-grade urothelial
carcinoma. High-grade urothelial carcinoma did not always
show a sufficient number of cells with marked atypia in cyto-
logic samples, and it was often difficult to make a differential
cytologic diagnosis of benign or low-grade lesions as shown
in Table 3; therefore, p16INK4a overexpression in indetermi-
inate or suspicious cases was a useful marker of high-grade
malignancy. Moreover, 57% of cytologic cases with negative
p16INK4a expression in group 4 probably resulted from deletion
of the 9p21 locus and were infiltrating carcinomas. This sugges-
tes that malignancy with marked cellular atypia and negative
p16INK4a expression in cytologic samples may be an indicator
of stromal invasion.

From a prognostic viewpoint, it is important to detect,
as early as possible, high-grade malignant lesions in the uri-
inary bladder that have stromal invasion or high potential of
invasion. Immunocyto logic analysis of p16INK4a expression
in cytologic samples is a useful ancillary tool for detecting
urothelial carcinoma and infiltrating potential.

References


