Research Article

Diagnostic significance of dual immunocytochemical staining of p53/cytokeratin20 on liquid-based urine cytology to detect urothelial carcinoma

Song-Yi Choi, Kyung-Hee Kim, Kwang-Sun Suh, Min-Kyung Yeo
Department of Pathology, Chungnam National University School of Medicine, Daejeon, Republic of Korea.

ABSTRACT

Background: Urine cytology is a noninvasive and inexpensive method; however, it is limited in low sensitivity for detecting and monitoring urothelial carcinoma (UC). To overcome limitation of cytology, several tests using urine samples have been attempted that immunocytochemical staining is an inexpensive and easy to perform ancillary technique. Dual immunocytochemical staining for p53 and cytokeratin 20 (CK20) is assessed in liquid-based urine cytology slides.

Materials and Methods: Liquid-based urine cytology samples collected between 2008 and 2013 and matched follow-up biopsy samples of high-grade UC (HGUC) (n = 44) and low-grade UC (LGUC) (n = 14) were analyzed.

Results: Urine cytology showing atypical cells was subjected to dual-color immunostaining for p53 and CK20. The sensitivity of urine cytology combined with p53 and CK20 immunostaining was 77.3% in HGUC and 52.9% in LGUC. Of 20 cases diagnosed with atypia by urine cytology, 13 (65%) were positive for p53 or CK20. Dual immunocytochemical staining for p53/CK20 improved the diagnostic accuracy of urine cytology.

Conclusions: The present results indicate that cytomorphology combined with p53/CK20 immunostaining is useful for the detection of HGUC and LGUC.

Keywords: Cytokeratin 20, Immunocytochemistry, p53, Urine cytology, Urothelial carcinoma

INTRODUCTION

Urothelial carcinoma (UC) is the fifth most common cancer worldwide and is most prevalent among men.\(^{1,2}\) Although UC has a good prognosis, it is characterized by frequent recurrence and progression to a high grade that continuous follow-up is required.\(^{1,3,4}\) Cystoscopy and urine cytology are the most widely used methods for the diagnosis and follow-up of UC.\(^{1,2,5,6}\) Cystoscopy with surgical biopsy is the gold standard for the diagnosis of UC; however, the invasiveness and high cost of this method limit its use during routine patient follow-up.\(^{7,8}\) In addition, although cystoscopy is sensitive for detecting papillary lesions, UC in situ is often missed.\(^{9}\)

Urine cytology, on the other hand, is a noninvasive, inexpensive, and highly specific method for screening and monitoring UC.\(^{15,7,9}\) Although urine cytology has high specificity (99%), it frequently shows low sensitivity (15.8%–84.6%) and interobserver variability, particularly for detecting low-grade UC (LGUC).\(^{5,6,8,10,11}\) Another issue in the cytocologic diagnosis of UC is the
category of “atypical urothelial cells” (AUCs). As reactive
cellular changes caused by inflammation, viral cytopathy,
and therapy-induced changes, for example, are often
diagnosed as atypical cells, ruling out UC can be difficult.[1]
A high incidence of AUCs is an important problem in urine
cytology, and the proportion of AUCs is >20% according
to large-scale studies.[1,12] AUCs category can be overused
as “wastebasket” that the proportion of AUCs needs to
be reduced to increase the clinical significance of urine
cytology.[12] The development of an ancillary test capable of
improving the efficacy of urine cytology would be of
value.

Several ancillary tests have been investigated and developed
to improve the accuracy of urine cytology. Although
immunocytochemistry (ICC) of urine samples is rarely
used, the method shows promise for improving diagnostic
accuracy when used as an adjunct to urine cytology.[5,11]
Several recent studies evaluated the usefulness of ICC using
biomarkers such as cytokeratin 20 (CK20), p53, CD44,
and hTERT.[5,14–16] Among them, immunohistochemical
staining for p53 and CK20 is a widely used and promising
method for the diagnosis of UC using surgical biopsy
samples.

CK20 is a cytoplasmic protein and is considered a useful
marker for detecting neoplastic changes in urothelial
cells. In the normal urothelium, CK20 is expressed only
in umbrella cells, whereas it is expressed in the entire
epithelial layer in UC.[17,18] In urine cytology, atypical cells
expressing CK20 may indicate neoplastic changes rather
than reactive changes.[14] p53 is the product of the tumor
suppressor gene TP53, and nuclear overexpression of
p53 indicates the presence of TP53 gene mutation. TP53
mutation is involved in several stages of UC development.
The expression of p53 characteristically shows a diffuse and
strong nuclear pattern in neoplastic changes, whereas its
expression is characterized by a focal and weak pattern in
reactive changes.[19,20] Strong positivity for p53 is a useful
marker of UC in urine cytology.

Most previous studies investigating the efficacy of ancillary
tests were performed using urine specimens obtained
separately from urine cytology. These studies analyzed
protein expression associated with molecular changes in
the urine without a morphological analysis based on urine
cytology. Previous studies analyzed the expression of CK20
using archival slides of urine cytology.[6,11,21] To the best of our
knowledge, there are no studies analyzing the expression of
p53 using urine cytology.

The aim of the present study was to evaluate the expression
of p53 and CK20 using dual immunocytochemical staining
of liquid-based cytology (LBC) slides. The usefulness of
these proteins for improving the diagnostic accuracy of urine
cytology for UC was analyzed.

MATERIALS AND METHODS

Patients and sample collection

Sixty-one liquid-based urine cytology specimens were
collected and histologically confirmed as UC between
January 2011 and December 2013, in Chungnam National
University Hospital. Patients for whom a period of
6 months or longer elapsed between cytology and biopsy
were excluded from the study because new lesions may
occur after 6 months.[22] Ten cases of urine cytology
confirmed as benign lesions by histologic biopsy were
used as negative controls. Urine cytology was processed
to LBC using the ThinPrep (Cytyc Co, Marlborough, MA,
USA) method according to the manufacturer's protocol.
Cytology specimens were stained using the staining method.
Cytologic diagnoses were divided into the following five
categories according to the Paris system: negative, AUCs,
suspicious for high-grade UC (HGUC), low-grade urothelial
neoplasia (LGUN), and HGUC. The three categories
suspicious for HGUC, LGUN, and HGUC were considered
as a positive result for the diagnosis of UC. Cytologic results
were reviewed by two pathologists (S. Y. C and M. K. Y) and
discrepancies were resolved by consensus.

Dual immunocytochemical staining for p53 and
cytokeratin 20

Papanicolau (PAP)-stained slides were photographed with
a digital camera (DP manager DP70, Olympus). Coverslips
were removed with xylene, and slides were destained using
an alcohol series. The slides were heated for 3 min at full
power in a pressure cooker containing 10 mmol/L sodium
citrate (pH 6.0) for antigen retrieval and incubated in 3% H2O2
in methanol at room temperature for 10 min. Nonspecific
protein-binding sites were blocked by incubation with
serum-free protein for 20 min. The slides were stained with
monoclonal antibodies against CK20 (Dako, Carpinteria,
CA, USA) and p53 (Dako, Carpinteria, CA, USA). Dual
staining for CK20 (cytoplasm, red, 1:250) and p53 (nucleus,
DAB/brown, 1:200) was performed using the BenchMark
XT automated stainer. A malignant immunoprofile pattern
was defined as the presence of at least five urothelial cells
positively stained for p53 or CK20.

RESULTS

A total of 61 urine samples diagnosed as UC by biopsy were
examined, including 17 LGUC and 44 HGUC cases. There
were 49 men and 12 women (ratio 4.1:1) with a median age of
72 years (range, 47–95 years). LBC specimens from the urine
of 61 patients with UC were diagnosed as negative (n = 19),
atypical (n = 20), suspicious for HGUC (n = 16),
LGUN (n = 2), and HGUC (n = 4). The sensitivity of urine
cytology for UC was 36%.  

CytoJournal • 2020 • 17(3) • 2
Choi, et al.: Expression of p53/cytokeratin20 on urine cytology

Dual staining for p53 and cytokeratin 20

The 61 destained urine cytology samples from patients with UC were evaluated by dual immunostaining for p53 and CK20. Ten cases confirmed as benign disease were also stained as negative controls. The results of immunostaining identified 38 (62.3%) p53-positive and 7 (11.5%) CK20-positive UC samples. The ten benign disease specimens demonstrated no reactivity for p53 or CK20. p53 expression showed a diffuse and strong nuclear staining pattern in scattered or clustered atypical cells [Figure 1]. CK20 was normally expressed in superficial urothelial cells with abundant cytoplasm and low nuclear-to-cytoplasmic ratio [Figure 2a and b]. CK20 expression detected in distinct superficial urothelial cells was excluded from positivity for UC. Of seven CK20-positive cases, six coexpressed p53 [Figure 3]. Only one case of LGUC showed cytoplasmic expression of CK20 without p53 expression [Figure 2c and d].

Diagnostic performance of p53 and cytokeratin 20 immunocytochemistry in urine cytology

The correlation between urine cytology and ICC for p53 and CK20 showed in Table 1. Of 19 cases diagnosed as negative by urine cytology, 8 (42.1%, p53+) and 1 (5.2%, CK20+) were classified as positive for UC by ICC. Of 20 cases diagnosed as atypical by urine cytology, 12 (60%, p53+) and 2 (10%, CK20+) were classified as positive for UC by ICC. Among 20 cases diagnosed as malignant by urine cytology, 4 (20%) were negative for both p53 and CK20.

The diagnostic sensitivity of urine cytology and ICC was analyzed according to the histologic grade of UC [Table 2]. To determine sensitivity, the categories suspicious for malignancy and malignant were classified as a positive result in urine cytology. The sensitivity of urine cytology was 36.1% (all cases), 29.4% (LGUC), and 38.6% (HGUC). The sensitivity of p53 ICC was 62.3% (all cases), 29.4% (LGUC), and 75.0% (HGUC). The sensitivity of urine cytology combined with p53 staining was 68.9% (all cases), 47.1% (LGUC), and 77.3% (HGUC). The sensitivity of urine cytology combined with p53 and CK20 dual staining was 70.5% (all cases), 52.9% (LGUC), and 77.3% (HGUC).

DISCUSSION

Urine cytology is widely used as a screening test for UC because it is inexpensive and easy. Urine cytology is also a noninvasive method and is useful for repeated and continuous surveillance of patients with UC. However, diagnosis by urine cytology is subjective, and it shows high specificity but low sensitivity.\(^\text{[11]}\) In particular, LGUC is difficult to diagnose by urine cytology. Previous studies reported a wide range of sensitivity values for urine cytology (15.8%–84.6%),\(^\text{[11]}\) and a meta-analysis reported an overall sensitivity of 42%.\(^\text{[21]}\) In the present study, the sensitivity of urine cytology was 36.1% for all cases, 29.4%
Several adjunctive tests were developed to improve the low sensitivity of urine cytology. Six ancillary tests (UroVysion, uCyt+, and tests for NMP22 and BTA) are approved by the FDA and are commercially available. Among these, UroVysion and uCyt+ are the most widely used, and these methods are based on urine sedimentation cells. However, these tests are costly, time-consuming, and require skilled technicians. An inexpensive, easy to perform, highly sensitive ancillary test to detect and monitor UC would be highly desirable.

Currently, ICC is rarely used for the diagnosis of UC using cytology. ICC has the advantage that it can be related to the morphology of cells in urine cytology. In the present study, dual immunostaining for p53 and CK20 was performed on archived urine cytology specimens to evaluate the diagnostic usefulness of adjunct ICC. Of 61 samples from patients with UC, 38 (62.3%) showed positive p53 expression. The combination of p53 ICC and urine cytology showed higher sensitivity (68.9%) than urine cytology alone (36.1%). The combination of urine cytology with p53 improved the sensitivity in both LUGC (from 29.4% to 47.1%) and HGUC (from 38.6% to 77.3%). Arville et al. reported the results of triple staining (CK20, p53, and CD44) using cell block samples of urine, and 67% and 79% of samples were p53 and CK20 positive, respectively. Two studies performing CK20 immunostaining of PAP slides of urine cytology showed 70.4% sensitivity for UC detection. Srivasta et al. reported that CK20 did not improve the detection of UC. Unlike the results of previous studies, we detected CK20 expression in 8.2% (5 of 61) of samples in this study. However, despite the low frequency of CK20 expression, the sensitivity of combining urine cytology with p53 and CK20 was >50% in LGUC. The diagnosis of LGUC by urine cytology has limitations, and the diagnostic value of cyto-morphological

| Expression | Cytologic diagnosis (n=61) |
|------------|---------------------------|
|            | Negative (n=19) | Atypical (n=20) | Suspicious (n=16) | LGUN (n=2) | HGUC (n=4) |
| p53        |               |               |               |            |            |
| Negative   | 11            | 8             | 2             | 2          | 0          |
| Positive   | 8             | 12            | 14            | 0          | 4          |
| CK20       |               |               |               |            |            |
| Negative   | 18            | 18            | 0             | 2          | 2          |
| Positive   | 1             | 2             | 0             | 0          | 2          |
| p53 and/or CK20 |       |               |               |            |            |
| Negative   | 11            | 7             | 2             | 2          | 0          |
| Positive   | 8             | 13            | 14            | 0          | 4          |

CK20: Cytokeratin 20, LGUN: Low-grade urothelial neoplasia, HGUC: High-grade urothelial carcinoma

| Cytology and ICC expression | All cases (n=61) | Sensitivity (%) | Low grade (n=17) | Sensitivity (%) | High grade (n=44) | Sensitivity (%) |
|-----------------------------|-----------------|----------------|------------------|----------------|------------------|----------------|
| Urine cytology              |                 |               |                  |                |                  |                |
| Negative                    | 49              | 36.1          | 12               | 29.4           | 27               | 38.6           |
| Positive                    | 22              | 5             | 5                | 17             |                  |                |
| p53                         |                 |               |                  |                |                  |                |
| Negative                    | 23              | 62.3          | 12               | 29.4           | 11               | 75.0           |
| Positive                    | 38              | 5             | 5                | 33             |                  |                |
| Cyto/cytology + p53         |                 |               |                  |                |                  |                |
| Negative                    | 19              | 68.9          | 9                | 47.1           | 10               | 77.3           |
| Positive                    | 42              | 8             | 8                | 34             |                  |                |
| Cyto/cytology + p53 + CK20  |                 |               |                  |                |                  |                |
| Negative                    | 18              | 70.5          | 8                | 52.9           | 10               | 77.3           |
| Positive                    | 43              | 9             | 9                | 34             |                  |                |

CK20: Cytokeratin 20
changes is still debatable. The combination of urine cytology with adjunct ICC can improve the accuracy of UC diagnosis.

Limitation of urine cytology is the high frequency of the diagnosis of atypical cells, which is reported at 2–23% in the literature. In these cases, the design of treatment is difficult for clinicians. As atypical cells are present in a variety of benign diseases, it is important to identify atypical cells associated with UC to reduce the rate of unnecessary cystoscopy. In the present study, 13 (65%) of 20 cases of atypia in urine cytology showed positivity for p53 or CK20. For patients diagnosed as atypia by urine cytology, additional staining for p53 and CK20 may be useful to identify patients with a high likelihood of UC, thereby facilitating treatment decisions by clinicians.

The application of immunochemical staining can be used as an adjunct to cytology-based diagnosis. However, extracellular materials for cell blocks or additional slides for immunochemistry are not always prepared. Furthermore, findings based on additional slides prepared for ICC can be inconsistent with those of the original urine cytology slides, and the amount of cells is likely to be insufficient. In the present study, ICC staining for p53 and CK20 was performed using destained archival slides of LBC. The main advantage of this method is that it allows simultaneous analysis of cytologic changes on PAP slides and the exclusion of nonspecific staining in normal cells.

In the present study, a small number of negative cases were enrolled because histologic confirmation was available only a few cases with negative cytology. It could limit the evaluation of specificity and predictive value of the ICC test. Urine cytology is one of screening test approaching urologic disorder. Nonetheless of this limitation, this study showed dual p53/CK20 ICC increased a diagnostic sensitivity of cytologic evaluation.

CONCLUSIONS

Combining urine cytology with p53 and CK20 ICC improved the diagnostic accuracy for both LGUC and HGUC. In addition, ancillary ICC staining for p53 and CK20 allowed discriminating between UC and benign diseases in patients diagnosed with atypia in urine cytology.

Acknowledgment

This work was supported by the Chungnam National University research fund (2015-1381-01).

COMPETING INTERESTS STATEMENT BY ALL AUTHORS

The authors declare that they have no competing interests.

AUTHORSHIP STATEMENT BY ALL AUTHORS

SYC: Conception and design of study, perform the experiments, collection and interpretation of data, drafting of manuscript
KKH: Collection and interpretation of data, literature search
KSS: Collection and interpretation of data and input in drafting manuscript
MKY: Conception and design of study, revising the manuscript
All the authors approved the final version of the manuscript.

ETHICS STATEMENT BY ALL AUTHORS

The study protocol was approved by the Institutional Review Board of Chungnam National University Hospital and complied with the tenets of the Declaration of Helsinki (CNUH August 31, 2016). The study was retrospective, and a waiver of consent was approved by the Institutional Review Board.

LIST OF ABBREVIATIONS (In alphabetic order)

CK20 – Cytokeratin 20
HGUC – High-grade urothelial carcinoma
ICC – Immunocytochemistry
LBC – Liquid-based cytology
LGUC – Low-grade urothelial carcinoma
PAP – Papanicolaou
UC – Urothelial carcinoma.

EDITORIAL/PEER-REVIEW STATEMENT

To ensure the integrity and highest quality of CytoJournal publications, the review process of this manuscript was conducted under a double-blind model (the authors are blinded for reviewers and vice versa) through automatic online system.

REFERENCES

1. Kumar P, Nandi S, Tan TZ, Ler SG, Chia KS, Lim WY, et al. Highly sensitive and specific novel biomarkers for the diagnosis of transitional bladder carcinoma. Oncotarget 2015;6:13539-49.
2. Anderlini R, Manieri G, Lucchi C, Raisi O, Soliera AR, Torricelli F, et al. Automated urinalysis with expert review for incidental identification of atypical urothelial cells: An anticipated bladder carcinoma diagnosis. Clin Chim Acta 2015;451:252-6.
3. Arville B, O'Rourke E, Chung F, Amin M, Bose S. Evaluation of a triple combination of cytokeratin 20, p53 and CD44 for improving detection of urothelial carcinoma in urine cytology specimens. Cytojournal 2013;10:25.
4. Halder S, Dey RK, Chowdhury AR, Bhattacharyya P, Chakrabarti A. Differential regulation of urine proteins in
urothelial neoplasm. J Proteomics 2015;127:185-92.
5. Zhou AG, Hutchinson LM, Cosar EF. Urine cytopathology and ancillary methods. Surg Pathol Clin 2014;7:77-88.
6. Wegelin O, Bartels DW, Trump E, Kuypers KC, van Melick HH. The effects of instrumentation on urine cytology and CK-20 analysis for the detection of bladder cancer. Urology 2015;86:772-6.
7. Chang S, Smith E, Levin M, Rao JY, Moatamed NA. Comparative study of ProEx C immunocytochemistry and UroVysion fluorescent in situ hybridization assays on urine cytology specimens. Cytojournal 2015;12:2.
8. Mbeutcha A, Lucca I, Mathieu R, Lotan Y, Shariat SF. Current status of urinary biomarkers for detection and surveillance of bladder cancer. Urol Clin North Am 2016;43:47-62.
9. Bhadia A, Dey P, Kumar Y, Gautam U, Kakkar N, Srinivasan R, et al. Expression of cytokeratin 20 in urine cytology smears: A potential marker for the detection of urothelial carcinoma. Cytopathology 2007;18:84-6.
10. Eissa S, Swellam M, Amin A, Balbba ME, Yacout GA, El-Zayat TM. The clinical relevance of urine-based markers for diagnosis of bladder cancer. Med Oncol 2011;28:513-8.
11. Soyuer I, Sofikerim M, Tokat F, Soyuer S, Ozturk F. Which urine marker test provides more diagnostic value in conjunction with standard cytology-immunoCyt/uCyt+or cytokeratin 20 expression. Diagn Pathol 2009;4:20.
12. Bostwick DG, Hossain D. Does subdivision of the "atypical" urine cytology increase predictive accuracy for urothelial carcinoma? Diagn Cytopathol 2014;42:1034-44.
13. Bölüm M, Schostak M, Hakenberg OW. Urinary immunocytochemistry – Promise or nonseller? A review with an opinion. Urol Oncol 2014;32:383-90.
14. Li HX, Li M, Li CL, Ma JH, Wang MR, Rao J, et al. ImmunoCyt and cytokeratin 20 immunocytochemistry as adjunct markers for urine cytologic detection of bladder cancer: A prospective study. Anal Quant Cytol Histol 2010;32:45-52.
15. Lin S, Hirschowitz SL, Williams C, Shintako P, Said J, Rao JY. Cytokeratin 20 as an immunocytochemical marker for detection of urothelial carcinoma in atypical cytology: Preliminary retrospective study on archived urine slides. Cancer Detect Prev 2001;25:202-9.
16. Khalbuss W, Goodison S. Immunohistochemical detection of hTERT in urothelial lesions: A potential adjunct to urine cytology. Cytojournal 2006;3:18.
17. Harnden P, Eardley I, Joyce AD, Southgate J. Cytokeratin 20 as an objective marker of urothelial dysplasia. Br J Urol 1996;78:870-5.
18. Harnden P, Mahmood N, Southgate J. Expression of cytokeratin 20 redefines urothelial papillomas of the bladder. Lancet 1999;353:974-7.
19. Mallocfré C, Castillo M, Morente V, Solé M. Immunohistochemical expression of CK20, p53, and Ki-67 as objective markers of urothelial dysplasia. Mod Pathol 2003;16:187-91.
20. McKenney JK, Desai S, Cohen C, Amin MB. Discriminatory immunohistochemical staining of urothelial carcinoma in situ and non-neoplastic urothelium: An analysis of cytokeratin 20, p53, and CD44 antigens. Am J Surg Pathol 2001;25:1074-8.
21. Srivastava R, Arora VK, Aggarwal S, Bhadia A, Singh N, Agrawal V. Cytokeratin-20 immunocytochemistry in voided urine cytology and its comparison with nuclear matrix protein-22 and urine cytology in the detection of urothelial carcinoma. Diagn Cytopathol 2012;40:755-9.
22. Ton Nu TN, Kassouf W, Ahmadi-Kaliji B, Charbonneau M, Auger M, Brimo F. The value of the "suspicious for urothelial carcinoma" cytology category: A correlative study of 4 years including 337 patients. Cancer Cytopathol 2014;122:796-803.
23. Hajdinjak T. UroVysion FISH test for detecting urothelial cancers: Meta-analysis of diagnostic accuracy and comparison with urinary cytology testing. Urol Oncol 2008;26:646-51.
24. Sullivan PS, Nooraie F, Sanchez H, Hirschowitz S, Levin M, Rao PN, et al. Comparison of ImmunoCyt, UroVysion, and urine cytology in detection of recurrent urothelial carcinoma: A "split-sample" study. Cancer 2009;117:167-73.
25. McCroskey Z, Pambuccian SE, Kletherms S, Antic T, Cohen MB, Barkan GA, et al. Accuracy and interobserver variability of the cytologic diagnosis of low-grade urothelial carcinoma in instrumented urinary tract cytology specimens. Am J Clin Pathol 2015;144:902-8.
26. Fowler LJ, Lachar WA. Application of immunohistochemistry to cytology. Arch Pathol Lab Med 2008;132:373-83.

How to cite this article: Choi SY, Kim KH, Suh KS, Yeo MK. Diagnostic significance of dual immunocytochemical staining of p53/cytokeratin20 on liquid-based urine cytology to detect urothelial carcinoma. CytoJournal 2020;17:3.