Title: High Apoptotic Index in Urine Cytology Is Associated with High-Grade Urothelial Carcinoma

Running Title: Association of Apoptosis with HGUC

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Precis: Excluding the ileal conduit specimens, the presence of frequent pyknosis or karyorrhexis in the urine cytology is significantly associated with high-grade urothelial carcinoma.

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ABSTRACT

Background: The significance of apoptosis and its association with high-grade urothelial carcinoma (HGUC) in the urine cytology has yet to be determined.

Methods: A computerized search of our laboratory information system was performed over a 3-year period for all urine cytology specimens processed by the SurePath liquid-based preparation technique. Only cases with correlating surgical pathology obtained within 6 months following the urine cytologic samples were included in the study. Cases from ileal conduit samples were excluded. A semiquantitative numerical scoring system (apoptotic index) was used to assess the amount of pyknosis or karyorrhexis: 0 (none), 1 (<10 per10 high-power fields), 2 (10–30 per 10 high-power fields), and 3 (>30 per 10 high-power fields). Statistical analysis using Pearson's Chi-square test was performed.

Results: A total of 228 cases including 105 cases of benign, 79 cases of HGUC and 44 cases of low-grade urothelial carcinoma (LGUC) diagnosed on followup surgical pathology were selected. Score 0 was observed in 70 benign, 11 HGUC and 8 LGUC cases; score 1 was observed in 31 benign, 21 HGUC and 23 LGUC cases; score 2 was observed in 3 benign, 27 HGUC and 9 LGUC cases and score 3 was observed in 1 benign, 20 HGUC and 4 LGUC cases. **Conclusions**: Excluding the cases of ileal conduit, the finding of high apoptotic index (score ≥2) with the presence of pyknosis or karyorrhexis ≥10 per 10 high-power fields in the urine cytology is significantly associated with HGUC ($P<0.05$).

Key words: urine cytology; karyorrhexis; apoptosis; urothelial carcinoma; SurePath

INTRODUCTION

Urinary cytology is a useful, noninvasive and inexpensive test for screening patients with hematuria, or high risk patients with prior chemical exposure. It is also used as a follow-up tool in conjunction with cystoscopy in patients with previous urothelial malignancy. Urologists continue to rely on urinary cytology as a first line screening test and the cytologic diagnosis of high-grade urothelial carcinoma (HGUC) is the most important indication for a patient to undergo cystoscopy. The most common and diagnostic cytologic features characteristic for the diagnosis of HGUC are hyperchromasia, high nuclear and cytoplasmic ratio, irregular nuclear membrane, irregular coarse clumpy chromatin, and anisonucleosis.[1-6] The less frequently observed cytologic features include India-ink nuclei (single cells with deep black and structureless nuclei), micronucleus, and cell cannibalism.[5-7] Apoptosis is a distinctive form of programmed cell death without accompanying any inflammatory reaction. Inappropriate apoptosis can occur in many types of human cancer. During the early process of apoptosis, cell shrinkage and pyknosis are visible by light microscopy. Pyknosis is the result of chromatin condensation and this is the most characteristic feature of apoptosis. On histologic examination, the apoptotic cell appears as a round or oval mass with dark eosinophilic cytoplasm and dense purple nuclear chromatin fragments. Karyorrhexis is defined as a stage of cell death that involves fragmentation of a cell nucleus. The nucleus breaks down into small dark beads of damaged chromatin and is likely to represent a late

stage of apoptosis in urothelial carcinomas usually following the pyknosis of the nuclei.[8-10] The apoptotic index of all grades urothelial carcinoma was noted to be significantly higher than that of the normal urothelium in a study of formalin-fixed paraffin embedded tissue in 45 bladder tumor specimens and the apoptotic index of urothelial carcinomas increased with increasing grade.[9] The significance of apoptotic index in urinary cytology and its association with HGUC has yet to be determined. In this study, we use a semiquantitative numerical scoring system to assess the amount of apoptosis including pyknosis and/or karyorrhexis in the urinary cytologic specimens and determine their association with HGUC. The specimens from ileal conduit urine were excluded from analysis due to the presence of numerous pyknotic nuclei, karyorrhectic debris, degenerative intestinal epithelia and histiocytes in these samples resulting from the higher turnover rate and abundant exfoliation of the intestinal epithelium cells compared to normal urothelial cells.[11]

MATERIALS AND METHODS

This study was approved by Indiana University Institutional Review Board. A computerized search of our laboratory information system was performed over a 3-year period from 2009 through 2011for all urine cytology specimens. Only cases with correlating surgical pathology (biopsies or resection specimens) obtained within 6 months following the urine cytologic samples were included in the study. All the urine samples were processed by the SurePath (BD-TriPath Imaging, Burlington, NC) liquid-based preparation technique. Cases of ileal

conduit urinary samples were excluded from the study. The original cytologic slides were reviewed by authors HHW and CSY. The cytologic diagnoses were classified based on the Johns Hopkins template as HGUC, low-grade urothelial carcinoma (LGUC), atypical urothelial cells cannot exclude high-grade urothelial carcinoma (AUC-H); atypical urothelial cells of undetermined significance (AUC-US), and negative for urothelial atypia or malignancy (NUAM). A semiquantitative numerical scoring system (apoptotic index) was used to assess the amount of pyknosis and/or karyorrhexis: 0 (none), 1 (seen occasionally, <10/10 high-power fields), 2 (10–30 per 10 high-power fields), and 3 (>30 per 10 high-power fields) (Table 1). The association of the score with the correlating histologic diagnosis was tabulated. Apoptotic cells are degenerative cells with dense cytoplasm and contained single (pyknosis) or multiple small, dark, nuclear fragments (karyorrhexis) (Figures 1 and 2). Statistical analysis using Pearson Chi-square test was performed (R Studio Version 0.98.1103, R Studio, Inc. Boston, MA, USA) for the association of high apoptotic index (score ≥2) with HGUC. The level of significance was set at 5%.

RESULTS

A total of 228 urine samples including 187 voided, 36 catheterized and 5 ureter washing, with correlating surgical pathology were identified. The follow-up surgical pathologic diagnoses include benign 105 (46%), HGUC 79 (35%) and LGUC 44 (19%). There were 67 female and 161 male patients ranging in age from 4 to 95 years. Apoptotic index score 0 was observed in

70 benign, 11 HGUC and 8 LGUC cases; score 1 was observed in 31 benign, 21 HGUC and 23 LGUC cases; score 2 was observed in 3 benign, 27 HGUC and 9 LGUC cases and score 3 was observed in 1 benign, 20 HGUC and 4 LGUC cases (Table 2). In total, there were 47 cases of HGUC versus 4 benign cases and 13 LGUC cases showing high apoptotic index (scored ≥2) (Table 3). Among 64 cases with high apoptotic index in urine samples, the corresponding cytologic diagnoses of biopsy-proven HGUC were HGUC 18, AUC-H 19, AUC-US 9 and NUAM 1, and the corresponding cytologic diagnoses of biopsy-proven LGUC were LGUC 1, AUC-H 1, AUC-US 5 and NUAM 6 (Table 4). Calculating with the Pearson Ch-square test, the presence of high apoptotic index (score ≥2) is significantly associated with HGUC ($P<0.05$) (Table 5).

DISCUSSION

The goal of urinary cytology is to identify HGUC. In addition to the well-defined cytologic features of HGUC, such as hyperchromasia, high nuclear and cytoplasmic ratio, irregular nuclear membrane, anisonucleosis, and irregular coarse clumpy chromatin, apoptosis with pyknotic nuclei and karyorrhexis are frequently seen in association with urinary cytologic examination of urothelial carcinoma.[1-6] Apoptosis was observed more often in HGUC than low-grade urothelial neoplasms in a small study of malignant atypical cells in urinary cytology.[6] In our study, high apoptotic index (≥10 apoptosis or karyorrhexis per 10 high-power fields) was observed in 47 of 79 (59%) cases of HGUC, 13 of 44 (29%) cases of

LGUC and only in 4 of 105 (3.8%) cases of benign lesions. Excluding ileal conduit specimen, high apoptotic index is highly associated with HGUC and shows statistical significance when comparing to LGUC ($P<0.002$) and benign lesions ($P<0.001$). Similar findings were noted in a study of histologic sections of 45 bladder tumors.[9] In this histologic study, the apoptotic index of normal transitional epithelium (0.06%) was significantly lower than that of all grades of urothelial carcinoma ($P=0.006$) and the apoptotic index of urothelial carcinomas increased with increasing grade, but the difference failed to achieve statistical significance.[9] When we reviewed the corresponding histologic sections of HGUC, frequent apoptosis was also noted in the tumors (Figure 3).

Hattori et al. suggested the presence of nucleus-fragmented cells could be applied as a parameter to improve diagnostic accuracy for low-grade urothelial tumors. The nucleus-fragmented cells represented the status of karyorrhexis. In Hattori's study, this parameter has been noted in 100% (10/10) of HGUC and 61.3% (19/31 cases) of LGUC in voided urine. The mean numbers of nucleus-fragmented cells per glass slide in the positive samples were much higher in HGUC than LGUC (16.9 versus 1.6).[10] Similarly, the number of karyorrhexis is much lower in LGUC in our study. Low apoptotic index (score <2) was more commonly observed in urine samples of LGUC (23/44 cases, 52%), than in benign lesions (31/105 cases, 29.5%) and HGUC (21/79 cases, 26.6%).

The urine cytologic diagnosis of AUC-H and HGUC are highly associated with

high-grade urothelial lesions.[2-4] In our previous study, more than one-half of patients (58%) who had biopsy-confirmed high-grade urothelial lesions had a preceding cytologic diagnosis of AUC-H or HGUC.[4] AUC-H and HGUC are associated with high-grade urothelial lesions in 80% and 90% of the cases and show statistical significance when compared with AUC-US or NUAM ($P<0.05$). In the current study, of the cytologic samples demonstrating high apoptotic index, 47 cases were confirmed as HGUC by follow-up surgical pathology and the corresponding cytologic diagnosis of either AUC-H or HGUC was noted in 37 cases (78.7%). However if we included AUC-US as an abnormal result, the rate of identifying HGUC increases to 97.8%. The addition of high apoptotic index into the traditional cytologic features of urothelial carcinoma will improve the sensitivity of urinary cytology in detecting HGUC.

In conclusion, excluding ileal conduit samples, the presence of high apoptotic index with increased number of pyknosis or karyorrhexis is a useful cytomorphologic feature for detecting HGUC. High apoptotic index can also serve as a surrogate maker in identifying HGUC in urine cytology, especially in voided urines containing only scant malignant cells which might be difficult to identify. The finding of high apoptotic index should alert the cytologist to search more thoroughly for diagnostic malignant cells, thereby increasing the sensitivity for the diagnosis of HGUC in urine cytology samples.

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Figure Legends:

Figure 1. Urine cytology demonstrates high apoptotic index with abundant pyknotic and karyorrhectic debris. SurePath, Papanicolaou stained, x400

Figure 2. Urine cytology of high-grade urothelial carcinoma shows cluster of malignant urothelial cells with frequent pyknotic and karyorrhectic debris in the background. SurePath, Papanicolaou stained, x400

Figure 3. Histology section of high-grade urothelial carcinoma demonstrates degenerative apoptotic cells containing dark eosinophilic cytoplasm and dense pyknotic nuclear fragments. H$E stained, x400

Table 1 Apoptotic index scoring system:

Apoptotic index	
0	No pyknotic or karyorrhectic nuclear fragments
1	Pyknotic or karyorrhectic nuclear fragments seen occasionally; <10 per 10 high-power field
2	10–30 pyknotic or karyorrhectic nuclear fragments per 10 high-power field
3	>30 pyknotic or karyorrhectic nuclear fragments per 10 high-power field

Table 2 Apoptotic index of urinary cytology with correlating histologic diagnoses

Histology Diagnosis	Benign	HGUC	LGUC	Total
Score 0	70	11	8	89
Score 1	31	21	23	75
Score 2	3	27	9	39
Score 3	1	20	4	25
Total cases	105	79	44	228

HGUC: High-grade urothelial carcinoma; LGUC: Low-grade urothelial carcinoma

Table 3 Apoptotic index of urine cytology with correlating histologic diagnoses

	Benign	HGUC	LGUC	Total
Score <2	101	32	31	164
Score ≥2	4	47	13	64
Total	105	79	44	228

HGUC: High-grade urothelial carcinoma; LGUC: Low-grade urothelial carcinoma

Table 4 Correlating cytologic and histologic diagnoses of cases with high apoptotic index (score ≥2)

	Cytologic Diagnosis					
Histology	NUAM	AUC-US	AUC-H	HGUC	LGUC	Total
HGUC	1	9	19	18		47
LGUC	6	5	1		1	13
Benign	4					
Total	11	14	20	18	1	64

NUAM, negative for urothelial atypia or malignancy; AUC-US, atypical urothelial cells of undetermined significance; AUC-H, atypical urothelial cells cannot exclude high-grade urothelial carcinoma; HGUC, high-grade urothelial carcinoma; LGUC, low-grade urothelial carcinoma.

Table 5 Statistic analysis using the Chi-square test for the association of high apoptotic index with HGUC

Apoptotic index ≥2	Chi-square statistic	P value	P<0.05 is significant
HGUC vs Benign	69.7711	<0.001	Statistically significant
HGUC vs LGUC	10.1446	<0.002	Statistically significant