The usefulness of nuclear area in the diagnosis of high-grade urothelial carcinoma cells in voided urine cytology

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Abstract

Objective: The Paris System for Reporting Urinary Cytology considered the nuclear-to-cytoplasmic (N:C) ratio as the most important cytomorphological feature for detecting high-grade urothelial carcinoma (HGUC) cells. Few quantitative studies were conducted on other features although quantitative studies on the N:C ratio were reported. Therefore, this study quantitatively analyzed the important cytomorphological features in distinguishing benign reactive cells from HGUC cells.

Methods: In this study we analysed 2,867 cells from the urine of 52 patients. A digital image analyzer was used to quantitatively measure the nuclear area, cell area, N:C ratio, and nuclear roundness for HGUC cells and benign reactive cells. Additionally, the diagnostic value of quantitative cytomorphologic criteria in HGUC cells was evaluated by the receiver operating characteristic curve.

Results: The area under the curve for HGUC cells prediction was in all cells and top five cells in the following order: nuclear area (0.920 and 0.992), N:C ratio (0.849 and 0.977), cell area (0.781 and 0.920), and nuclear roundness (0.624 and 0.605). The best cut-off value of the N: C ratio to differentiate HGUC cells from benign reactive cells was 0.438, and using the N: C ratio of 0.702, the positive predictive value obtained was 100%.

Conclusions: Our study indicated that nuclear area is more important cytomorphological

criterion than the N:C ratio for HGUC cell detection. Moreover, extracted data of the top five cells were more valuable than all cells' data, which can be helpful in the routine practice and future criteria definition in urine cytology.

Key words: urine cytology, high-grade urothelial carcinoma, nuclear area, nuclear-to-cytoplasmic ratio, liquid-based cytology, Paris System

INTRODUCTION

Urothelial carcinoma is the twelfth most common malignancy worldwide, arising from the bladder, the renal pelvis, and ureters [1]. Urothelial carcinoma consists of two subtypes, including low-grade urothelial carcinoma (LGUC) and high-grade urothelial carcinoma (HGUC). LGUC frequently recurs but has a good prognosis, whereas HGUC more frequently recurs with stromal invasion, and has a poor prognosis [2-4]. Urine cytology has high sensitivity and specificity for HGUC; thus, it has been widely used for urothelial carcinoma diagnoses and follow-up. However, urine cytology has low sensitivity and specificity for LGUC [5-8].

The diagnostic criteria and reporting of the urine cytology were not standardized in the past, thereby causing confusion among urologists, despite the differences in prognosis and usefulness of urine cytology between LGUC and HGUC as described above [9,10]. Therefore, The Paris System for Reporting Urinary Cytology (TPS 1.0) was published in 2016, as a standardized reporting system, including diagnostic criteria for detecting HGUC, followed by the second edition (TPS 2.0) in 2022 [11,12]. With the introduction of TPS 1.0, the accuracy of urine cytology has been reported to be better than before in most studies [13-16]. Although TPS 2.0 has some differences from TPS 1.0 regarding the diagnostic approach, the critical cytomorphologic features (the nuclear-to-cytoplasmic

[N:C] ratio, hyperchromasia, coarse chromatin, and irregular chromatinic rim) remain unchanged. In addition, among these features, the N:C ratio is the most important cytomorphologic feature, and the critical values of the N:C ratio (0.5 and 0.7) are also maintained [17]. Although, since the TPS 1.0 was published, quantitative studies of the N:C ratio have been examined using the HGUC cells [18-21], and few quantitative studies on other cytomorphologic features have been conducted. Additionally, few studies compared the quantitative cytomorphologic features of reactive urothelial cells (RUCs) and HGUC cells in urine cytology [18]. Furthermore, TPS barely mention renal tubular cells, and no data have compared the quantitative cytomorphologic features of reactive renal tubular cells (RRTCs) and HGUC cells. Therefore, this study assessed whether quantitative cytomorphologic features (nuclear area, cell area, N:C ratio, and irregular nuclear borders), based on the TPS criteria, as assessed using a digital image analyzer, could distinguish benign reactive cells such as RUCs and RRTCs from HGUC cells.

To our knowledge, this is the first report to assess the quantitative differences in cytomorphological features among RRTCs, RUCs, and HGUC cells.

MATERIALS AND METHODS

Patients, urine samples, and SurePath slides

This study used the urine of 19 patients (8 men and 11 women, mean age = 52.7 ± 21.7 years) histopathologically diagnosed with glomerular disease using renal biopsy in Kagawa University Hospital, 18 patients (14 men and 4 women, mean age = 56.5 ± 15.8 years) with urinary calculus clinically diagnosed in Fujita Health University Hospital, and 15 patients (10 men and 5 women, mean age = 75.2 ± 6.7 years) histopathologically diagnosed with HGUC using biopsy or surgical specimens in Kobe University Hospital and Fujita Health University Hospital.

The samples were voided urine, and catheterized urine and bladder washing urine were not included. Urine cytology slides were prepared using the modified SurePath manual protocols (Becton, Dickinson, Franklin Lakes, NJ, USA) [22]. Subsequently, routine Papanicolaou staining was performed on all SurePath slides.

Digital image analysis

Images of RRTCs, RUCs, and HGUC cells on SurePath slides were captured by two medical technologists (KM and CO) using a microscope at an objective magnification of ×100 and cellSence software (Olympus Corporation, Tokyo, Japan). RRTCs, RUCs, and HGUC cells were selected according to the previously reported cytomorphological features [5,8,11,12,23]. The criteria for selecting RRTCs, RUCs, and HGUC cells were clear

cytoplasmic and nuclear membrane borders. Therefore, cells with unclear or overlapping borders were excluded from this study. A maximum of 100 cells were evaluated on SurePath slides. If fewer than 100 cells were present, all cells on the slide were evaluated [21].

For digital image analysis, we used ImageJ software (US National Institutes of Health, Bethesda, MD, USA), and images were handled as red–green–blue images with 8 bits of resolution. The boundary between the cytoplasm and nucleus was manually traced for all cell images using XP-Pen Star G640 (XP-Pen, Shenzhen, China) by two medical technologists (KS and KM) (Figure 1). Using the ImageJ measuring tool, the cell area, nuclear area, and nuclear roundness were obtained. The N:C ratio was calculated by dividing the nuclear area by the cell area. Nuclear roundness was a surrogate for irregular nuclear shapes. Comparisons were made among the RRTCs, RUCs, and HGUC cells concerning the above four cytomorphological features. In addition, a comparison among the three groups was made by selecting five cells with the highest values (nuclear area, cell area, and N:C ratio) or the lowest values (nuclear roundness) for four cytomorphological features on each slide, referring to previous studies [20,21]. Although hyperchromasia and coarse chromatin are also crucial criteria for the TPS, they were excluded from this study because there were differences in the staining intensity of nuclei

among the three hospitals.

Statistical analysis

The Mann–Whitney U test was used where appropriate. The $p < 0.05$ indicated statistical significance. The diagnostic value of quantitative cytomorphologic criteria in HGUC cells was evaluated by the receiver operating characteristic (ROC) curve, and the cutoff value was detected. All analyses were performed using StatFlex software (version 7.0; Artec Inc., Osaka, Japan).

Ethical considerations

This study was approved by the ethics committees of Kagawa University Hospital (No. H26-111) and Fujita Health University (No. HM19-265 and HM20-209), and written informed consent was obtained from all patients in these institutions. Similarly, this study was approved by the ethics committee of the Kobe University Graduate School of Medicine (No. B190148), which waived the requirement for informed consent because of the retrospective study design. This study was conducted in accordance with the principles of the Declaration of Helsinki.

RESULTS

Number of cells analyzed

The total numbers of analyzed RRTCs, RUCs, and HGUG cells were 532, 1154, and 1180, respectively. The average numbers of RRTCs, RUCs, and HGUC cells per patient were 28.1, 64.1, and 78.7, respectively (Figure 2).

Nuclear area

HGUC cells (170.3 μm^2 and 365.1 μm^2) had a significantly larger mean nuclear area than RRTCs (74.2 μm^2 and 107.4 μm^2) and RUCs (79.1 μm^2 and 108.3 μm^2) in all and the top five cells ($p < 0.001$). RUCs mean nuclear area was significantly larger than RRTCs in all cells ($p < 0.001$), but with no significant difference between them in the top five cells ($p = 0.787$) (Figure 3).

Cell area

HGUC cells (326.3 μm^2 and 674.4 μm^2) had a significantly larger mean cell area than RRTCs (185.7 μm^2 and 272.1 μm^2) and RUCs (208.7 μm^2 and 298.6 μm^2) for all cells and the top five cells ($p < 0.001$). RUCs were significantly larger than RRTCs in both all cells and the top five cells in benign reactive cells ($p < 0.001$ and $p = 0.035$).

N:C ratio

The mean N:C ratio was significantly higher in HGUC cells (0.53) than in RRTCs (0.42) and RUCs (0.39) in all cells ($p < 0.001$). Mean N:C ratio in HGUC cells (0.72) was significantly higher than in RRTCs (0.52) and RUCs (0.52) in the top five cells ($p < 0.001$).

Nuclear roundness (Irregular nuclear borders)

The mean nuclear roundness was significantly higher in HGUC cells (0.90) than in RRTCs (0.87) and RUCs (0.89) in all cells ($p < 0.001$). Conversely, mean nuclear roundness in HGUC cells (0.78) was significantly lower than in RUCs (0.82) in the top five cells ($p < 0.001$). Namely, in all cells, the nuclei of HGUC cells were closer to circular than RRTCs and RUCs, whereas the nuclei of HGUC cells showed more irregular shapes than RUCs in the top five cells ($p < 0.001$).

ROC curve analysis

In all cells, the area under the curve for HGUC cell prediction was higher in the order of the nuclear area (0.920), N:C ratio (0.849), cell area (0.781), and nuclear roundness (0.624). The best cutoff value of the nuclear area and N:C ratio to differentiate HGUC

cells from benign reactive cells was 98.8 μm^2 (sensitivity 88.7%, specificity 79.5%) and 0.438 (sensitivity 82.9%, specificity 69.9%), respectively. In addition, when we used the cutoff value of the nuclear area of 253.9 μm^2 (sensitivity 15.9%, specificity 100%) and the N:C ratio of 0.702 (sensitivity 7.5%, specificity 100%), the positive predictive value was 100% (Figure 4) (Table 1).

The area under the curve for HGUC cell prediction was higher in the order of the nuclear area (0.992), N:C ratio (0.977), cell area (0.920), and nuclear roundness (0.605) in the top five cells. The best cutoff values of the nuclear area and N:C ratio for differentiating HGUC cells from benign reactive cells in the top five cells were 169.6 μm^2 (sensitivity of 96.0%, specificity of 95.1%) and 0.619 (sensitivity of 92.0%, specificity of 95.1%), respectively. Additionally, the positive predictive value was 100% using a cutoff value of 236.6 μm^2 for the nuclear area (sensitivity of 74.7%, specificity of 100%) and 0.698 for the N:C ratio (sensitivity of 69.3%, specificity of 100%).

DISCUSSION

Our study results illustrated that the mean number of cells eligible for analysis differed among RRTCs, RUCs, and HGUC cells. Although we excluded large clusters from this analysis, they were present on the slides of HGUC cells and RUCs. The HGUC cells and

RUCs group clusters were divided broadly into large clusters and isolated cells. Conversely, RRTCs mainly consisted of small clusters of 2–20 cells. The finding of the highest number of cells in HGUC could be a consequence of the loss of intercellular junction (E cadherin expression) in HGUC cells [24]. In addition, the finding of a similar number of cells in the HGUC and RUC groups could be attributable to cell detachment caused by the mechanical stimulus of calculi [25]. Conversely, the small number of cells in RRTCs could have plausibly resulted from their simple cuboidal epithelium and few influences of mechanical stimuli.

The present study revealed that the nuclear area, cell area, N:C ratio, and nuclear roundness differed significantly between HGUC cells and benign reactive cells (RUCs and RRTCs) on SurePath slides.

Our results showed that the mean N:C ratio was significantly higher in HGUC cells than in benign reactive cells for all cells and the top five cells. Additionally, we constructed the ROC curve in this study to discover the best cutoff value for the N:C ratio to detect HGUC cells. The results showed that the best cutoff value of the N:C ratio to differentiate HGUC cells from benign reactive cells were 0.438 for all cells, and 0.619 for the top five cells. Furthermore, when we used the N:C ratio at the cutoff value of 0.702 in all cells, and 0.698 in top five cells, the positive predictive value was 100%. Using the

above cutoff values, the sensitivity was 7.5% for all cells, compared to 69.3% for the top five cells, a nearly 10-fold increase. The sensitivity of the top five cells better reflects the actual situation in clinical practice, especially since cells with high N:C are extracted for diagnosis. Furthermore, although slightly inferior to nuclear area, N:C exhibited an extremely high area under the curve (all cells 0.849, and top five cells 0.977), indicating that N:C will remain useful for detecting HGUC cells. Hang et al [18]. reported using SurePath slides that the HGUC cells or carcinoma in situ cells mean N:C ratio was 0.53 and 0.43 of atypical urothelial cells. They also showed a maximum average N:C ratio of > 0.5 as the best cutoff value. In our study using all cells, the mean N:C ratio was 0.53 for HGUC cells, 0.42 for RRTCs, and 0.39 for RUCs, and the best cutoff value of the N:C ratio to differentiate HGUC cells was 0.438. Therefore, our data mostly agree with previous data. As a result, our quantitative study proved the validity of the N:C cutoff value (0.5 and 0.7) in TPS, which was determined based on expert experience. Previous studies reported that the mean N:C ratio did not significantly differ between SurePath slides and ThinPrep slides or between ThinPrep slides and Cytospin slides [20,21]. Therefore, regardless of the processing method used, the N:C ratio 0.5 and 0.7 effectively detects HGUC cells.

In both all and the top five cells, the mean of nuclear areas was significantly higher, in

order of HGUC cells, RUCs, and RRTCs. Additionally, compared with the benign reactive cell group, the nuclear area of HGUC cells was 2.2 times as large in all cells and 3.4 times as large in the top five cells. A previous study that quantitatively compared the nuclear area of HGUC cells and RUCs using tissue specimens revealed a 2.4 times greater mean nuclear area of HGUC cells than that of RUCs, consistent with our all cell data [26]. McIntire et al. quantitatively measured bladder-derived HGUC cells that appeared in urine cytology specimens and reported a mean nuclear area of 158.2 μm^2, which we believe is almost consistent with the present results of all cells (170.3 μm^2) [27]. To our knowledge, no previous study quantitatively compared the nuclear area of HGUC cells and benign reactive cells including RRTCs on urine cytology specimens. However, Vosoughi et al. reported that a qualitative review of cases classified as atypical urothelial cells in TPS revealed the highest positive predictive value (95.8%) and the highest correlation between HGUC cells over other findings when the nuclei of the atypical urothelial cells were three times larger than the lymphocytes [10]. The present results revealed that the nuclear area showed the highest area under the curve (all cells 0.920, and top five cells 0.992) in detecting HGUC cells, especially in the top five cells, and the positive predictive value was extremely high at 100% (sensitivity of 74.7%, specificity of 100%) when the cutoff value was 236.6 μm^2. Therefore, focusing on the nuclear area

is important in detecting HGUC cells. A study using tissue specimens to measure nuclear area quantitatively revealed the top 25% (upper quartile) largest nuclei to be the best discriminative factor between normal urothelium, urothelial dysplasia, and carcinoma in situ [28]. Thus, the diagnosis should be made by extracting cells with a particularly large nuclear area, rather than by averaging all cells.

As for cell area, the area under the curve was as high as 0.920 for the top five cells, and the cutoff value of 572.1 μm^2 resulted in a positive predictive value of 100% (sensitivity of 49.3%, specificity of 100%), which is considered useful for detecting HGUC cells, although inferior to the nuclear area and N:C ratio.

The nuclei of HGUC cells were closer to circular than benign reactive cells in all cells, whereas, the HGUC cells showed irregular nuclear shapes than RUCs in the top five cells in the mean nuclear roundness (surrogate for irregular nuclear borders). These different results are likely to be due to the so-called intra-tumour heterogeneity. Tumour cells are known to exhibit genetic and morphological variation among individuals in tumour tissue of a certain size, and undifferentiated tumour cells are larger and have more morphological atypia, such as irregular nuclear shape [29-30]. Thus, the nuclear shape was averaged out by many round nuclei in all cells, while the top five cells may have been particularly pronounced because the most irregular nuclear shape was extracted.

The strength of this study is that it used quantitative data, which may help develop future cytomorphologic features and automated cytodiagnosis systems. However, digital image analysis in this study is time-consuming, making it difficult to use for daily microscopic examinations. Therefore, we need to clarify the useful qualitative index of the nuclear area to detect HGUC cells in future studies. Additionally, other methods, such as ThinPrep and Cytospin, may have different nuclear area sizes although the SurePath method was used in this study; thus, quantitative studies of HGUC cells and benign reactive cell nuclei using those specimen preparation methods are necessary.

CONCLUSION

The study results revealed that the nuclear area is more helpful in detecting HGUC cells than the N:C ratio. Additionally, the data of the top five cells were more valuable than all cells data in the analyzed four cytomorphological features. We use the cytomorphology with particularly strong atypia to make the final diagnosis in actual cytodiagnosis. Therefore, data from the top five cells is more consistent with the actual situation.

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Figure legends

Figure 1. Traced high-grade urothelial carcinoma cell nucleus on the ImageJ application window (Papanicolaou stain, original magnification × 100).

Figure 2. (a) Reactive renal tubular cells (RRTCs). (b) Reactive urothelial cells (RUCs). (c) High-grade urothelial carcinoma (HGUC) cells (Papanicolaou stain, original magnification ×100).

Figure 3. Comparison of cytomorphologic features in each disease group.

*HGUC: high-grade urothelial carcinoma, RRTCs: reactive renal tubular cells, RUCs: reactive urothelial cells

Figure 4. ROC curve analysis of cytomorphologic features to detect HGUC cells.

*AUC: area under the curve, ROC: receiver operating characteristic, HGUC: high-grade urothelial carcinoma

Author contributions

Methodology: Ohsaki H. **Formal analysis**: Sakumo K., Morihashi K., Nakamura A. and Ohsaki H. **Software**: Sakumo K. and Nakamura A. **Visualization**: Sakumo K. and Ohsaki H. **Resources**: Nukaya T., Sumitomo M., Sofue T., Haba R., Itoh T., and Ohsaki H. **Writing-original draft**: Ohsaki H. **Writing-review and editing**: Sakumo K., Nakamura A., Nakamura M, Kamoshida S. and Ohsaki H. **Supervision**: Ohsaki H

Data availability statement

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

Inside this month's Cytopathology

Nuclear-to-cytoplasmic ratio is considered the most important cytomorphological feature in The Paris System for reporting urine cytology. However, evaluation of other cytomorphological features is lacking. This study compared various cytomorphological features of high-grade urothelial carcinoma cells and benign reactive cells using a digital

image analyser. Quantitative analysis revealed the usefulness of the nuclear area, which has not been reported until now.

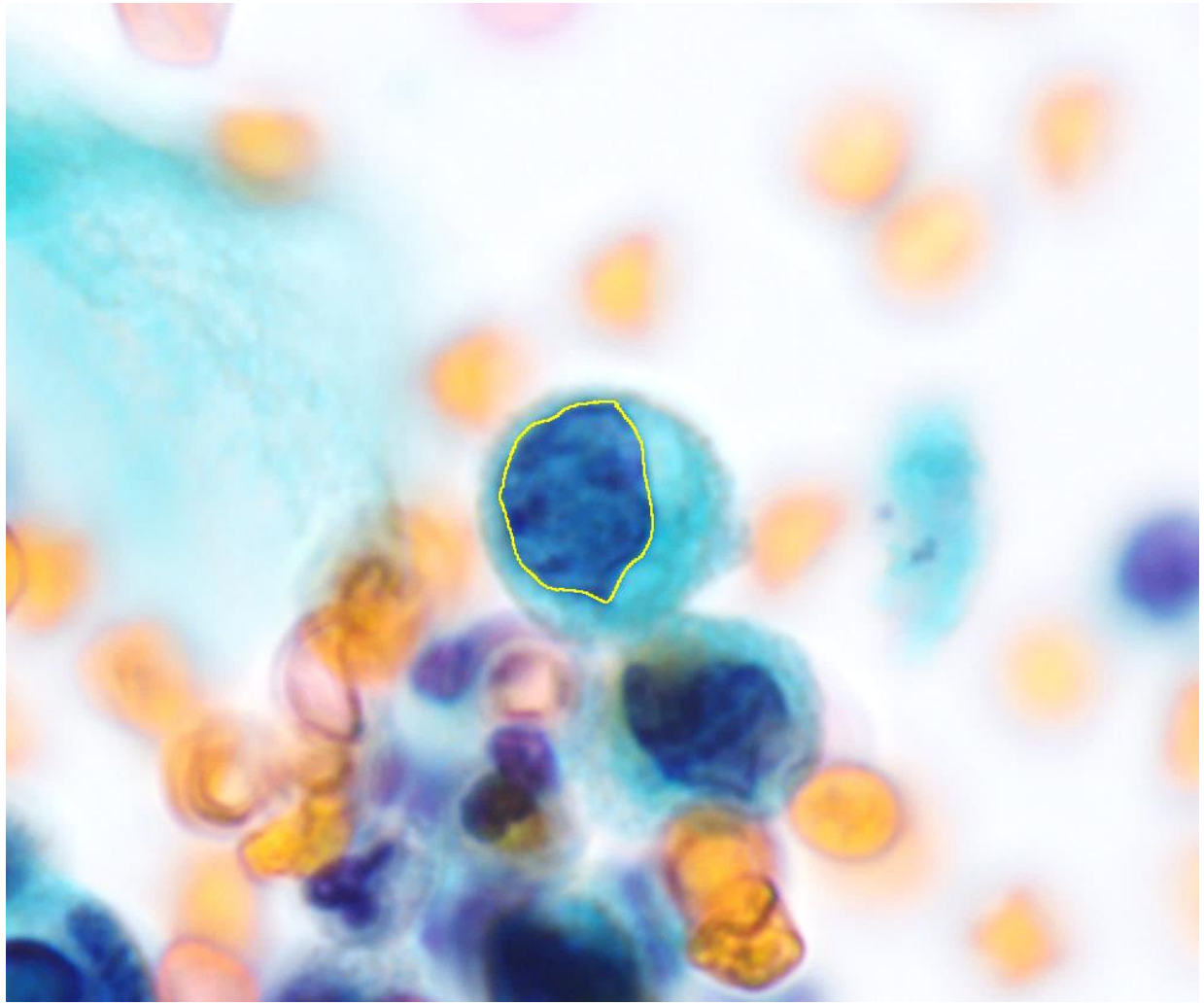

Figure 1. Traced high-grade urothelial carcinoma cell nucleus on the ImageJ application window (Papanicolaou stain, original magnification × 100).

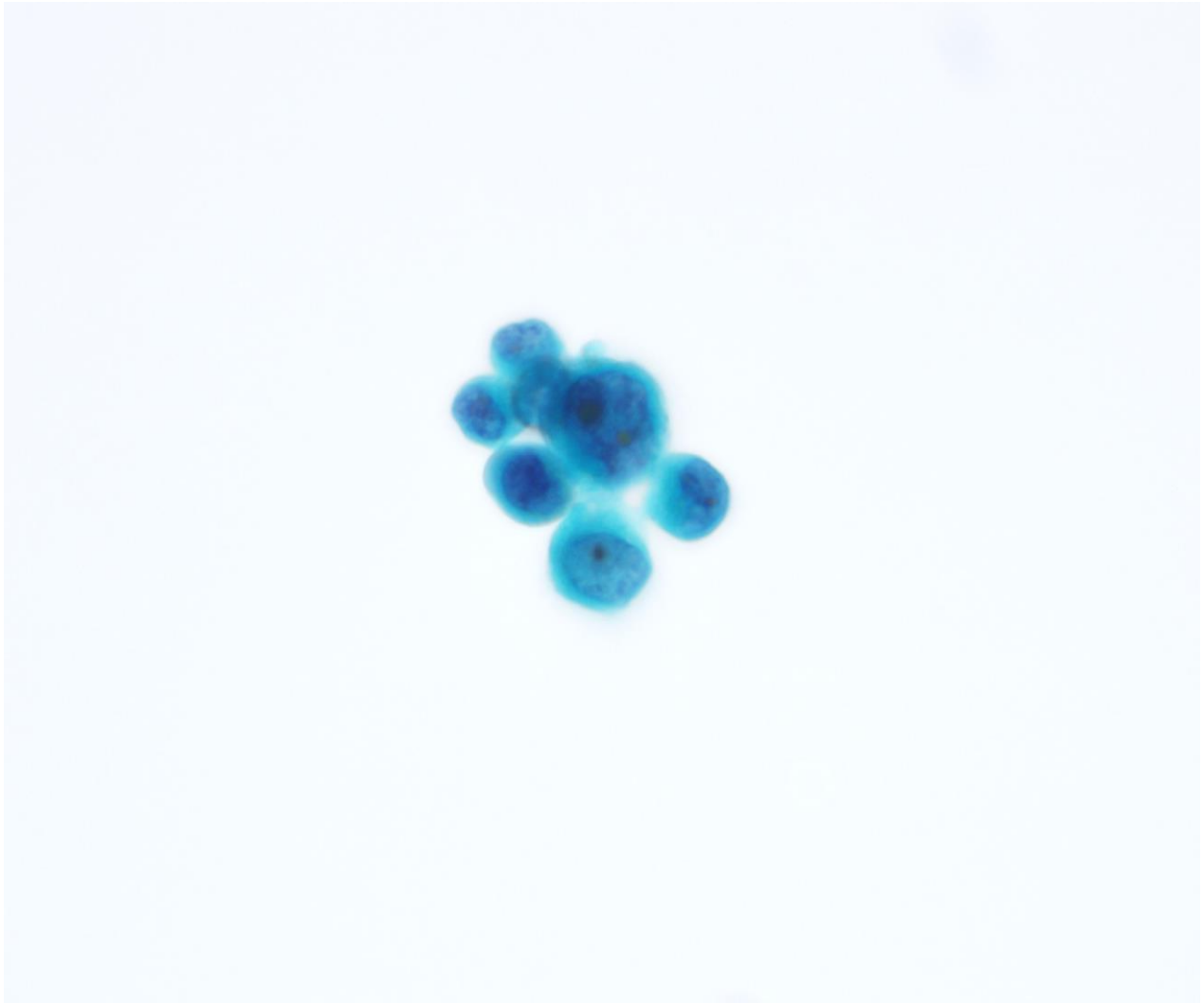

Figure 2. (a) Reactive renal tubular cells (RRTCs)(Papanicolaou stain, original magnification ×100).

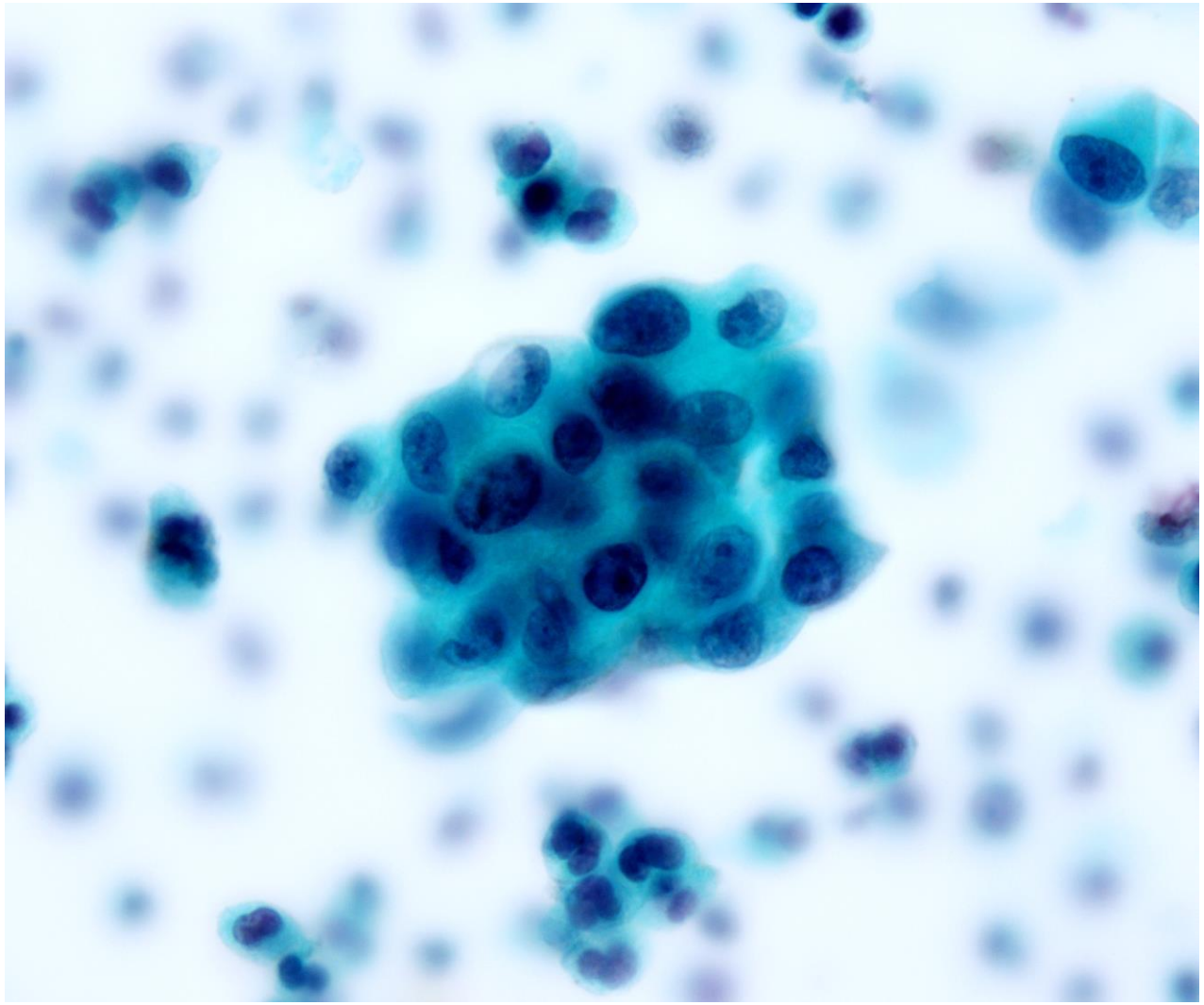

Figure 2. (b) Reactive urothelial cells (RUCs). (Papanicolaou stain, original magnification ×100).

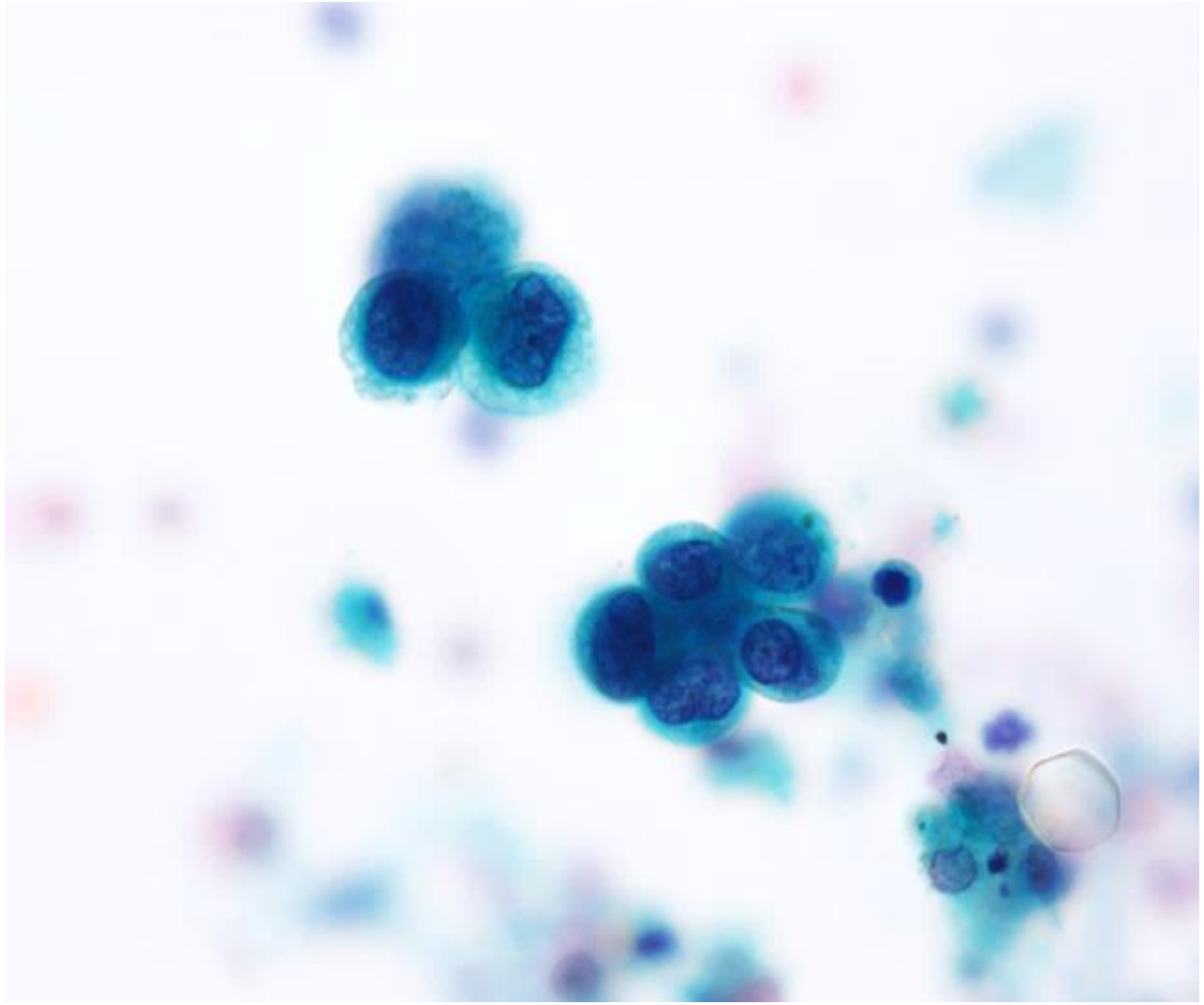

Figure 2. (c) High-grade urothelial carcinoma (HGUC) cells. (Papanicolaou stain, original magnification ×100).

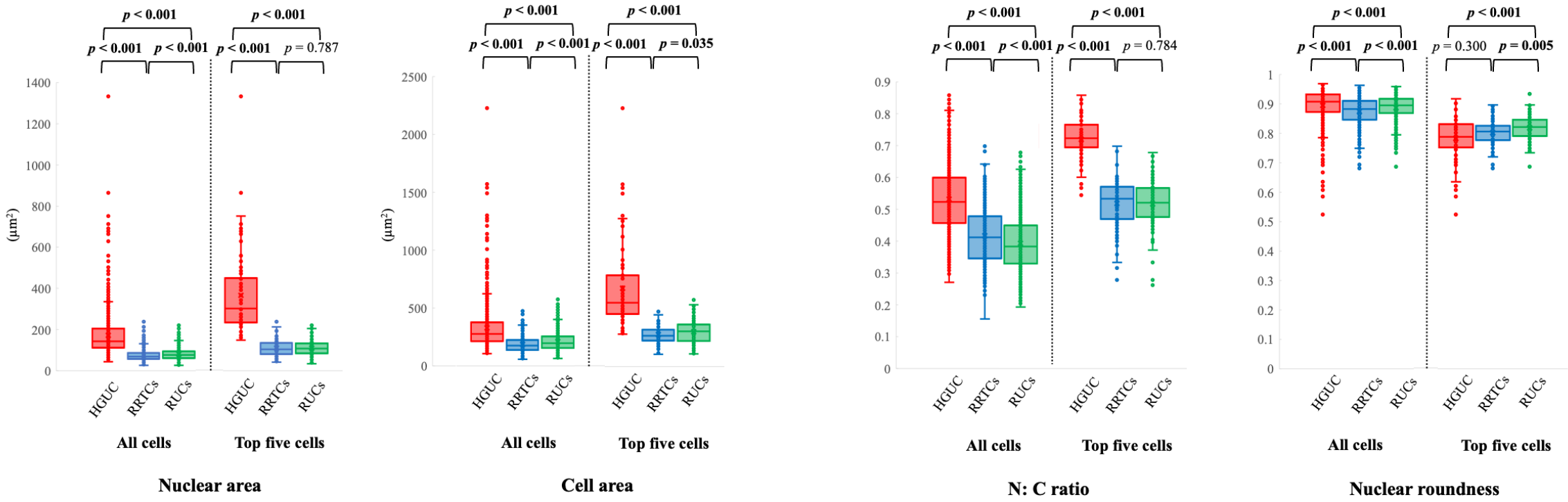

Figure 3. Comparison of cytomorphologic features in each disease group.

*HGUC: high-grade urothelial carcinoma, RRTCs: reactive renal tubular cells, RUCs: reactive urothelial cells

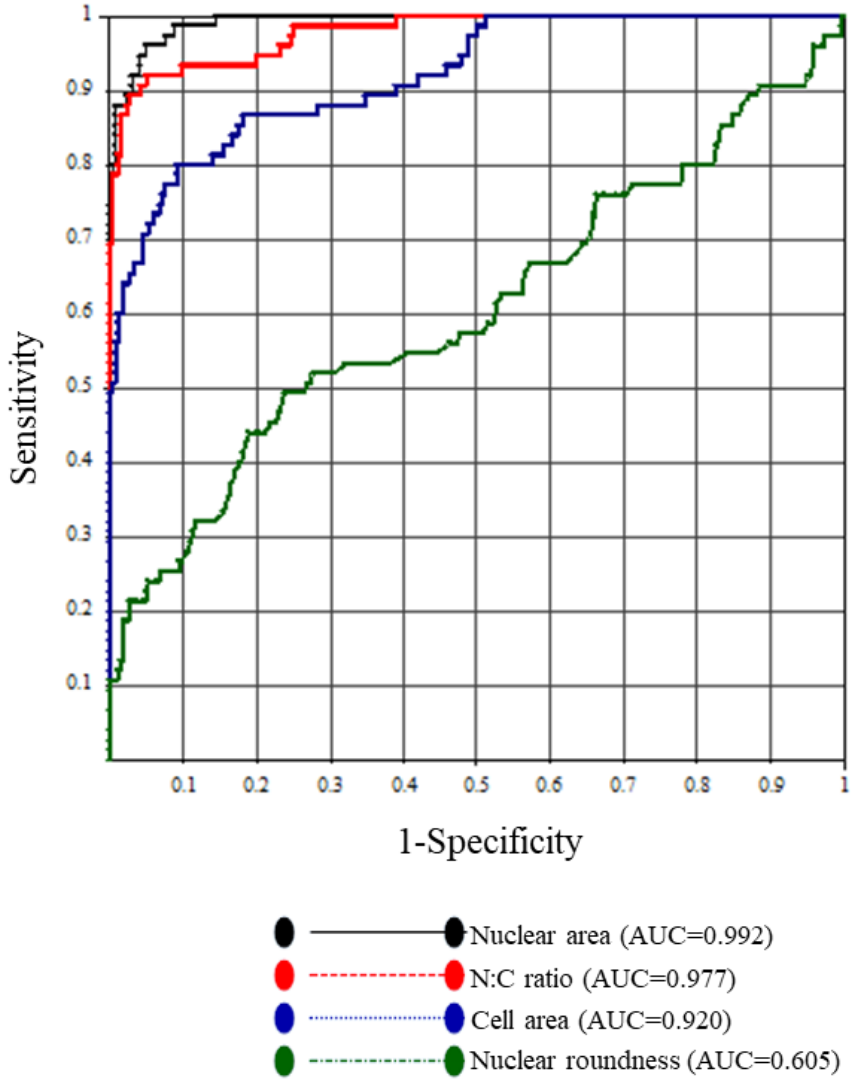

Figure 4. ROC curve analysis of cytomorphologic features to detect HGUC cells.

*AUC: area under the curve, ROC: receiver operating characteristic, HGUC: high-grade urothelial carcinoma

Table 1. Comparison of cytomorphological criteria to distinguish between HGUC cells and benign atypical cells

	HGUC cells	BRCs	AUC	SE	Youden index maximum / PPV 100%		
					Cut off value	Sensitivity	Specificity
All cells							
Nuclear area	1180	1687	0.920	0.005	98.8 / 253.9	0.887 / 0.159	0.795 / 1.000
Cell area	1180	1687	0.781	0.009	229.7 / 613.9	0.733 / 0.079	0.671 / 1.000
N: C ratio	1180	1687	0.849	0.007	0.438 / 0.702	0.829 / 0.075	0.699 / 1.000
Nuclear roundness	1180	1687	0.624	0.011	0.928 / 0.963	0.302 / 0.004	0.900 / 1.000
Top five cells							
Nuclear area	75	265	0.992	0.003	169.6 / 236.6	0.960 / 0.747	0.951 / 1.000
Cell area	75	265	0.920	0.018	424.9 / 572.1	0.800 / 0.493	0.909 / 1.000
N: C ratio	75	265	0.977	0.008	0.619 / 0.698	0.920 / 0.693	0.951 / 1.000
Nuclear roundness	75	265	0.605	0.042	0.785 / 0.680	0.493 / 0.107	0.762 / 1.000

*HGUC: high-grade urothelial carcinoma, BRCs: benign reactive cells, AUC: area under the curve, SE: standard error, PPV: positive predictive value