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Mechanical characterization of benign and malignant urothelial cells from voided urine

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This study investigates whether mechanical differences exist between benign and malignant urothelial cells in voided urine. The Young's modulus of individual cells was measured using the micropipette aspiration technique. Malignant urothelial cells showed significantly lower Young's modulus values compared to benign urothelial cells. The results indicate that Young's modulus as a biomechanical marker could possibly provide additional information to conventional urinary cytology. We hope that these preliminary results could evoke attention to mechanical characterization of urine cells and spark interest in the development of biomechanical approaches to enhance non-invasive urothelial carcinoma detection. © *2013 American Institute of Physics*. [http://dx.doi.org/10.1063/1.4798495]

Bladder cancer is the most common tumor of the urinary system in North America, representing the 4th most common malignancy in men and the 10th most common in women.[1] More than 350 000 individuals are diagnosed with bladder cancer per year worldwide, including more than 70 000 per year in the United States.[2] Urothelial carcinomas (UCs) or transitional cell carcinoma (normal cells from the lining of bladder undergo genetically modification that causes the uncontrolled cell growth) is by far the most common type of bladder cancer (~90%) in the United States.[3] 70% of UCs recur, and 10%–30% of recurrent UCs progress to invasive cancer, necessitating lifelong monitoring of patients.[4] Cystoscopy is the gold standard for the detection of urothelial carcinoma. It is highly invasive and causes significant patient discomfort, especially for the patients who need periodical monitoring due to the high recurrence rate of the disease.[5,6]

Considerable efforts have been devoted to the development of biochemical and genetic markers for non-invasive bladder cancer detection on voided urine. These tests utilize expensive markers and reagents and rely on somewhat subjective interpretation by highly trained personnel; therefore, their application for regular urine screen has been limited. Urinary cytology, which morphologically evaluates urothelial cells exfoliated or shed from the lining of urinary tract into voided urine, is a convenient, non-invasive, and relatively inexpensive technique for regularly monitoring recurrent patients.[7] Urinary cytology has high specificity and relatively high sensitivity for the detection of UCs. However, its overall usefulness is limited since it is purely based on cell morphologies, and the morphological overlap between malignant and benign cells can cause significant false positive detection.[7,8]

The past two decades have seen substantial research in understanding biomechanical properties of cancer cells.[9,10] The cytoskeleton of a cell constitutes a scaffold determining the shape and mechanical rigidity of the cell. Cancer cells reprogram their growth, division, and mobility by modifying the cytoskeletal proteins and their interactions.[11] A number of studies have shown that mechanical property changes are crucial for cancerous cells to alter their gene expression and invade tissue and organs.[10,12,13] It was demonstrated that ovarian cancer cells are generally softer and display lower intrinsic variability in cell stiffness than non-malignant ovarian epithelial cells;[14] non-tumorigenic human cancer cell lines are less deformable and more viscous than tumorigenic cancer cell lines.[15]

In this report, we present the results of a feasibility study in which we attempted to determine mechanical differences of benign and malignant urothelial cells present in voided urine. Urothelial cells were obtained from voided urine samples of urothelial carcinoma negative and positive donors. The Young's modulus was measured using the micropipette aspiration method.[16] The results indicate that Young's modulus as a biomechanical marker can possibly provide additional information to conventional urinary cytology for non-invasive detection and monitoring of urothelial carcinoma.

Details of the working mechanism and operation procedure of the micropipette aspiration system are described elsewhere.[16] For samples from healthy donors, 200 ml of voided urine was collected and divided into four 50 ml centrifugal tubes. Due to the extremely low cell density in voided urine from healthy donors (typically <100 cells per 200 ml), a large volume of 200 ml of urine was collected. Sample was then centrifuged at 400 g for 5 min. The very thin layer of sediment on the bottom of the tubes was re-suspended in 1 ml PBS after carefully aspirating out all the supernatant. Urine samples were also obtained from cancer patients, who have been confirmed with urothelial carcinoma by Toronto General Hospital. Sample preparation was almost identical to the treatment of healthy urine samples. Since the cell density in cancerous samples was higher, only 20–50 ml urine was collected. All samples were tested within 3 h after collection.

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Three types of urothelial cells were identified in voided urine.[7,17] Basal cells (Fig. 1(a)), which rest on the basal membrane of the bladder lining, are small and cuboidal, typically having a hyperchromatic nucleus. Intermediate cells constitute the majority of the urothelium, displaying a clear and amphophilic cytoplasm and possessing a round to oval nucleus (Fig. 1(b)). The most superficial cells of the bladder lining are umbrella cells (Fig. 1(c)), which are characterized by an eosinophilic and wide cytoplasm and large rounded nucleus, sometimes exhibiting bi-nucleation and even multi-nucleation and usually displaying prominent nucleoli.[17] Malignant urothelial cells usually possess a higher nucleus to cytoplasm (NC) ratio and have granulous nucleus and cytoplasm (Fig. 1(d)). Since basal cells and umbrella cells are rarely present in healthy urine samples and morphologically distinguishable from malignant cells, we focused only on characterizing benign intermediate cells and malignant cells in this study.

In urine cytology, a high nucleus-to-cytoplasm ratio plus granular nuclear membrane is a clinically accepted criterion for identifying bladder cancer cells. Urine cytology has poor sensitivity (29%-84% depending on tumor grades) but high specificity (94%-98%).[4,5,7] The definitions of these two metrics are sensitivity = TP/(TP + FN) (note: 100% sensitivity indicates testing detects cancer in all patients truly with bladder cancer) and specificity = TN/(TN + FP) (note: 100% specificity indicates testing never detects cancer in a healthy patient), where TP denotes true positive, FP denotes false positive, TN denotes true negative, and FN denotes false negative. High specificity directly translates to high accuracy when urine cytology confirms that a cell is a bladder cancer cell. However, when urine cytology concludes a sample is negative, the accuracy can be low (i.e., poor sensitivity). Instead of using samples that are unknown positive or negative, cancer cell samples used in this study were donated by patients who have been diagnosed with urothelial carcinoma with cystoscope and biopsy. Out of the 70 tested cells in this work, we only kept data from those cells for which urine cytology verification had no ambiguity in confirming as cancer cells, utilizing the high specificity nature of urine cytology. Immunostaining of our tested cells could have provided additional evidence that these selected cells were truly cancer cells; however, staining involves a number of washing steps, and the cell number in voided urine is extremely low, making our attempts to immunostain voided urine cells unsuccessful.

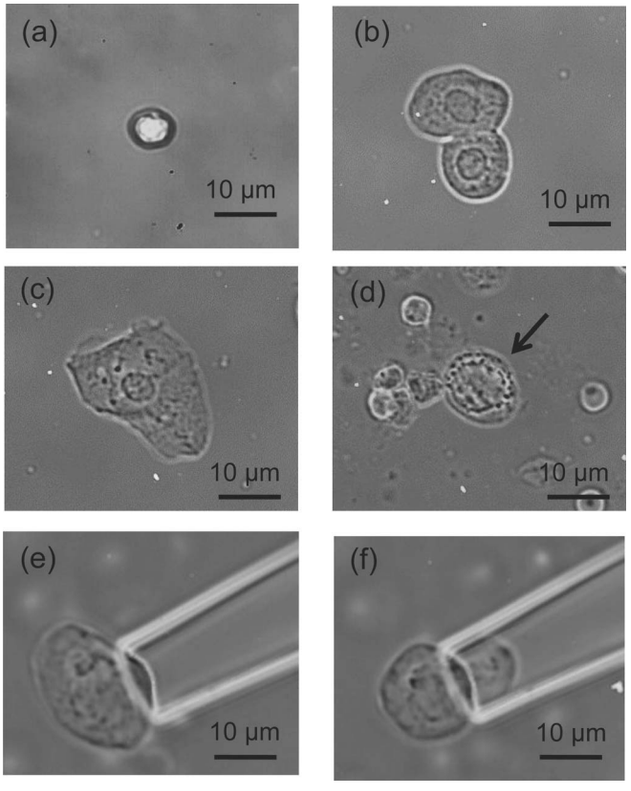

FIG. 1. Different types of urothelial cells present in human voided urine. (a) Basal cell, (b) two intermediate cells, (c) umbrella cell, (d) malignant urothelial cell that possesses a high nucleus to cytoplasm (NC) ratio and granulous nucleus membrane, and (e),(f) micropipette aspiration of a cancer cell. Part of the cell is aspirated into the micropipette opening due to negative pressure application (enhanced online) [URL: http://dx.doi.org/10.1063/1.4798495.1].

During micropipette aspiration experiments, intermediate urothelial cells were identified morphologically and selected for mechanical characterization. Images of tested cells were also verified by experienced professional specializing in urinary cytology. Data from those cells whose type cannot be confidently classified were excluded.

The Young's modulus of benign intermediate cells and malignant urothelial cells was measured using micropipette aspiration. All cells were prepared according to the procedure described above. It is known that cell nucleus is stiffer than cytoplasm.[18] Therefore, when the cell nucleus occupies most of the space inside a cell, the measured Young's modulus value of the cell tends to be higher due to the effect from aspirating the cell nucleus. As a result, within the cancer cell group, the malignant samples with higher NC ratios (0.496 ± 0.10) and the malignant samples with lower NC ratios (0.213 ± 0.062) showed different response to the aspiration pressure and different Young's modulus values (see Figs. 2(a) and 2(b)). The Young's modulus (mean $\pm$ s.e.) measured from benign intermediate cells ($n = 13$) was 457.8 ± 53.6 Pa, while the Young's modulus of malignant urothelial cells (including the malignant cells with higher and lower NC ratio, $n = 17$) was 196.4 ± 42.4 Pa, as shown in Fig. 2(c).

A two-sample independent t-test conducted on the malignant and benign cell populations showed that the population mean values were significantly different at a 95% confidence level ($P = 5.8037 \times 10^{-4}$). However, when comparing the benign cell population and the malignant cell population with high NC ratios (Fig. 2(b)), we found that Young's modulus values of these two populations are not significantly different ($P = 0.0654$). It cannot be concluded, however, that the measured Young's modulus of the malignant cells with high NC ratios is not significantly lower than that of benign urothelial cells. The nucleus effect is difficult to avoid using the micropipette aspiration technique. It is possible that experimental methods, such as atomic force microscopy,[19] with a better control for small cell indentation can better mitigate the effect from the nucleus, which we plan to pursue as the next step.

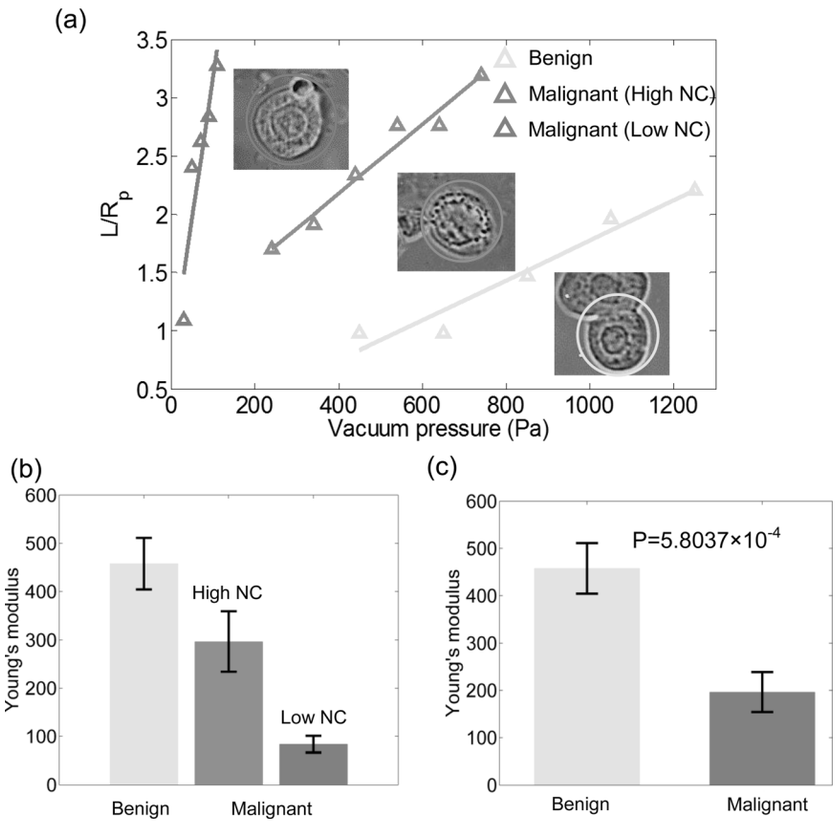

FIG. 2. (a) Experimental micropipette aspiration data (normalized aspiration length (L/R_p) vs. aspiration pressure) collected from benign intermediate cells (cyan), malignant urothelial cells with high NC ratios (purple), and malignant urothelial cells with low NC ratios (red), L and R_p represent aspiration length and micropipette diameter, respectively. Note that even the low NC ratio malignant cells have a slightly higher NC ratio than benign urothelial cells. (b) Young's modulus of benign intermediate cells (457.8 ± 53.6 Pa, $n = 13$), malignant urothelial cells with high NC ratio (296.5 ± 62.3, $n = 8$), and malignant urothelial cells with low NC ratio (83.8 ± 17.3 Pa, $n = 9$). (c) Young's modulus of benign intermediate cells (457.8 ± 53.6 Pa, $n = 13$) and malignant urothelial cells (196.4 ± 42.4 Pa, $n = 17$). Error bar represents standard error.

Fig. 3 shows a scatter plot of NC ratio vs. Young's modulus of benign and malignant urothelial cells. For the benign cells and the malignant cells with high NC ratios, their morphological difference is distinct and readily distinguishable even to trained graduate students. However, morphologically distinguishing the malignant cells with lower NC ratios from benign urothelial cells can sometimes be challenging. This might be where Young's modulus measurement can add bolstering value to urinary cytology for identifying malignant urothelial cells in voided urine.

According to these tests on cells from voided urine, malignant urothelial cells appear generally more deformable than benign urothelial cells. This finding is consistent with the previous report which found stiffness changes of malignant cells compared with their healthy counterparts.[9,10,20] This report demonstrated that cancer cells present in voided urine can possibly be identified by testing their mechanical stiffness differences from benign urothelial cells. Compared to other sources of clinical samples (e.g., pleural fluid), urine samples can be truly non-invasively obtained and tested. Different from existing costly biochemical markers for bladder cancer detection, which require complex preparation procedures and complex interpretation, further technology development for more accurate, higher-throughput measurement of urothelial cells' mechanical properties can provide practically useful information to complement traditional urinary cytology. The number of characterized cells in this study is limited. The number of cells in voided urine is extremely low (<150 cells per cancerous sample; tens of cells per healthy sample). Sample preparation such as centrifuge further reduced cell numbers. Within the remained cells, only those cells with intact membrane were suitable for mechanical testing. We tested a total of 70 cells from voided urine samples. In data analysis, we only included those cancer cells that clinical urine cytology verification had zero ambiguity in confirming as cancer cells. Despite the very low testing throughput of micropipette aspiration, choosing this technique for testing voided urine cells was intentional in this proof-of-principle study since it permits careful selection of cells that are suitable for mechanical testing (e.g., intact membrane, cell size, nucleus-to-cytoplasm ratio, and granular nuclear membrane).

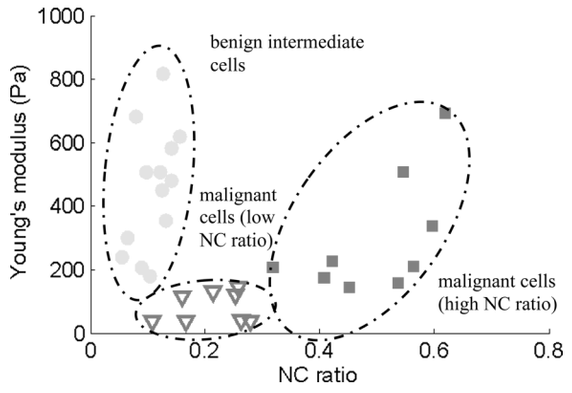

FIG. 3. Scatter plot of nucleus to cytoplasm (NC) ratio vs. Young's modulus of benign intermediate cells (cyan, $n = 13$) and malignant urothelial cells (red, $n = 17$) with high NC ratio (0.496 ± 0.10) and low NC ratio (0.213 ± 0.062).

Next steps of research should include the development of an easier-to-use technique permitting rapid mechanical measurement of urothelial cells from voided urine for testing a larger cell sample size. Microfluidic technologies[20–22] can possibly provide options for achieving these objectives. We hope that the preliminary results reported here could evoke

attention to this promising topic and spark interest in the development of biomechanical approaches to enhance urothelial carcinoma detection.

Malignant urothelial cells and benign intermediate urothelial cells from voided urine were mechanically tested. Our preliminary results suggest that malignant urothelial cells could feature a decrease in cell stiffness. Retrieving these cells from voided urine is truly invasive to patients, and biomechanical testing in concept is an inexpensive, label-free approach that can provide bolstering information to traditional urinary cytology, particularly when morphological differences are insufficient to distinguish benign intermediate urothelial cells and malignant urothelial cells. Further research in mechanical characterization of cells in voided urine will result in better understanding of biomechanical differences among cell types and could provide useful diagnostic cues.

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