Diagnostic approach for cancer cells in urine sediments by 5-aminolevulinic acid-based photodynamic detection in bladder cancer

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Bladder urothelial carcinoma is diagnosed and followed up after transurethral resection using a combination of cystoscopy, urine cytology and urine biomarkers at regular intervals. However, cystoscopy can overlook flat lesions like carcinoma in situ, and the sensitivity of urinary tests is poor in low-grade tumors. There is an emergent need for an objective and easy urinary diagnostic test for the management of bladder cancer. In this study, three different modalities for 5-aminolevulinic acid (ALA)-based photodynamic diagnostic tests were used. We developed a compact-size, desktop-type device quantifying red fluorescence in cell suspensions, named “Cellular Fluorescence Analysis Unit” (CFAU). Urine samples from 58 patients with bladder cancer were centrifuged, and urine sediments were then treated with ALA. ALA-treated sediments were subjected to three fluorescence detection assays, including the CFAU assay. The overall sensitivities of conventional cytology, BTA, NMP22, fluorescence cytology, fluorescent spectrophotometric assay and CFAU assay were 48%, 33%, 40%, 86%, 86% and 87%, respectively. Three different ALA-based assays showed high sensitivity and specificity. The ALA-based assay detected low-grade and low-stage bladder urothelial cells at higher rate (68-80% sensitivity) than conventional urine cytology, BTA and NMP22 (8-20% sensitivity). Our findings demonstrate that the ALA-based fluorescence detection assay is promising tool for the management of bladder cancer. Development of a rapid and automated device for ALA-based photodynamic assay is necessary to avoid the variability induced by troublesome steps and low stability of specimens.

The standard methods for detection and follow up of bladder tumors are cystoscopy and urine cytology. Although exophytic tumors are easily recognized by cystoscopy, flat lesions, including carcinoma in situ (CIS) and small lesions, such as recurrent tumors, are frequently overlooked. Urine cytology or other various urine-based tests can provide some aid in diagnosing these lesions (1). Although urinary cytology presents a high specificity, it shows significantly low sensitivity for low-grade urothelial carcinoma (UC). In addition, other urine-based tests are limited by poor sensitivity, specificity or cost-effectiveness (1,2).

Over the past two decades, 5-aminolevulinic acid (ALA)-induced fluorescence cystoscopy (AFC) has been established with the aim of detecting flat and/or small lesions that are barely visible under white-light cystoscopy, leading to the decreased rate of tumor recurrence (3,4). The principle of AFC is based on the metabolism of ALA, a precursor of the heme biosynthesis pathway (5). Administration of ALA can induce tumor-selective accumulation of protoporphyrin IX (PPIX). This phenomenon is explained by the increased uptake of ALA, the altered activity of certain enzymes such as porphobilinogen deaminase and ferrochelatase activity, and the altered intracellular redistribution and storage of PPIX in malignant cells. Intracellular PPIX is the decisive metabolite allowing red fluorescence detection at an excitation wavelength of approximately 400 nm and an emission wavelength at 635 nm. After instilling 5-ALA into the urinary bladder for 1–2 h, UC tissues show a selective red fluorescence under blue-violet light excitation.

AFC provides great benefits over white-light cystoscopy for the detection of non-muscle invasive bladder cancer (3,4). The benefits are remarkable, particularly for CIS, with a 1.5-fold increase in the detection rate compared to white-light cystoscopy (6). AFC is an important tool in the detection of flat lesions and the confirmation of resection margin during transurethral resection of bladder tumors (TURBT). The clinical value of AFC-assisted TURBT has been validated in large prospective randomized clinical trials. Recurrence-free survival rates at 1 year with and without AFC assistance are 66–90% and 39–74%, respectively (7). The difference in outcome between the two techniques extended over 8 years (8).

AFC-assisted surgery improves clinical outcome. However, AFC is still an invasive and expensive diagnostic tool used on outpatients for the screening and follow up of bladder cancer.

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Materials and Methods

Cell culture and chemical compounds. The T24 cell line, which originated from advanced high-grade bladder cancer, was purchased from ATCC (Manassas, VA, USA) and maintained in RPMI-1640 growth medium (Nissui, Tokyo, Japan) supplemented with 10% FBS (ICN Biomedicals, Aurora, OH, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Grand Island, NY, USA) in a standard humidified incubator at 37°C in an atmosphere of 5% CO2. A stock solution of ALA (Sigma-Aldrich, St. Louis, MO, USA) was prepared in deionized water at a concentration of 10 mM and stored at −20°C. PPIX (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in dimethylsulfoxide and used as a control solution.

Patients. Preoperative urine samples were obtained from patients. Two different cohorts were used in this study. Patients who underwent TURBT from January 2008 to April 2013 in the Department of Urology, Nara Medical University Hospital and those with pathologically confirmed UC were enrolled in the present study. All participants received study information and signed a written informed consent form. The protocol for the research project was approved by the ethics committee of the institution within which the study was undertaken, in accordance to the provisions of the Declaration of Helsinki (1995). A total of 58 patients with bladder tumors were eligible to participate in the study. Resected tumors were histologically examined, staged and graded according to the 2002 TNM classification and the 2004 World Health Organization classification system. No pathologist was involved in the evaluation of the following fluorescence detection tests. The mean age of the patients was 72.6 years (range 34–91 years). Urine samples obtained from 20 benign cases were used as assay controls in this study. The cases consisted of 13 patients with benign prostatic hyperplasia (BPH), three with overactive bladders, three with urinary tract infections, three with chronic kidney disease (stage III) and two with idiopathic hematuria. The mean age of the control benign patients was 70.4 years (range 50–90 years).

Collection of urine samples and treatment with 5-ALA. Before TURBT, 200–300 mL of voided urine was obtained for conventional cytology, a urine BTA test, a urine NMP22 test and fluorescence cytology. Fluorescence cytology was performed within 2 h after sample collection using the following procedures (Fig. 1). Voided urine samples obtained from 20 benign cases were used as controls. Voided urine was centrifuged for 5 min at 400g. The cell pellet was washed using PBS. After centrifugation, the pellet was resuspended in serum-free culture medium, RPMI-1640 containing 1 mM of ALA. The cell suspension was then incubated for 2 h at 37°C to allow cells to produce and accumulate intracellular PPIX. After treatment with ALA, subsequent procedures were performed under light-protected conditions.

Cytological test using fluorescence microscopy. The medium was replaced with 1 mL of PBS, and intracellular fluorescence was visualized using a Provis AX-70 fluorescence microscope (Olympus, Tokyo, Japan). A mercury lamp was used as the illumination source. In a previous report, human urothelial cells harbored autofluorescence ranging from green to orange, which would be an obstacle for a fluorescence detection test and would decrease the test specificity. To eliminate autofluorescence of urine sediments and specifically detect the red fluorescence emitted by PPIX, a fluorescence filter block with band-pass filter 370–410 nm for the excitation, dichroic mirror 495 nm and band-pass filter 612–644 nm for the emission.
were purchased from Olympus. Voltage gain, exposure time and sensitivity (contrast and brightness) were fixed for all fields and images. Erythrocytes are known to present a red autofluorescence,\(^{(16)}\) therefore, red fluorescence detected in erythrocytes was eliminated in the fluorescence microscopic analysis.

Using this method, PPIX red fluorescence was localized in the cytoplasm and/or nucleus of urothelial cells. Identification of UC cells was based on the morphologic criteria of malignancy, such as enlarged polymorphous cells with prominent lobulated nucleus and high nucleus/cytoplasm ratio. In each test, T24 cells treated with ALA for 2 h were used as a positive control. Exposure time and gain were adjusted so that T24 cells treated with ALA looked bright red and the background was completely black (Fig. 2, upper panels). Fluorescence cytology was considered positive if the red fluorescence was detected in urothelial cells (Fig. 2). The results were compared with the tumor histology, stage and findings of conventional cytological examination. The examiner had no clinical information prior to the examination.

**Evaluation of ALA-induced fluorescence using a fluorescence spectrophotometer.** We used 50 mL of voided urine for each of the ALA-treated sediments and ALA-untreated sediments. After the treatment with ALA in serum-free media, urine sediments were obtained by centrifugation and resuspended in 500 µL of PBS. The resuspended sediments in PBS were transferred in triplicate (100 µL each) to a flat-bottom transparent 96-well plate. In each urine sample, ALA-treated sediments and untreated sediments were subjected to the spectrophotometric assay. Fluorescence emission spectra were recorded using a microplate spectrophotometer (Infinity 200M PRO; Tecan, Männedorf, Switzerland) equipped with i-control version 1.8 software. The excitation wavelength was 400 nm, and the photomultiplier tube voltage was fixed. A series of spectra obtained from each specimen (Fig. 3) and the fluorescence intensity at 635 nm was measured. The values of ALA-treated sediments and those of the ALA-untreated control were compared. If all of the three intensity values of ALA-treated samples were higher than any values of ALA-untreated controls, the sample was considered positive for the spectrophotometric assay.

**Development of a novel fluorescence detector specific for 5-ALA-induced fluorescence.** With the goal of establishing an easy, rapid and objective modality for fluorescence urine cytological testing, we have developed a novel device quantifying red fluorescence emitted from intracellular PPIX. The compact-size desktop-type device was produced by MICRONIX (Kyoto, Japan) and named Cellular Fluorescence Analysis Unit (CFAU) (Fig. 4a). Operation and data collection for the CFAU assay are performed with a Windows XP computer incorporated with operating software provided by MICRONIX. The urine sediment suspension remaining after fluorescence microscopic and spectrophotometric analysis was subjected to CFAU analysis. In 23 of 58 urine samples from bladder cancer and 19 of 24 samples from control cases, the remnant urine sediments were sufficient for the analysis. The unit consists of four sections: a sample tube rack, an elastic aspiration capillary, a fluorescence detection box and a collection tube rack.

The cell suspension is placed in the sample tube rack. The suspension in the sample tube is aspirated into a plastic syringe electronically, and it flows through the aspiration capillary to the fluorescence detection box. In the CFAU, the detection box section plays the most important role in the test accuracy (Fig. 3b). The detection box contains a light-emitting diode (LED) that produces stable excitation at a single wavelength of 400 nm. In this box, the cell suspension flows through a quartz capillary. The fluorescence intensity of each sample is determined by the integral value for 5 s. In the preliminary test, 1 ng/mL of PPIX solution in PBS was subjected to CFAU, and the integral value of red fluorescence at a wavelength of 635 nm was detected successfully (Fig. 3c).
Results

Detection of urothelial cancer cells with a fluorescence microscope. The voided urine before TURBT was successfully obtained, processed and analyzed. Table 1 depicts the results of conventional cytology, BTA, NMP22 and three different fluorescence cytological tests (i.e. fluorescence microscopy, fluorescence spectrophotometric assay and CFAU assay). For fluorescence microscopic examination, ALA-treated sediment suspension in PBS was transferred onto a fluorescence-free microscope slide and covered using a cover slip. Fluorescence-positive epithelial cells were detected in 50 (87%) out of 58 patients (Table 1). Whereas the sensitivity of conventional cytology was 16% in low-grade tumors and 20% in pTa tumors, fluorescent microscopic cytology allowed for the detection of cancer cells in 81% of low-grade tumors and 80% of pTa tumors. No control sample was positive for fluorescence microscopic cytology.

Detection of ALA-induced fluorescence in the urine sediments using a fluorescence spectrophotometer. Fluorescence emission spectra at 635 nm by excitation at a 400-nm wavelength were recorded in representative cases. A control case with BPH showed almost no difference in the fluorescence intensity at 635 nm between ALA-treated sediments and untreated sediments (Fig. 3, left panel). In contrast, the analysis of urine from bladder cancer patients showed statistically higher intensity in ALA-treated sediments compared to untreated sediments (Fig. 3, middle and right panels). The difference in the fluorescence intensity at 635 nm of other urine samples was also tested. The fluorescence was detected in 48 out of 58 patients (Table 1), indicating a sensitivity of 86% for the fluorescence spectrophotometer analysis. This method allowed for an increased sensitivity, especially in low-grade and low-stage bladder tumors as compared to conventional cytology and two tested biomarkers, without decreasing the assay specificity. The results were similar to those of fluorescence cytology.

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Sensitivity of Cellular Fluorescence Analysis Unit for detecting red fluorescence of urine sediments. To determine the detection sensitivity of CFAU, we carried out a preliminary experiment using T24 cells. Non-cancerous cells are supposed to be negative for red fluorescence after ALA exposure. In this experiment, untreated T24 cells were used as simulated non-cancerous cells without red fluorescence. ALA-treated T24 showed almost no difference in the fluorescence intensity at 635 nm between ALA-treated sediments and untreated sediments (Fig. 3, left panel). In contrast, the analysis of urine from bladder cancer patients showed statistically higher intensity in ALA-treated sediments compared to untreated sediments (Fig. 3, middle and right panels). The difference in the fluorescence intensity at 635 nm of other urine samples was also tested. The fluorescence was detected in 48 out of 58 patients (Table 1), indicating a sensitivity of 86% for the fluorescence spectrophotometer analysis. This method allowed for an increased sensitivity, especially in low-grade and low-stage bladder tumors as compared to conventional cytology and two tested biomarkers, without decreasing the assay specificity. The results were similar to those of fluorescence cytology.

Table 1. Diagnostic sensitivity comparison between six different urinary tests

| Diagnostic test | Bladder cancer | | | | | Control (%) |
|-----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| | Tumor grade (%) | Pathological T stage (%) | Total (%) | |
| | Low | High | Ta | T1 | T2 ≤ Tis | Patients (n) | |
| Conventional cytology | 26 (16) | 32 (75) | 5 (20) | 9 (56) | 9 (82) | 5 (83) | 28 (48) | 0 (0) |
| BTA | 2 (8) | 17 (53) | 2 (8) | 5 (31) | 10 (91) | 2 (33) | 19 (33) | NA |
| NMP22 | 3 (12) | 20 (63) | 3 (12) | 7 (43) | 10 (91) | 3 (50) | 23 (40) | NA |
| Fluorescence cytology | 21 (81) | 29 (91) | 20 (80) | 15 (94) | 11 (100) | 4 (67) | 50 (86) | 0 (0) |
| Spectrophotometric assay | 19 (73) | 29 (91) | 17 (68) | 14 (88) | 11 (100) | 5 (100) | 48 (86) | 0 (0) |
| CFAU assay (n = 23)† | 6/8 (75) | 14/15 (93) | 6/8 (75) | 8/9 (89) | 8/8 (100) | 5/5 (100) | 20/23 (87) | 0/19 (0)‡ |

†Out of the 58 cancer cases, 23 were available for the CFAU assay. ‡Out of the 24 control cases, 19 were available for the CFAU assay. CFAU, Cellular Fluorescence Analysis Unit; NA, not analyzed.
cells were used as fluorescence-positive cells (i.e. cancerous cells). The cells were suspended in PBS at a concentration of $10^5$ cells/mL. Series of cell suspensions consisting of fluorescence-negative cells (untreated T24 cells) and fluorescence-positive cells (ALA-treated T24 cells) at various ratios were prepared as follows: 100:0, 50:50, 25:75, 10:90, 5:95 and 0:100. The cell suspensions were subjected to CFAU assay, and the spectra of emitted fluorescence were recorded by the equipped computer. No peak at the 635-nm wavelength was observed in a sample containing only untreated T24 cells (Fig. 5a). However, an apparent peak at the wavelength of 635 nm could be detected by CFAU in samples containing more than $1.0 \times 10^4$ cells/mL of fluorescence-positive cells in a final concentration of $10^5$ cells/mL. Subtle peaks were observed in samples containing $0.5 \times 10^4$ cells/mL of fluorescence-positive cells. According to the preliminary experiments using the cell line, the lower detection limit ranged from 0.5 to $1.0 \times 10^4$ cells/mL.

In this assay, the detection of subtle changes in fluorescence signal at the wavelength of 635 nm seemed to be important. Compared to the experimental control using the cell line, a higher background was observed in clinical samples (Fig. 4b, upper panels). To achieve high sensitivity in the CFAU test, clinical samples were analyzed by comparison of fluorescence spectra of ALA-treated and untreated cells. The detection of a peak representing red fluorescence at the wavelength of 635 nm was defined as a positive sample (Fig. 5b). A peak at 635 nm was detected in 20 (87%) of the 23 urine sediments treated by ALA, indicating the presence of bladder UC. The sensitivity of CFAU was similar to that of the two other fluorescence-based assays. No positive result was observed with the control sample.

**Comparison of the results from conventional cytology, urine biomarkers and fluorescent cytology tests.** The overall sensitivity values for conventional cytology, BTA, NMP22, fluorescence cytology, fluorescent spectrophotometric assay and CFAU assay were 48%, 33%, 40%, 86%, 86% and 87%, respectively (Table 1). BTA and NMP22 were less sensitive compared to conventional cytology and fluorescence-based assays. Three different fluorescence-based assays showed similar sensitivity. Next, the results of conventional cytology, fluorescence cytology and fluorescent spectrophotometric assay were compared using contingency tables (Fig. 6). Fluorescence cytology detected 34 cancers that conventional cytology failed to detect (Fig. 6a). Only two cases were detected by conventional cytology that fluorescence cytology failed to detect. Fluorescence spectrophotometric assay detected 32 cases that conventional cytology failed to detect (Fig. 6b). The results of fluorescence cytology and fluorescent spectrophotometric assay were compared, and a high concordance (86%, 48 of 58 cases) was demonstrated between the two different assays (Fig. 6c).

**Discussion**

Although several studies have made efforts to establish detection assays targeting intracellular fluorescence induced by treatment with ALA, hexaminolevulinate or other photosensitizers, no assay is clinically available to date. One of the major challenges is to improve the detection rate of bladder cancer using fluorescence-based assays.
problems is that some of the photodynamic detection methods lack objectivity and specificity. To solve this problem, we have developed a novel photodynamic detection device, CFAU. This device takes advantage of the working principle of flow cytometry (Fig. 4). In addition, we carried out fluorescence cytology and a fluorescence spectrophotometric assay using urine samples from patients with bladder cancer or benign urological disorders to compare their diagnostic accuracy. This study is the first to address the comparison of three different photodynamic detection modalities: fluorescence cytology, fluorescence spectrophotometer assay and flow cytometry assay.

In fluorescence microscopic analysis, autofluorescence of various cells could decrease the specificity, and results are likely to be affected by the experience of the observers, as in conventional cytology (9,17). Hematuria, leukocyturia and bacteriuria can result in false positives. A couple of strategies were applied to avoid false positives. One was the use of a custom-made filter block, which enabled the detection of intracellular PPIX specifically by fluorescence cytology. Another was the introduction of a background fluorescence subtraction method in the spectrophotometric assay and the CFAU assay (Figs 3, 5b). In the 58 cases with bladder cancer, no correlation was observed between the result of fluorescence-based assays and the presence of hematuria, leukocyturia or bacteriuria (data not shown). No case showed false positive results in the fluorescence-based assays (Table 1).

ALA-based photodynamic detection assays for urine samples could be clinically applied to a screening test. Patients undergoing a screening test comprise those with chronic kidney disease, chronic urinary infection, lower urinary tract syndrome and asymptomatic hematuria. The assay specificity was checked using control patients with a variety of urological disorders. However, only a few control patients with hematuria (4) and leukocyturia (3) were enrolled in this studied population. To assess the specificity more precisely, the inclusion of more patients with inflammation, infection, dysplasia, hyperplasia and other preneoplastic lesions should be considered.

The diagnostic accuracy of ALA-based photodynamic detection assays is sufficiently high to be applied to the clinical setting. However, the sensitivity in low-grade and low-stage tumors is relatively low. Assay sensitivity could depend on the quantity of PPIX accumulated in exfoliated UC cells. A previous report demonstrated that higher grade and higher stage bladder tumors can produce and accumulate higher levels of PPIX after ALA administration (17). Suppressed expression of ferrochelatase and increased expression of porphobilinogen deaminase in malignant cells have been thought to be possible factors contributing to the intracellular accumulation of PPIX (18). Hagiya et al. (18) reveal that the upregulation of peptide transporter gene PEPT1 and the downregulation of ABCG2 are among the key molecular mechanisms. Several factors seem to be involved in the mechanisms underlying the increased accumulation of PPIX in higher grade tumors. The number of UC cells exfoliated into the urine would be another possible factor. Gene alterations such as loss of E-cadherin and β-catenin are frequently observed in high-grade tumors compared to low-grade tumors (19). Loss of cell adhesion in high-grade and high-stage tumors leads to a tendency to exfoliation into urine, resulting in many UC cells in voided urine.

In conclusion, the ALA-based photodynamic assay detected low-grade and low-stage bladder UC cells at a higher rate than conventional urine cytology, BTA and NMP22. Our findings demonstrated that the ALA-based fluorescence detection assay is a promising tool for the management of bladder cancer. Because the present study was conducted only to evaluate the feasibility of detection methods using ALA, there was a bias in the selection of cases. We intend to develop a second generation CFAU, which enables automated photodynamic detection and is more subjective by reducing the variability induced by troublesome steps and the poor stability of specimens. Further large-scale studies are required to consolidate the clinical usefulness and potential as a screening test of the automated device described herein.

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Disclosure Statement

The authors have no conflict of interest.

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