DOI:10.31557/APJCB.2026.11.3.721

RESEARCH ARTICLE

Comparative Evaluation of Urinary Biomarkers *BLCA1*, *BLCA4*, *5-ALA*, and *HAL* in Liquid Biopsy for Early Bladder Carcinoma Detection

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Abstract

Introduction: Bladder cancer is associated with structural alterations in the cell nucleus. This study analyzed urine samples from patients with bladder cancer and healthy controls to assess nuclear matrix proteins (NMPs), specifically BLCA-1 and BLCA-4, which reflect tumor-related cellular changes. In addition, the study introduces a novel non-invasive liquid-biopsy approach using 5-aminolevulinic acid (5-ALA)-induced flow cytometry, alongside hexaminolevulinate (HAL)-induced flow cytometry and fluorescent cytology. Cancerous cells preferentially absorb these agents, leading to altered enzyme activity and intracellular accumulation of protoporphyrin IX (PPIX), which produces detectable red fluorescence in urine samples. **Materials and Methods:** The study involved 80 participants: 50 with confirmed bladder cancer (via imaging and histopathology) and 30 controls hospitalized for non-cancerous urological issues. Urine samples were collected in five 10 mL portions per participant. This initial study compared the diagnostic effectiveness of urine-based ELISA assays for BLCA-1 and BLCA-4 with 5-ALA-induced fluorescent cytology, HAL, and 5-ALA-induced flowcytometry. **Results:** The BLCA-1 showed 96% sensitivity and 90% specificity. While BLCA-4 demonstrated a sensitivity of 98% and a specificity of 93.3%, The 5-ALA-induced fluorescent cytology exhibited 93.3% sensitivity and 86.6% specificity. Both HAL and 5-ALA-induced flowcytometry achieved 80% sensitivity and specificity. The combined evaluation of BLCA-1 and BLCA-4 biomarkers demonstrated remarkable increase in sensitivity 97% and specificity 91.67 % respectively. **Conclusion:** The findings suggest that BLCA-1, BLCA-4, HAL, and 5-ALA are highly sensitive and specific non-invasive markers for bladder cancer detection, effectively distinguishing between high-grade and low-grade urothelial carcinoma. These marker panels could be integrated into routine screening protocols, potentially minimizing the need for invasive diagnostic procedures.

Keywords: Bladder cancer- biomarkers- non-invasive- liquid biopsy- sensitivity- specificity

Asian Pac J Cancer Biol, **11 (3)**, 721-728

Submission Date: 03/27/2026 Acceptance Date: 05/17/2026

Introduction

Bladder cancer (BC) ranks as the tenth most common cause of cancer-related fatalities worldwide, with a higher prevalence in men compared to women. According to the American Cancer Society, approximately 84,870 new cases of bladder cancer are anticipated in 2025, with 65,080 cases in men and 19,790 in women, along with an estimated 17,420 deaths (12,640 men and 4,780 women). [1]. In India, bladder cancer makes up 3.9% of all cancers, with 5.6% in men and 1.8% in women, resulting in about 18,921 new cases and 10,231 deaths annually [2]. Bladder cancer is categorized into non-muscle-invasive (NMIBC) and muscle-invasive bladder cancer (MIBC), each requiring distinct treatment strategies [3]. A recent study conducted in the United States reported approximately

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82,000 new cases of bladder cancer, the majority of which were classified as non-muscle invasive bladder cancer (NMIBC). [4] Muscle invasive bladder cancer (MIBC) affects about 25% of patients and is associated with a consistently poor prognosis [5]. Over 80% of initial diagnoses are NMIBC, with recurrence and progression rates of approximately 70% and 15%, respectively [6]. The frequent recurrence leads to increased financial and physical burdens due to repeated imaging and cystoscopies [6]. Although cystoscopy and urine cytology are gold standards, cytology lacks sensitivity, particularly for low-grade tumors, and cystoscopy is both invasive and expensive [7, 8]. Around 90% of bladder cancers are transitional cell carcinoma (TCC), which is known for high recurrence and mortality in advanced stages [9]. Accurate diagnosis is challenging due to tumor heterogeneity and varied clinical presentations [3]. This has spurred interest in non-invasive biomarkers to improve early detection and monitoring. Liquid biopsy, using urine samples, presents a promising non-invasive method [10]. The urothelium's direct contact with urine allows for the detection of tumor-related proteins, nucleic acids, and metabolites [11]. Notably, biomarkers such as BLCA-1, BLCA-4, 5-ALA, and HAL have shown significant potential in enhancing diagnostic sensitivity and specificity [15]. BLCA-1 is not found in healthy cells and is highly specific to bladder cancer, contributing to tumor progression through nuclear structural changes [12]. BLCA-4, expressed in early cancer stages, aids in distinguishing between benign and malignant conditions [13]. Photodynamic biomarkers like 5-ALA and HAL also show promise in diagnostics. 5-ALA accumulates in malignant cells and emits red fluorescence under blue light, enabling detection via fluorescence cytology [15]. HAL, a derivative of 5-ALA, is absorbed by cancer cells and converted into fluorescent porphyrins, enhancing tumor visualization during cystoscopy [16]. In this study, we introduced a novel, non-invasive 5-ALA–induced flow cytometry approach using liquid biopsy as a rapid and automated method to assess 5-ALA–labeled tumor cells in urine. This technique enables clear differentiation between benign and malignant cells by quantifying fluorescence intensity at the single-cell level, thereby offering enhanced diagnostic throughput and sensitivity. Hence, the present study aims to evaluate and compare the diagnostic performance of multiple urinary bladder cancer detection methods, including ELISA-based assays for BLCA-1 and BLCA-4, HAL-based flowcytometry, and traditional fluorescence cytology, with a novel 5-ALA–induced flowcytometry technique.

Materials and Methods

Patient Population

This cross-sectional study was conducted with approval from the institutional ethical committee (Ref No. MDC/JNMCIEC/370 dated 1/7/2024). The study group comprised of patients of age above 18 years and attending urology clinics with imaging and histopathology confirmed bladder cancer. The control group included patients aged above 18 years admitted to the urology department with non-cancerous conditions such as radiographic cystitis, urinary tract infections and urinary stones.

Sample collection, storage and processing

A total of 50 mL freshly voided urine samples were collected from the patients (50 Bladder cancer and 30 control) attending the urology clinic of a single tertiary care centre. The samples were aliquoted in tubes with preservatives and stored at -80 ^{o}C for further experiments.

Detection of BLCA-1 and BLCA-4 using ELISA kit based method

The detection of BLCA-1 and BLCA-4 was done using the GENLISA™ Human BLCA-1 ELISA and GENLISA™ Human Bladder Cancer-Associated Nuclear Matrix Protein 4 (BLCA-4) ELISA kit (Krishgen Biosystems) respectively as described by the manufacturer's instruction. The absorbance of each sample was measured at a wavelength of 450 nm using an Epoch BioTek reader with Gen5 software. Results were expressed as ng/ml for BLCA-1 and pg/ml for BLCA-4, providing a quantitative assessment of the biomarker in the urine sample.

5-ALA Fluorescent Cytology

The 5-ALA fluorescent cytology was performed only for 15 non-cancerous and 15 cancer patients following the protocol outlined by Rangrez et al. (2020). Urine samples were initially centrifuged at 1,500 rpm for 5 minutes, after which the supernatant was discarded. The resulting pellet was resuspended in minimum essential medium (MEM) containing 5-ALA hydrochloride (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) at a final concentration of 200 μg/mL. The suspension was then incubated in the dark at 37°C for 2 hours. Subsequently, the sample was centrifuged again under the same conditions, and the pellet was resuspended in MEM. Protoporphyrin IX (PpIX) fluorescence was finally assessed using a Nikon ECLIPSE Ni fluorescent microscope (Nikon Corporation, Tokyo, Japan) with excitation at 405 nm and emission detection between 600–650 nm. Bright intense red fluorescence indicated a positive result, while absence or dim fluorescence indicated negative cytology.

5-ALA and HAL Flowcytometry

The flowcytometry for 5-ALA and HAL was conducted on five noncancerous and five cancer patients as per the protocol described by Cunderlikova et al 2007. Urine samples were first centrifuged at 3,000 rpm for 10 minutes, and the resulting pellet was treated with trypsin for 10 minutes. The trypsin activity was neutralized using RPMI-1640 medium supplemented with fetal bovine serum (FBS). The cells were subsequently centrifuged and washed twice with serum-free medium. They were then incubated with a HAL stock solution of 6.33mM/mL in RPMI1640 medium for 1 hour at 37°C. After incubation, the samples were centrifuged again, and the final pellet was resuspended in phosphate-buffered saline (PBS). Similarly, to evaluate 5-ALA assay the urine samples were

centrifuged at 3000 rpm for 10 minutes. The cells were washed twice with a serum-free medium. The pellet was then suspended in a 5-ALA stock solution of 1.193 M/mL in MEM. This mixture was incubated in dark at 37°C. Finally, the sample was centrifuged, and the resulting pellet was resuspended in serum-free medium.

Cells were diluted with PBS to maintain data acquisition below 370 events/second. Samples with adequate cellularity and low inflammatory cell content (≥10,000 events) were included. Incubation was done for one hour with HAL and two hours with 5-ALA. 5-aminolevulinic acid and Hexaminolevulinate induced a distinct upward shift in red fluorescence in cancer cells, indicating a strong signal response. Further, the samples were analyzed using a Beckman Coulter Cytoflex flow cytometer with a 488 nm argon laser to stimulate PpIX fluorescence. Red fluorescence (510–540 nm and 670 nm filters), FSC, and SSC were captured. Histograms were used to assess fluorescence intensity and distinguish urothelial carcinoma cells.

Comparison with Histopathology: All patients with imaging-confirmed tumors underwent cystoscopy, transurethral resection, and biopsy. Histopathological findings such as Low grade Papillary TCC and high grade papillary urothelial carcinoma were used to validate the diagnostic accuracy of BLCA-1, BLCA-4, 5-ALA induced fluorescent cytology and HAL and 5-ALA induced flowcytometry.

Statistical Analysis

To examine the data, the independent t-test was used. $p<0.05$, is considered statistically significant. The statistical software SPSS version 27.0 was used to conduct the analysis.

Results

A total of 80 subjects including 50 cases confirmed by imaging and histopathology and 30 noncancerous were included in the study. Table 1 represents the clinical and demographic data of the study subjects. The Mean age of control was 44.13±8.27 years and cases was 65±12 years. The noncancerous group included 2 patients with chronic renal failure, 8 with urinary stones, 12 with benign prostatic hyperplasia, and 8 patients (26.67%) hospitalized for reconstructive surgery. None of these conditions are known to be associated with the parameters under investigation. Table 2 summarizes the diagnostic performance of BLCA-1 and BLCA-4 using ELISA assays.

BLCA-1 was negative in 27/30 noncancerous group and positive in 48/50 cases group whereas BLCA-4 was negative in 28/30 noncancerous group and positive in 49/50 cases. For BLCA-1, the limit of quantification was less than 0.25 ng/mL in noncancerous indicating negative for BLCA-1 and greater than 0.25 ng/mL in cases indicating positive for BLCA-1. Similarly, for BLCA-4, it was less than 120.5 pg/mL in noncancerous indicating negative for BLCA-4 and greater than 120.5 pg/mL in cases indicating positive for BLCA-4, the concentrations of BLCA-1 and BLCA-4 are higher in cases compared to noncancerous. Additionally, the data highlight differences in expression levels between high-grade urothelial carcinoma (HGUC) and low-grade urothelial carcinoma (LGUC) cases. These biomarkers can be used to differentiate between bladder cancer and non-cancer patients, as well as HGUC and LGUC. The BLCA-1- ELISA assay had 90% specificity, and 96% sensitivity and BLCA-4 ELISA assay had a sensitivity of 98% and a specificity of 93.3%. According

Table 1. Clinical Demographic Characteristics

Sl. No	Variables	Controls (n=30) (%)	Bladder tumour (n=50) (%)
1	Age (years)		
	<40	15 (50)	6 (12)
	41-50	10 (33.33)	8 (16)
	51-60	5 (16.67)	10 (20)
	61-70	-	15 (30)
	71-80	-	6 (12)
	81-90	-	6 (12)
2	Gender		
	Male	24 (80)	40 (80)
	Female	6 (20)	10 (20)
3	Cancer Type		
	Non-invasive Bladder cancer	-	35 (70)
	Male	-	30 (60)
	Female	-	5 (10)
	Invasive Bladder cancer	-	15 (30)
	Male	-	10 (20)
	Female	-	5 (10)
4	Co-Morbidities	-	
	Hypertension & Type II Diabetes Mellitus	5 (16.67)	30 (60)

Table 2. Concentrations of BLCA-1 and BLCA-4 Measured in Urine Samples of High-grade Urothelial Carcinoma (HGUC), Low-grade Urothelial Carcinoma (LGUC), and Negative Controls

Biomarker	HGUC	LGUC	Normal Control	p-value
BLCA-1	5.79 ± 1.01	3.32 ± 1.57	0.23 ± 0.20	0.0001
BLCA-4	1605.05±412.77	823.51 ± 274.06	95.03 ± 17.09	0.0001

Table 3. Combined Evaluation and Comparison of Sensitivity and Specificity for BLCA-1, BLCA-4, 5-ALA Cytology Biomarkers in Bladder Cancer

Biomarkers	BLCA-1	BLCA-4	BLCA-1 & BLCA-4	BLCA-1 & BLCA-4 in LGUC	BLCA-1 & BLCA-4 in LGUC	5-ALA fluorescent cytology
Controls (30)	27	28	55	55	55	15
Bladder cancer (50)	48	49	97	78	22	15
Sensitivity	96%	98%	97%	100%	86%	93.30%
Specificity	90%	93.30%	91.67%	91%	91%	86.60%

to our study, the levels of BLCA-1 and BLCA-4 are comparatively elevated in HGUC in contrast to LGUC and noncancerous (Table 3).

The 5-ALA fluorescent cytology was positive for 2/15 in noncancerous group and 14/15 in cancer group. The sensitivity and specificity of fluorescence cytology recorded by 5-ALA assay was 93.3% and 86.6% respectively, which also provides the definition of positive and negative cytology as illustrated in (Figure 1 a-d).

The Flow cytometric analysis of 5-ALA and HAL-induced demonstrated a clear upward shift along the red fluorescence (abscissa) in the stained cases compared to their respective stained controls and unstained cases showing a higher rate of positivity in bladder tumor samples compared with controls. Positive fluorescence signals were observed in 4 out of 5 bladder tumor cases, indicating a strong association with malignant urothelial cells. In contrast, only 1 out of 5 control samples showed a positive signal, suggesting a low background or false-positive rate in non-tumor specimens. Specifically, the 5-ALA–stained case exhibited an upward shift after 2 hours of incubation, while the HAL-stained case showed a similar shift after 1 hour of incubation. In contrast, the 5-ALA–stained control, HAL-stained control, unstained control, and unstained case samples showed minimal or no shift in red fluorescence intensity. This upward shift indicates increased red fluorescence intensity, reflecting enhanced intracellular accumulation of protoporphyrin IX (PpIX) following incubation with 5-ALA and HAL. The absence of a comparable shift in control and unstained samples confirms that the observed fluorescence increase is specifically attributable to the photosensitizer-induced metabolic activity rather than background or auto fluorescence. Overall, these findings support the ability of both 5-ALA– and HAL-based flow cytometry to preferentially detect bladder tumor cells, with greater positivity in tumor cases than in controls. Histograms were analyzed to assess fluorescence intensity

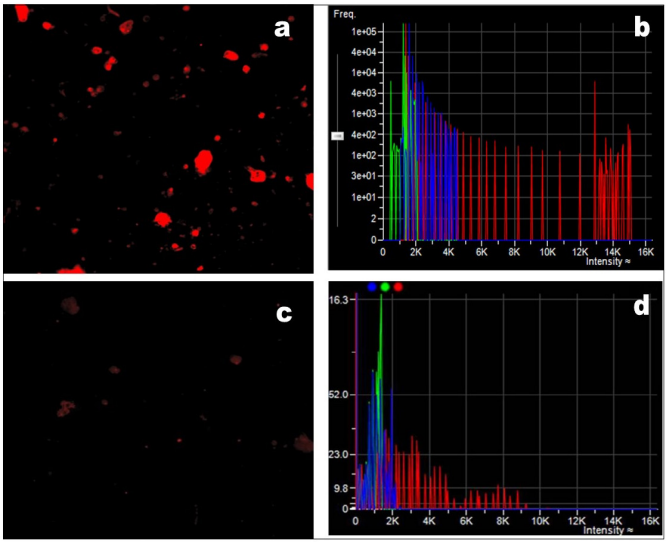

Figure 1. a. Under ×200 magnification, cells exhibiting strong red fluorescence a sign of cancerous cells were seen. b. A histogram showing the distribution of a malignant tumor cell's frequency (X-axis) versus intensity (y-axis). c. Cells that were visible with × 200 magnifications were showing a slight red fluorescence, which is indicative of a non-cancerous cell. d. A histogram showing the distribution of a non-malignant cell's frequency (X-axis).

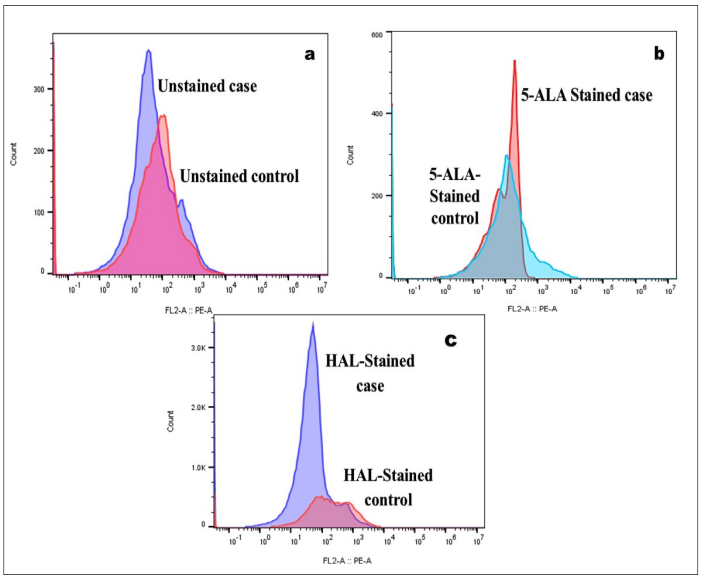

Figure 2. a. Histogram of Unstained Control and Case. b. 5- ALA PpIX fluorescence intensity histogram of case and control. c. HAL PpIX fluorescence intensity histogram of case and control.

and to distinguish urothelial carcinoma cells (Figure 2 a-c). 5-ALA and HAL have confirmed that the BLCA-1 and BLCA-4 are specific indicators for bladder cancer detection as shown in (Figure 3 a-b). The histological findings of the bladder cancer patients corroborate with the findings of BLCA-1 and BLCA-4 as well as 5-ALA-induced fluorescence cytology, HAL and 5-ALA-induced flowcytometry.

Discussion

Early and accurate detection of bladder cancer remains a clinical challenge, particularly in distinguishing malignant from non-malignant conditions and in stratifying tumor grade [14]. In this context, Liquid biopsy is gaining attention as a significant non-invasive technique, allowing for the detection of tumor-derived elements in body fluids [15]. It offers benefits over traditional methods, such as reduced invasiveness, a better representation of tumor heterogeneity, and the potential for early detection and personalized treatment strategies [16, 17]. To aid this, urinary biomarkers and fluorescence-based cytology have gained increased attention as adjuncts to conventional diagnostic modalities [18].

In our cohort, BLCA-1 was negative in the majority of noncancerous samples (27/30) and positive in most bladder cancer cases (48/50). The defined limits of quantification further strengthened diagnostic discrimination, with BLCA-1 concentrations below 0.25 ng/mL and BLCA-4 concentrations below 120.5 pg/mL reliably identifying noncancerous samples, while values above these thresholds were strongly associated with malignancy, recording a sensitivity of 96% and specificity of 90%. This supports its potential as a non-invasive diagnostic marker, which correlates with a study by Zaharan et al., 2023 that demonstrates whether BLCA-1 can be detected in the serum of patients with non–muscle-invasive bladder cancer (NMIBC) and whether elevated serum levels are associated with an increased risk of disease, the results showed that serum BLCA-1 is associated with NMIBC, showing sensitivities of 61.5% and 79.4% and specificities of 70% and 73% for Ta and T1 NMIBC, respectively. These findings suggest that BLCA-1 may play a meaningful role in bladder tumorigenesis and has potential utility as a serum-based biomarker [19, 20]

Similarly, BLCA-4 was negative in 28/30 noncancerous

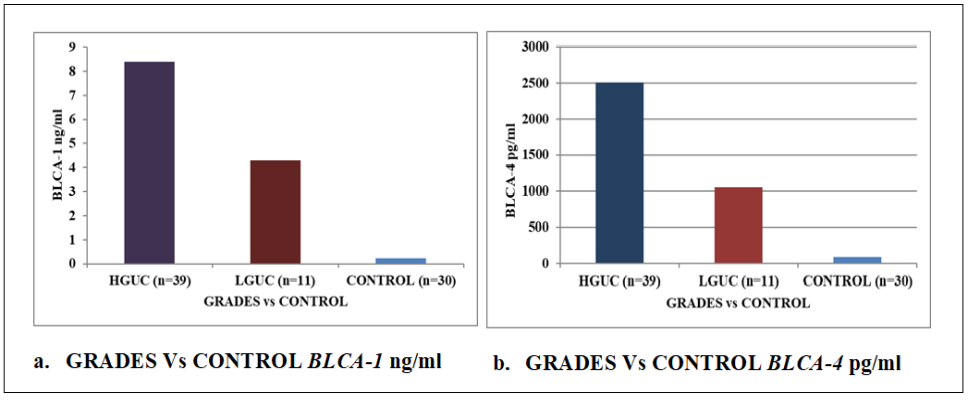

Figure 3. a and b. The graph shows the relationship between BLCA-1ng/ml and BLCA-4 pg/ml concentration with bladder cancer grades and control. Type the following correctly: Higher concentration of BLCA-1 and BLCA-4 levels were detected in HGUC compared to LGUC.

samples and positive in 49/50 cancer cases with sensitivity of 98% and specificity of 93.3%, underscoring the test's specificity. Our results were in the same direction as the study reported by Cai et al., 2015 where they explored the diagnostic effect of urine BLCA-4 in bladder cancer with a sensitivity of 93% and specificity of 97% [21]. The findings suggest that BLCA-4 assays could enhance early detection and complement existing diagnostic methods. These results are consistent with earlier studies demonstrating that BLCA-1 and BLCA-4 are preferentially expressed in bladder cancer and are largely absent in benign urothelial conditions [22]. Overall, both biomarkers showed significantly higher concentrations in cancer patients compared to noncancerous controls.

Fluorescence-based diagnostic approaches have emerged as valuable adjuncts to conventional cytology for the detection of bladder cancer, particularly in improving sensitivity while maintaining acceptable specificity [23]. In 2020, Rangrez et al assessed the diagnostic efficacy of 5-ALA-induced fluorescence cystoscopy, known for detecting mild bladder lesions due to increased 5-aminolevulinic acid uptake, altered enzyme activity, and unique PpIX handling by tumor cells, enabling red fluorescence detection. 5-ALA cytology demonstrated 100% sensitivity and specificity of 96% (25 cancer patients, 75 controls), significantly surpassing traditional cytology, which showed 64% sensitivity overall [16]. The present study also observed that 5-ALA fluorescent cytology was negative for 13/15 control group and positive for 14/15 cases, showing a sensitivity of 93.3% and specificity of 86.6% Our findings demonstrate that these modalities preferentially identify malignant urothelial cells and show strong concordance with histopathological diagnoses. Thus, suggesting fluorescence cytology, a promising, highly sensitive non-invasive method for bladder cancer detection and may reduce reliance on frequent cystoscopies.

In 2007, Cunderlikova and colleagues introduced a new technique to reduce the subjectivity of standard cytology by detecting bladder cancer cells in urine through protoporphyrin IX (PpIX) accumulation after incubation with hexaminolevulinate (HAL), using flow cytometry. An artificial model using TCCSUP bladder cancer cells and normal bladder epithelial cells (NBECs) demonstrated that only TCCSUP cells produced PpIX after HAL incubation, allowing for their detection and separation. The method could identify TCCSUP cells even when they constituted just 5% of the mixture. In urine samples from 19 patients with confirmed bladder cancer, results were consistent with conventional cytology. These findings support flow cytometry with HAL as a simple, accurate, and objective tool for bladder cancer detection [24].

In parallel, flow cytometric analysis using both 5-ALA and HAL demonstrated an increased proportion of positivity findings in bladder tumor samples compared with controls. However, the positive fluorescence signals were observed only in 4 out of 5 tumor cases, whereas only 1 out of 5 control samples showed a positive signal, suggesting a low background fluorescence and limited false-positive results in non-malignant specimens. Notably, HAL produced a distinct upward shift in red fluorescence in cancer cells, indicating a strong and easily detectable signal response. These findings align with earlier studies reporting that HAL, due to its higher lipophilicity and improved cellular uptake, may offer enhanced fluorescence intensity and tumor-to-background contrast [17, 24].

Importantly, the fluorescence-based findings correlated well with biomarker expression. Both 5-ALA and HAL confirmed the specificity of BLCA-1 and BLCA-4 as indicators of bladder cancer. The concordance between BLCA-1 and BLCA-4 positivity, fluorescence cytology, flow cytometry, and histopathological examination strengthens the validity of our results and supports a multimodal diagnostic approach. Histological findings in bladder cancer patients were consistent with elevated BLCA-1 and BLCA-4 levels as well as positive 5-ALA- induced fluorescent cytology. and HAL- and 5-ALA–based flow cytometric results, suggesting that these methods reflect underlying tumor biology.

Overall, our study highlights the complementary roles of BLCA-1, BLCA-4, 5-ALA induced fluorescent cytology, HAL, and 5-ALA–induced flow cytometry, as key specific cancer biomarkers in improving diagnostic accuracy through liquid biopsy. While fluorescent cytology offers high sensitivity at the cellular level, flow cytometry provides objective and quantitative assessment of fluorescence signals, and biomarker analysis adds molecular specificity. Integration of these approaches into clinical practice may enhance early detection, reduce false-negative rates, and support more accurate diagnosis and monitoring of bladder cancer [25]. Further studies with larger cohorts are warranted to validate these findings and to define optimal combinations of these modalities within diagnostic and surveillance pathways. Their presence in urine samples allows for improved disease monitoring, precise detection and personalized treatment planning. This represents a major advancement over traditional diagnostic methods by providing a more comprehensive and precise approach that can overcome key limitations such as tumor heterogeneity, which often leads to incomplete or inaccurate assessments when relying on conventional techniques.

In conclusion, the study demonstrates that urine-based BLCA-1 and BLCA-4 ELISA assays, 5-ALA-induced fluorescence cytology, HAL-induced flowcytometry, and 5-ALA-induced flowcytometry are highly sensitive, non-invasive methods for bladder cancer detection. These techniques can accurately identify cancer in urine samples, potentially reducing the need for invasive cystoscopy. Importantly, they can differentiate between low-grade and high-grade urothelial carcinoma, aiding in prognosis and treatment planning and supporting personalized care. The short study duration limits the evaluation of long-term diagnostic performance, and the single-center design may introduce biases related to patient population, and clinical practices. Further validation through larger, multi-center studies across diverse regions and healthcare systems is necessary to help assess the robustness of these assays, refine diagnostic protocols and deploy its utility in the

broader clinical setting.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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