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Fluorometric optical sensor arrays for the detection of urinary bladder cancer specific volatile organic compounds in the urine of patients with frank hematuria: a prospective case-control study

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Abstract: This study outlines a simple fluorometric optical sensor system for the sensitive, real time measurement of volatile organic compounds (VOCs) as biomarkers of urinary bladder cancer in patients presenting with frank hematuria and confirmed to have the disease on histopathology. Arrays of 24 sensor points based on fluorescence VOC sensitive materials were made. Urine samples of 38 consecutive patients with pathologically confirmed bladder tumours and 41 age and gender matched healthy controls were recruited and analysed using this sensor array. This system correctly classified 68 out of 79 urine samples with 84.21% sensitivity and 87.80% specificity; the system also achieved 66.67% sensitivity and 75.00% specificity for classification of high-grade and low-grade bladder cancer patients. This study showed promising results in the detection of urinary bladder cancer as well as to classify high grade versus low grade bladder cancers.

1. Introduction

Bladder cancer is the 10th most common cancer in the world and 2nd in urological cancers [1], it is 4th commonest in men (27 per 100,000 population) and 7th commonest cancers in women (8 per 100,000 population) [2]. Despite a significant morbidity and mortality caused by the disease, at present population-based screening for bladder cancer is not recommended due to: low accuracy of biomarkers for early stage diagnosis, challenges in identifying the high risk population, and lacks of non-invasive diagnostic tools [3].

In recent years, bladder cancer has become one of the most expensive cancer types due to its high recurrence rate and progression and requires frequent endoscopic surveillance. Currently the gold standard for bladder cancer diagnosis and surveillance is cystoscopy and biopsy, which is invasive, time-consuming, and expensive. According to National Institute for Health and Care Excellence (NICE) guidelines for bladder cancer management, the treatment for each newly diagnosed bladder cancer patient costs £1480 at the beginning, with £248 for each follow-up check procedure. The number of follow-up check procedures can vary depending on the grade and risk stratification of non-muscle invasive urinary bladder cancer [4].

Diagnosis of urinary bladder cancer using volatile organic compounds (VOCs) is not new. In 2004, Willis et al published a study using six trained detection dogs to diagnose bladder cancer using smell of urine from patients and healthy controls, the dogs successfully identified 22 out of 54 (41%) cancer patients; by chance alone this would have been 14%" [5]. In recent years, studies using gas-chromatography and mass-spectroscopy (GC-MS) have found that there are over 200 bladder cancer volatile organic compounds (VOCs) biomarkers in human urine,

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including aldehydes, ketones, alcohols, aromatics, and hydrocarbons [6]. The main source of biogenic VOCs cancer biomarkers is the lipid peroxidation induced by loss of balance of reactive oxygen species (ROS) in cancer cells. Normal cells are under dynamic balance of generation and clearance of ROS, while in cancer cells, the ROS levels are abnormally higher, resulting peroxidation damages to lipid membranes, and the end products are usually low molecular weight VOCs [7].

The relationship between biogenic VOCs and the presence of cancer provides an opportunity for non-invasive diagnosis of bladder cancer using patients' urine samples. In recent years, several attempts have been made to reproduce and improve the mammalian sense of smell using chemical sensors [8–10]. Sensory arrays used in the e-nose technology and other detection methods are mostly metal-oxide semiconductor sensors (MOS and MOSFET), conducting polymeric sensors (CP) and mass sensors (QMB and SAW), each type having different levels of answer to VOCs released by human body and associated advantages and disadvantages (Table 1). Point-of care diagnosis, cost-effectiveness of these devices and miniaturization of technology remain major issues of translational research and to address some of these issues. To address these issues, we have developed a urinary bladder cancer VOC biomarkers detection system using novel fluorometric optical sensory arrays as reported recently [11]. Compare to other e-nose sensing technology, optical sensors have unique advantages including lower detection limits (see Table 1), higher sensitivity, easier to manufacture and maintain, therefore lower the unit cost for the final test.

Table 1. Comparison of sensing techniques commonly used in e-nose. MOS: Metal-oxide-semiconductor; MOSFET: Metal oxide semiconductor field effect transistor; CP: Conducting polymer; QMB: Quartz microbalances; SAW: Surface acoustic wave. ppmV: parts per million by volume; ppbV: parts per billion by volume; pptV: parts per trillion by volume.

Type	Pros	Cons	Detection limit
MOS	wide response range low unit costs	high working temperature baseline shifting	ppmV level
MOSFET	very sensitive	expensive complicated to fabricate	low ppmV- to ppbV- level
CP	low working temperature	sensitive to water baseline shifting	low ppmV level
QMB	structure simplicity good sensitivity	cross-responding issues	ppbV level
SAW	good sensitivity	low signal-to-noise ratio	low ppbV level
Optical	very sensitive good signal-to-noise ratio	expensive cross-responding issues	ppbV- to pptV- level

Previous e-noses are based on the cross-reactive sensor arrays that depend on the changes of their properties when exposed to the analytes, mainly mass (polymer coated SAW/QMB sensors) and conductivity (CP, MOS) [12]. On the level of molecular forces, these sensors rely on van der Waals interactions to bring VOC molecules with their sensitive materials closer to each other. For optical VOC sensor, there are two fundamental requirements: each sensitive material must have a centre to interact strongly with the VOCs and each interaction centre must be coupled to an intense fluorophore [13]. The first requirement means the interactions could be not only the physical absorption and van der Waals interaction, but also π-π interactions and Bronsted and Lewis acid/base interactions. The second requirement means the sensitive materials should be fluorophores that have large conjugate structures that allow for interaction with the VOC molecules. In general, three classes of dyes fulfil the abovementioned requirements: Lewis acid/base dyes, such as porphyrins and metalloporphyrins; dyes responding to Bronsted acidic or

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basic pH indicators; dyes with large permanent dipoles, such as zwitterionic solvatochromic dyes [14].

In our previous study [11], we have assessed its performance in detection of four known urinary bladder cancer VOC biomarkers and preliminary classification of urine samples from 5 bladder cancer with 5 healthy controls. This study will further assess the diagnostic performance of this system in the detection of bladder cancer specific volatile organic compounds in patients presenting with frank hematuria using a case-control study design methodology.

2. Materials and methods

2.1. Participant recruitment

38 consecutive patients with pathological confirmed urinary bladder cancers and presenting with frank hematuria were recruited from hematuria clinic in a tertiary cancer centre. Ethic approval for this study was obtained through the East of Scotland Research Ethics Service (REC: 17/ES/0003). 41 age and gender matched healthy controls were recruited from the same department of the hospital. The exclusion criteria for controls were presence of urinary tract infection (UTI) or any other types of cancer. The urine samples of the participants were collected in a sterilized urine beaker and stored under −20 °C within 1 hour of collection.

All participant received participant information sheet and signed consent form on the day of recruitment. The demographic details of the participants are shown in the Table 2.

Table 2. Patient demographics, PVD: Peripheral vascular disease, CKD: Chronic kidney disease.

Characteristic	Cancer (n = 38)	Control (n = 41)
Age (Years):		
Means	72.74	67.73
Range	33-92	25-85
Gender:		
Male	31 (81.6%)	35 (85.4%)
Female	7 (18.4%)	6 (14.6%)
Grading:		
G1	1 (2.6%)	
G2-Low	19 (50.0%)	
G2-High	9 (23.7%)	
G3	9 (23.7%)	
Staging:		
pTa	27 (71.0%)	
pT1	6 (15.8%)	
pT2	5 (13.2%)	
Co-morbid conditions:		
Diabetes	6 (15.8%)	
Hypertension	18 (47.4%)	
Respiratory diseases	8 (21.1%)	
Cardiac diseases	13 (34.2%)	
PVD	8 (21.1%)	
CKD	3 (7.9%)	

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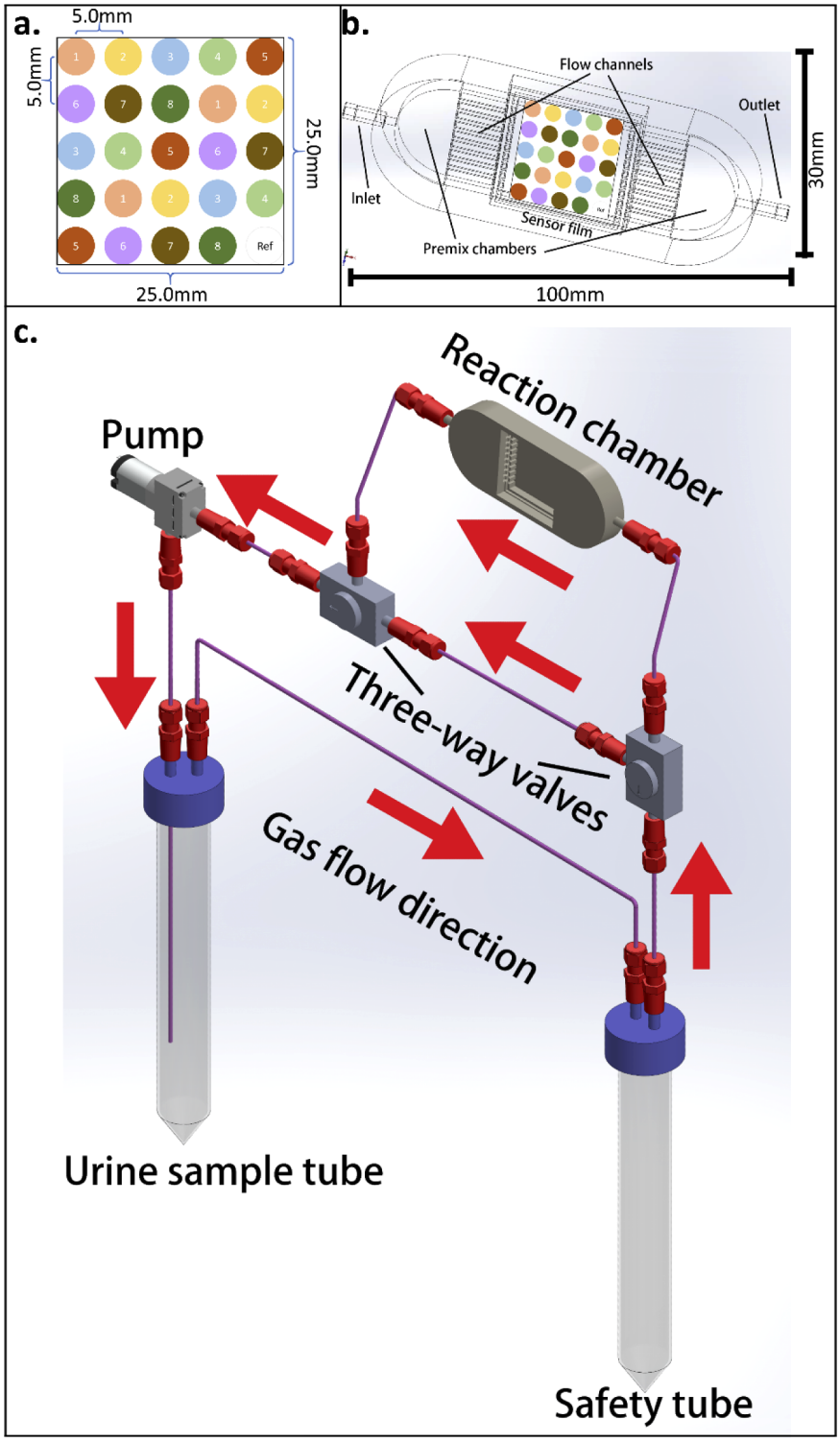

Fig. 1. a. Layout of fluorescence sensor array on PVDF film; b. Sketch of reaction chamber; c. Sketch of system components and workflow.

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2.2. System workflow

The system design used in this study has been previously described [11]. In brief, a dedicated designed fluorescence sensitive optical array was made by spotting eight VOC sensitive materials (porphyrins, metalloporphyrins, pH indicators and solvatochromic dyes) on the Polyvinylidene fluoride (PVDF) films. These sensory films have been proven to show specific responses to various kinds of bladder cancer related urinary VOCs. The layout of the sensory film is shown in Fig. 1(a), each film has 24 sensor element points (8 sensor types × 3 repeats) and 1 blank reference point, the excitation lights are UV (365nm), blue (450nm) and green (532nm). A gas delivery system (Fig. 1(c)) was used for providing a stable urinary vapour flow in the reaction chamber (Fig. 1(b)) while the sensory film was placed right in the middle of the chamber. The detecting system was able to read the fluorescence signals of each elements of the sensory array and pass the data to computer for analysis.

Before each test, the urine sample was thawed under 37°C water bath and 2.5mL of the urine sample were then transferred to a 15mL centrifuge tube (Fisher-Science, UK) and treated with 2.5mL 1M NaOH solution for 20 minutes, this process is to enhance release of urinary VOCs and has been widely used in urinary VOCs analysis previously [15–17]. At the beginning of each test, the initial fluorescence readings of each element on the sensory film were measured and recorded as "before" test, then the gas delivery system was started, and the urine vapour were circulated within the urine container for 2 min until the VOCs reach the gas-liquid dynamic equilibrium. Afterwards the urine vapour was guided through the reaction chamber and come in contact with the sensory film for 2 min. At the final step, the gas flow was stopped, and the fluorescence signals of each element were measured and saved as "after" test.

2.3. Data analysis

Fluorescence signals of each elements of the sensory array were read by the fluorescence spectrometer, then being processed by a series of algorithms [11]: removing backscattering, normalization, de-noising, and serialization. After serialization, the data gathering from each sensory film formed a unique signal sequence. Each film generated two signal sequences from "before" test and "after" test, respectively. The differences between "before" signal sequence and "after" signal sequence were calculated and stored as differential signal sequence and to be identified by Partial least squares Discriminant Analysis (PLS-DA).

PLS-DA is a linear classification algorithm that combines the partial least squares regression with the discrimination analysis for classification purposes. The PLS-DA using Latent Variables (LVs) to describe the input features of the algorithm, which are the linear combination of original features. Loadings are the coefficients of original features in the linear combination, while scores are the coordinates of each samples in the LV projection hyperspace. LVs, loadings, and scores are the most commonly used parameters in visualizing the results of PLS-DA classification [18]. The PLS-DA was performed using third-class Classification toolbox for MATLAB version 4.2 developed by Milano Chemometrics and QSAR Research Group [19].

To verify the performance of the PLS-DA model, cross-validation was used. Each time the algorithm randomly separated the dataset into two parts: "training" set and "testing" set, the "training" set was used for training the model and the "testing" set was used for validating the ability of the model to classify the correct sample into the right group. In leave-one-out cross-validation, each iteration only one sample was retained for testing set while in 20% Random Monte Carlo cross-validation, 16 samples (20% of all sample size) were randomly selected and retained each time for validation.

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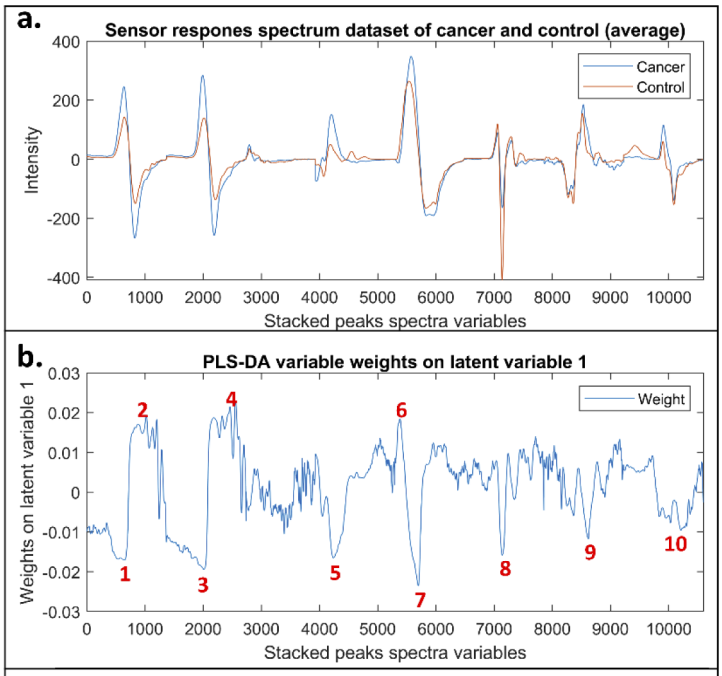
3. Results

Figure 2 showed the responses of sensor array to urine samples from urinary bladder cancer patients and healthy control volunteers. The differential signal sequence was shown in Fig. 2(a), the overall responses of sensors to urine vapour are similar, the differential signal sequences of both cancer and control groups showed blueshifts in sensor type 1, 2, 3, 5 and 8, redshifts occurred in sensor type 4 and 7, and intensity changes appeared in all three peaks of sensor type 6. Compared to healthy controls, the sensor responses of cancer group have generally higher



c.

Region	Spectral characters
1 & 2	Peak shifts and intensity changes of sensor type 1 on 654←694nm region
3	Peak shifts and intensity changes of sensor type 2 on 653←697nm region
4	Peak shifts of sensor type 3 on 575←600nm region
5	Peak shifts of sensor type 4 on 550→567nm region
6 & 7	Peak shifts and intensity changes of sensor type 5 on 611←684nm region
8	Peak intensity changes of sensor type 6 on 666nm
9	Peak shifts and intensity changes of sensor type 7 on 661→673nm region
10	Peak shifts of sensor type 8 on 651→692nm region

Fig. 2. a. sensor responses spectrum dataset (differential signal sequence) of urine samples from bladder cancer patients and healthy controls; b. Weight of each variables in contribution to PLS-DA latent variable 1; c. Spectral characters of top 10 most weighted variable regions labelled in b.

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levels of shifts and intensity changes of all the sensor types except type 6, which is expected considering cancer patient's urine has more signature VOCs that can bond to sensitive materials on the sensor array and leading to larger shifts/intensity changes. Figure 2(b) showed the weight of each variables in contribution of PLS-DA latent variable 1, which represented the importance of each spectral region among the whole differential signal sequence in achieving discrimination between cancer and healthy groups. The top 10 most weighted variable regions labelled in Fig. 2(b) were explained in Fig. 2(c), those regions made the biggest contribution to the latent variable in PLS-DA classification model, which unexpected are the regions in the differential signal sequence that have biggest variance between bladder cancer and healthy control groups.

Figure 3 showed the responses of sensor array to urine samples from bladder cancer patients with high-grade (blue) and low-grade (red) tumor. The differential signal sequence shown very similar pattern to Fig. 2(a), with smaller peak shifts in sensor 1 and 2 (9nm smaller on peak shifting), smaller peak intensity changes and shifts in sensor 6 and 8. In general the high-grade group has higher peak intensity changes and shifts than low-grade group, which is expected considering high-grade cancer patient's urine has higher concentration and more variance signature VOCs appear in their urine.

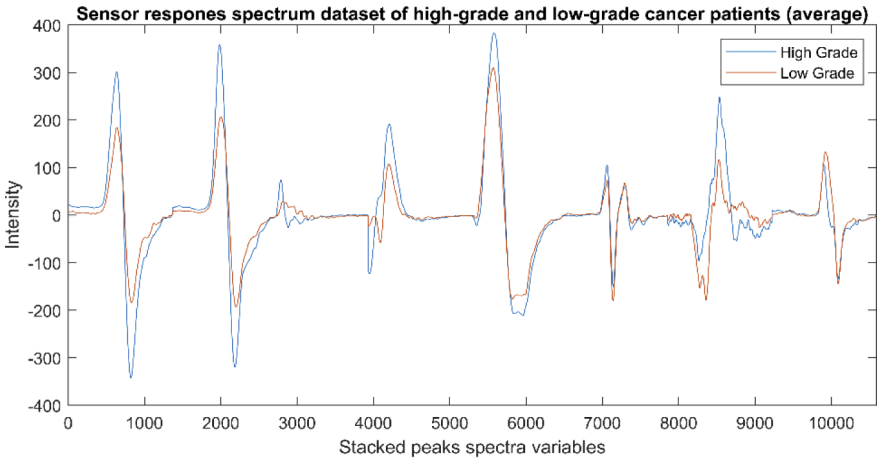

Fig. 3. sensor responses spectrum dataset (differential signal sequence) of urine samples from bladder patients with high-grade(blue) and low-grade(red) tumor.

Figure 4 showed the classification of cancer/healthy control and high-grade/low-grade cancer groups. The PLS-DA score plot (Fig. 4(a) and Fig. 4(d)) represented the score of each differential signal sequence in the PLS-DA latent variables space. Both score plots showed clear separation of groups with minimal inter-group overlaps. Both "low responding" groups (controls and low-grade cancer) in Fig. 4(a) and Fig. 4(d) showed larger intra-group spreading in the score plot than the "high responding" groups, this was because the low levels but broader non-specific bonding of urinary VOCs and sensitive materials occurred in "low responding" groups while "high responding" groups had higher levels of specific bonding of cancer VOCs and such bonding dominated the signal responses of the sensors.

For distinction of bladder cancer and healthy controls, the PLS-DA algorithm correctly classified 68 out of 79 samples with 84.21% sensitivity and 87.80% specificity in leave-one-out cross-validation (Fig. 4(b)). To further verify the performance of the PLS-DA model, a 20% Random Monte Carlo cross-validation was used, this method will randomly pick 80% of the sample data from the dataset and use them as "training" dataset for building the model, while the remaining 20% will be used as "testing" dataset for validating the prediction ability of the model, and such process will repeat 1000 iterations, because each iteration are individually performed

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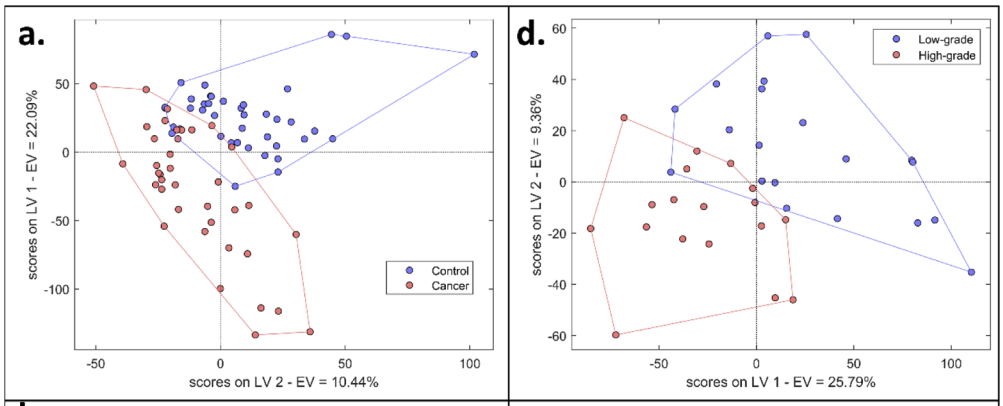

b.

Diagnosed / Predicted	Bladder Cancer BC 38	Health Control HC 41
Predicted BC 37	True positive 32	False positive 5
Predicted HC 42	False negative 6	True negative 36
Total population 79	**Sensitivity** 84.21%	**Specificity** 87.80%

e.

Diagnosed / Predicted	High Grade HG 18	Low Grade LG 20
Predicted HG 17	True positive 12	False positive 5
Predicted LG 21	False negative 6	True negative 15
Total population 38	**Sensitivity** 66.67%	**Specificity** 75.00%

c.

Diagnosed / Predicted	Bladder Cancer BC 7742	Health Control HC 8258
Predicted BC 7165	True positive 5994	False positive 1171
Predicted HC 8835	False negative 1748	True negative 7087
Total population 16000 of 16000	**Sensitivity** 77.42%	**Specificity** 85.82%

f.

Diagnosed / Predicted	High Grade HG 2545	Low Grade LG 3036
Predicted HG 2502	True positive 1735	False positive 767
Predicted LG 3079	False negative 810	True negative 2269
Total population 5581 of 8000	**Sensitivity** 68.17%	**Specificity** 74.74%

Fig. 4. a. score plot of PLS-DA classification model of bladder cancer and healthy control groups (4 latent variables); b. leave-one-out cross-validation confusion matrix of PLS-DA classification model of cancer and non-cancer; c. 20% Random Monte Carlo cross-validation confusion matrix of PLS-DA classification model of cancer and non-cancer; d. score plot of PLS-DA classification model of high-grade and low-grade bladder cancer group (10 latent variables); e. leave-one-out cross-validation confusion matrix of PLS-DA classification model of high-grade and low-grade bladder cancer group; f. 20% Random Monte Carlo cross-validation confusion matrix of PLS-DA classification model of high-grade and low-grade bladder cancer group

without influence from previous iterations, therefore this cross-validation method was considered as stronger prevention of overfitting than leave-one-out cross-validation. As shown in Fig. 4(c), the sensitivity and specificity of same PLSDA model under 1000 iteration of 20% Random Monte Carlo cross-validation showed very little drops of sensitivity (84.21%→77.42%) and specificity (87.80%→85.82%).

Figure 4(d) showed the classification results of PLS-DA model of urine samples from high-grade (G2-High to G3) and low-grade (G1 to G2-Low) bladder cancer patients. The model achieved 66.67% sensitivity and 75.00% specificity in leave-one-out cross-validation (Fig. 4(e))

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but failed to pass the 1000 iteration of 20% Random Monte Carlo cross-validation (see Fig. 4(f), only 5581 out of 8000 testing samples success assigned groups), suggested that the sample size is not big enough for support the statistically significant classifications for cancer grading. Current test failed to classify the staging of the cancer group (data not shown), this was due to the dominate number of urine samples from non-invasive (low stage) bladder cancer than invasive (high stage) cancer patients (27 vs. 11, see Table 2) that did not achieve balance enough for building a statistical significant classification model, this could be a future area of study to evaluate if the device could identify the stages of the bladder tumour by urine tests.

4. Discussion

This study shows promising results of using low-cost miniaturized fluorescence gas sensor array in distinguish between urine samples of bladder cancer patients and healthy people and distinguish between urine samples of high-grade and low-grade bladder cancer patients. Compare to our previous study [11], this study have included more urine samples and redesigned the system to accommodate the requirement for urine test, as well as improved

The freeze-thaw process has been proven to have no effect on the release of cancer-related VOCs from urine samples [10,16]. The effect of pH adjustment of urinary VOCs release was also reviewed in previous literature [15,17]. In general, adding acid or alkaline can increase the release of VOCs by increasing the ionic strength of the urine, lowering pH value can promote the release of acids and sulfur-containing molecules, while higher pH will increase the release of alcohols, amines, ketones and N-heterocyclic compounds. Depending on the natural of bladder cancer specific VOCs (rich in aldehydes, ketones and alkanes) and the composite of the sensor array, we use alkaline adjustment for urinary VOC release enhancement.

For the classification of cancer and non-cancer, the system achieved 87.80% specificity, which is comparable to the specificity of some most commonly used urinary biomarker tests like BTA(60–92%) and NMP22(60-90%) [20]. The sensitivity was 84.21%, which is also higher than those urinary biomarker tests mentioned. This system also showed potential in classification of urine samples from high- and low-grade bladder cancer patients and achieved 66.67% sensitivity and 75.00% specificity respectively. Although this study was only proof-of-principle study and the proposed diagnostic method needs further evaluation using larger population, nevertheless, the results here have shown promising prospects for a low-cost non-invasive diagnosis tool for bladder cancer using photonic technology.

As shown in Table 3, compared to other similar studies using e-nose or equivalent sensing techniques for detecting urinary VOCs biomarkers for diagnosing bladder cancer, our study showed competitive sensitivity and specificity, and extra ability in classification of high-grade and low-grade cancer patients. However, the biggest advantage of using fluorescence gas sensor optical arrays for bladder cancer diagnosis is the cost-efficiency, the device costs of current laboratory setup was as low as £3500, with sensory film for only £0.5 per test, and the costs can be further reduced in mass productions in the future. In comparison, in the UK, particularly in the National Health Services (NHS), the mean cost per test for Photodynamic diagnosis (PDD) was £1371, for White light cystoscopy (WLC) was £937, for flexible cystoscopy (CSC) was £441, urine cytology costed £92, Nuclear matrix protein 22 (NMP22) costed £39, ImmunoCyt costed £54 and Fluorescence in situ hybridization (FISH) costed £55 [21]. In contrast to current expensive personalized diagnosis assays, this method described in the present study doesn't need complex sampling and storing gadgets, nor expensive analytical equipment. The diagnostic results are real-time, visualized, and intuitive, which makes the diagnostic method ideal for point-of-care diagnosis and surveillance purposes. At the current stage, none of the urinary VOC tests were used in point-of-care practice and the method described in this study is expected to improve the situation.

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Table 3. Comparison of other studies of urine-based diagnosis of bladder cancer. N/A: Not available; CV: cross-validation; EV: external validation; HPLC: High Performance Liquid Chromatography; MS: Mass Spectrometry; GC: Gas Chromatography; MOS: metal-oxide-semiconductor; CP: conducting polymer

Study	Study size n (cancer + control)	Techniques	Sensitivity	Specificity	Note	Estimated costs (per test)
[5]	144 (36 + 108)	Detection dogs	0.41	N/A		N/A
[22]	89 (48 + 41)	HPLC-MS	1.00	1.00	No CV	~£200
[6]	277 (72 + 205)	GC-MS	0.90	0.88		~£100
[23]	75 (24 + 51)	GC-ToFMS	1.00	1.00	Non-VOC	
[24]	85 (50 + 35) training	GC-MS	0.86	0.86	CV	
	96 (59 + 37) testing		0.70	0.70	EV	
[8]	89 (30 + 59)	GC + e-nose	0.62	0.70		~£40
[9]	98 (24 + 74)	GC-MOS	0.96	0.95		
[10]	60 (30 + 30)	CP	0.75	0.86	No CV	
Mean	126.6		0.80	0.87		
OURS	79 (38 + 41)	fluorescence	0.84	0.88		£5

There are still some limitations of this study: we only recruited newly diagnosed bladder cancer patients with hematuria but did not recruit any recurrence or following-up patients, a further study can focus on those patients for investigating the application of this device in surveillance of patients with known previous bladder cancer. Using analytical chemistry tools like GC-MS in parallel of fluorescence array will be helpful to better understand the mechanism and application of the urinary VOC sensors and guide the future development and improvement works. Furthermore, a larger multi-centre cohort study will be helpful in determining external validity of this diagnostic device in correctly identifying grades and stage of urinary bladder tumours.

5. Conclusion

In conclusion, the fluorescence urinary VOCs detection system successfully classified urine samples of bladder cancer patients and those of healthy controls with good sensitivity and specificity. This system also showed potential in the classification of high-grade and low-grade bladder cancer patients, but further experiments are needed to confirm this. These results indicated that using low-cost fluorescence gas sensor arrays for urinary bladder cancer diagnosis is feasible. Future development of novel low-cost portable devices for bladder cancer diagnosis and surveillance could benefit from this technology.

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Disclosures

The authors declare no conflicts of interest.

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