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A study for the detection of kidney cancer using fluorescence emission spectra and synchronous fluorescence excitation spectra of blood and urine

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ABSTRACT

In this study, we compared different types of biomolecular markers in kidney cancer patients and in normal healthy controls, using fluorescence emission spectra and synchronous fluorescence excitation spectra. We were able to provide an accurate classification of the spectral features of kidney cancer patients relative to that of normal controls, in terms of the concentration ratios of biomolecules (viz., tryptophan, NADH, FAD, basic porphyrin, and acidic porphyrin) based on the intensity of their spectral peaks. The specificity and sensitivity of the method were 90%. The rationale of our current approach is to evolve an innovative protocol for the spectral characterization of in vitro optical analyses suitable for both small clinics and hospitals.

1. Introduction

Kidney cancer is one of the ten most common cancers in both men and women worldwide. The risk of developing life-threatening kidney cancer is about 1.6%. Smoking is a main contributing cause of kidney cancer development, with men being at higher risk than women [1].

Other causes of kidney cancer include obesity, excessive alcohol consumption, hypertension, and low physical activity [2]. Early detection of the disease could improve survival rates [3]. Several techniques have been used to diagnose kidney cancer, such as routine blood and urine tests, and ultrasonography, magnetic resonance imaging (MRI), computed tomography (CT), intravenous pyelogram, and angiography are the established protocols for visualization of the tumors. However, these techniques are quite expensive and most involve exposure to ionizing radiation. MRI has the highest accuracy rate with sensitivity and specificity at 95%, but it is more expensive, rendering it inaccessible for small hospitals and clinics in developing countries [4].

Cancer detection using fluorescence spectroscopy based on endogenous and exogenous fluorophores of tumor tissue has already been established for a few decades. It has shown potential for the characterization of biological and morphological properties of cancer tissue from each patient [5].

Alfano et al. in an early study identified laser-induced fluorescence properties of biomolecules, such as tryptophan, collagen, elastin, nicotinamide adenine dinucleotide (NADH), and flavin, for normal and cancerous human tissue [6]. Schomacker et al reported the application of laser-induced fluorescence methods to distinguish neoplastic from hyperplastic and normal colonic tissue. The fluorescent biomarkers each had a specific peak; for example, at 390 nm due to collagen and at 460 nm due to NADH [7]. Lin et. al. investigated the auto fluorescence spectral features between normal and cancerous nasopharyngeal tissues to extract the potential information leading to the diagnosis of nasopharyngeal carcinoma (NPC). They studied the variation in their integrated fluorescence intensity $I_{455 \pm 10\ nm}$and $I_{380 \pm 10\ nm}$ on the basis of ratios between collagen and NAD(P)H In order to diagnose NPC tissues at 340 nm, they calculated the fluorescence intensities using a two-peak ratio algorithm [8]. In another study that employed the Xenon ion laser (365 nm), cancer tissue manifested emission peaks at 630 nm due to porphyrin [9].

Most cancer detection studies have been done on tumor tissues but not on body fluids. Leinier and his research team investigated plasma and urine for specific fluorescent biomolecules (tryptophan, tyrosine, flavin adenine dinucleotide (FAD), etc.) and found that they were markedly different for cancer patients [10–12]. Our group published a set of research papers on quantification of porphyrin in the acetone extract of cellular components as well as of the above-mentioned biomarkers of blood plasma and urine [13–15].

From this point of view, this paper introduces a new diagnostic fluorescence spectroscopy technique based on spectra of biomolecules found in blood plasma and urine samples. Being simple, rapid, and inexpensive, this technique is suitable for small clinics and hospitals.

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2. Materials and methods

The instrumentation and techniques employed in this study were very similar to those previously described [13–18]. In order to highlight major differences in spectral features of the blood components, blood plasma, red blood cell, and urine samples were obtained from normal controls and from kidney cancer patients

A total of 20 subjects were considered for the study, 10 of whom had kidney cancer (age: 40–55 years) and 10 were healthy control individuals adjusted for age who had no specific disease (normal control group). All volunteers in the normal control group were regular employees of the King Saud University and the King Khalid University Hospital (Saudi Arabia). The protocols of the study were explained to the subjects in order to obtain their informed consent. The Institutional Review Board (IRB) of King Khalid University Hospital had approved this study on human sample under approval no 14/4028/IRB. In the current study, the population size of the kidney cancer patient was very small. There were three patients on stage 2, three on stage 4 and the rest were also on different stages of cancer severity. The results presented in this study were preliminary for the proof of concept only.

Intravenous blood (5 mL) was drawn from each subject and collected in a vial coated with ethylenediaminetetraacetic acid (EDTA) on the inner wall. Each tube was gently inverted five times to mix the contents evenly, and then centrifuged (3000 rpm, 15 min) to separate the cellular components from the plasma. Approximately 1 mL of the supernatant plasma, a greenish-yellow liquid, was transferred by pipetting into a sterile glass tube. The remainder of the top buffy coat in the EDTA vial was carefully discarded by pipetting, leaving behind the thick jelly-like residue that contained cellular components, mainly erythrocytes. Next, a 0.5 mL suspension of erythrocytes was lysed using 1.5 mL of spectroscopic-grade acetone. The mixture was shaken well for efficient extraction of biomolecules from the erythrocytes. This was then centrifuged (3000 rpm, 15 min), and the clear supernatant, containing mostly fluorescent biomolecules, was used for spectral analysis. The total time required for blood sample preparation was 20 min. The method used for the preparation of urine samples was as follows. The first requirement was to collect first voided urine sample under 48 h condition of non-essential medicines (including herbal and ayurvedic), spicy meals, and meats in a sterile vial. Finally, a 2 ml of urine sample in a quartz cuvette was used for the study under 400 nm excitation wavelengths and the fluorescence emission spectra (FES) was recorded in the wavelength range from 425 nm to 700 nm.

When a biomolecule absorbs a photon, it becomes excited and remains in this state for a few nanoseconds, during which most of the excitation energy is dissipated as heat but a small part is emitted as fluorescence, which acts as a fingerprint for a particular set of molecules and their environment. Out of a large number of biomolecules, such as proteins or amino acids, up to 12 molecules emit fluorescence in the range of 200–800 nm. The relative proportion of such fluorescent molecules acts as a biomarker for a certain set of well-defined diseases [13–15].

The PELS 55 spectrophotometer (PerkinElmer LS 55, USA) used in this study is equipped with two diffraction gratings for emission, excitation, or synchronous scans. The excitation grating can be preset to select light of a particular wavelength (e.g., 400 nm), and when used to excite a sample (e.g., acetone extract of erythrocytes), the emission grating is scanned from 425 to 700 nm to map the fluorescence profile of a set of biomolecules in that range. The results are called fluorescence emission spectra (FES) in the wavelength range from 425 nm to 700 nm. Alternatively, when the excitation and emission gratings are set at a 10 nm offset and rotated simultaneously, synchronous fluorescence excitation spectra (SFXS) in the wavelength range from 200 nm to 600 nm are obtained. The other different offsets like 20 nm, 30 nm, and 40 nm were also tried but the SFXS at an offset of 70 nm provided a good discrimination between the samples of healthy and kidney cancer patient. This –is logical because 70 nm corresponded to the excitation peak (290 nm) and emission peak (360 nm) of tryptophan. These set of molecules obtained during SFXS include tryptophan, NADH, FAD, and porphyrin, which have partially overlapping emission profiles in complex systems such as the blood plasma. It is important to emphasize that FES and SFXS are variants of fluorescence spectroscopy [6,13]. Such SFXS are also known as Stokes' shift spectra [6].

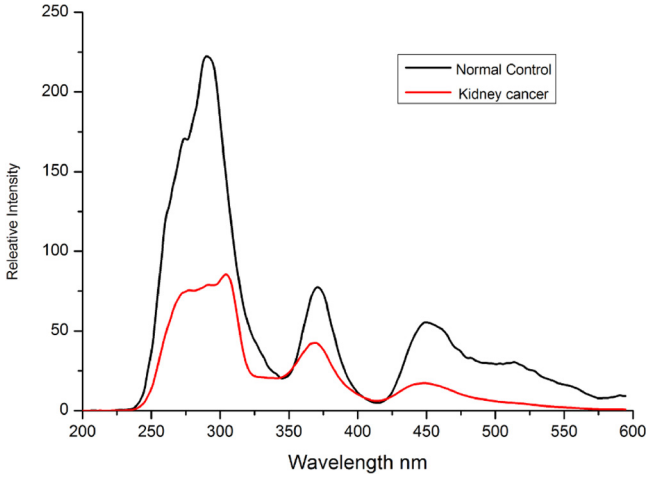

Fig. 1. shows the synchronous fluorescence excitation spectra of the plasma of normal control (a) and synchronous fluorescence excitation spectra of kidney cancer patients (b).

3. Results

Fig. 1(a) shows the SFXS of normal kidney plasma. This spectrum has three main bands, one at 290 nm (due to tryptophan), a second at 360 nm (due to NADH), and a third at 450 nm (due to FAD). We defined the ratio *R290/360 (Plasma)*, where the ratio of the intensity of the peaks at 290 and 360 nm was calculated to be about 2.60 for the normal control, and *R448/360 (Plasma)* was about 0.83. In contrast *R290/360 (Plasma)* and *R448/360 (Plasma)* for kidney cancer patients were about 1.30 and 0.34, respectively. That is, the intensity of the 290 nm peak was almost three times greater for the normal controls than for the kidney cancer patients (Fig. 1(a), (b)). For the normal controls, the mean value for *R290/360 (Plasma)* was 2.60 ± 0.29 and for *R448/360 (Plasma)* was 0.83 ± 0.05 (Table 1). In the case of the kidney cancer patients, the mean *R290/360 (Plasma)* was 1.30 ± 0.39 and the mean *R448/360 (Plasma)* was 0.34 ± 0.09. In addition, plasma samples from the kidney cancer patients had a three times lower tryptophan peak intensity than that of the normal controls [13,16,17].

Fig. 2(a) shows the FES of the acetone extract of cellular components from the normal control group. This spectrum showed two wavelength bands, one at 585 nm (due to basic porphyrin) and a second at 630 nm (due to acidic porphyrin). Based on the ratio R630/585 (cellular component), the fluorescence of acidic porphyrin at 630 nm relative to basic porphyrin at around 585 nm was 1.01 for normal controls (SD: 15.0%). All the cellular measurements were quantitative and

Table 1
Ratio Parameters of Normal against Kidney cancer patients.

Parameter	ID	Study Population	Mean	Std. Deviation ±
R290/360 (*Plasma*)	Normal	10	2.60	0.29
	Cancer	10	1.30	0.39
R448/360 (Plasma)	Normal	10	0.83	0.05
	Cancer	10	0.34	0.09
R630/585 (cellular component)	Normal	10	1.01	0.15
	Cancer	10	2.20	0.24

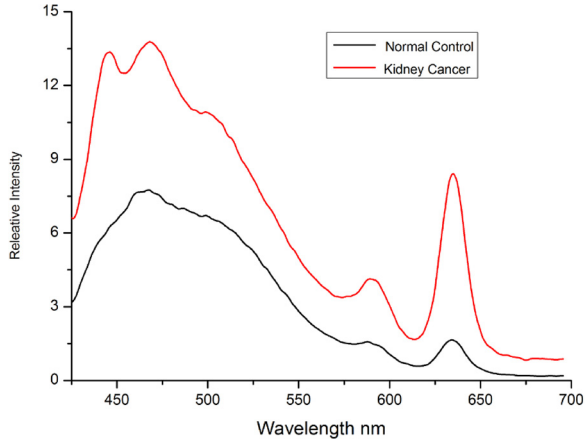

Fig. 2. shows the FES plasma of normal control (a) and FES of kidney cancer patients (b).

repeatable.

Fig. 2(b) represents the FES of the acetone extract of cellular components of the kidney cancer patients. It is important to note that the wavelength band at 630 nm (due to acidic porphyrin) was significantly elevated for kidney cancer patients (by 4 times relative to the controls) [16]: this is to be compared with the elevation of 585 nm band by 1.8 times. Altogether, the ratio R630/585 gets enhanced by 2.2 times for kidney cancer patients. The other peak variations were marginal and hence ignored.

Fig. 3 shows the SFXS of urine of the normal controls (graph (a)) and kidney cancer patients (graph (b)). It was obvious that the peak at 365 nm (due to NADH) was elevated relative to the 450 nm band (due to FAD) in the normal controls. In the case of the kidney cancer patients, the wavelength band was blue shifted, showing a peak at 330 nm (due to tryptophan or its closely related amino acids).The peak of 330 nm was completely absent in the SFXS of urine of the normal controls.

In Fig. 4(a) and (b), the FES of urine from the normal controls and kidney cancer patients, respectively, are shown. Two broad peaks were observed that might be due to emission at 475 nm (due to NADH) and the other at 525 nm (due to FAD). The peaks of both biomarkers were elevated by almost 50% in the normal controls relative to those of the kidney cancer patients. In conclusion, the intensity of the entire spectrum was higher in normal controls than in kidney cancer patients

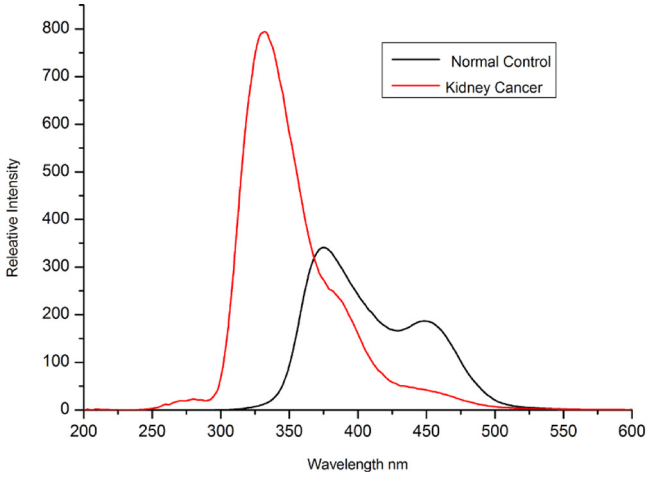

Fig. 3. shows the SFXS urine of normal control (a) and SFXS of kidney cancer patients (b).

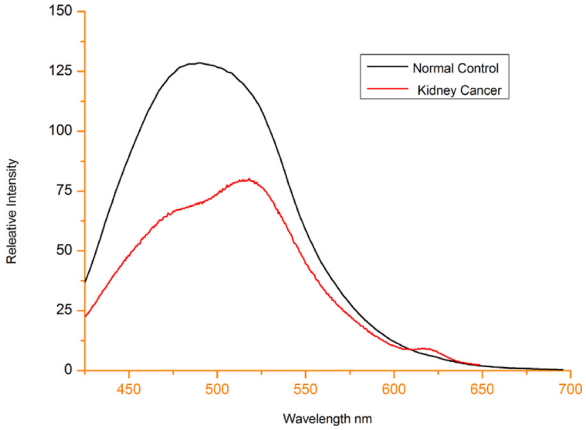

Fig. 4. shows the FES urine of normal control (a) and FES of kidney cancer patients (b).

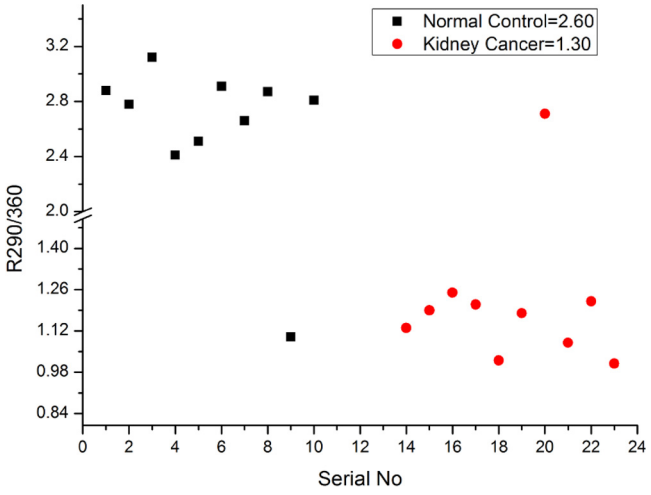

Fig. 5. Scatter plot of the ratio R290/360 (*Plasma*), as obtained from Fig. 1. a-Normal controls b- Kidney cancer patients.

indicating that there was an increased emission from both of the peaks.

Fig. 5 shows the scatter plot for *R290/360 (Plasma)* for the normal individuals and kidney cancer patients. It was clearly evident from the experimental results that *R290/360 (Plasma)* had classified all normal controls and kidney cancer patients correctly, with an average of 2.60 for the normal controls and 1.20 for the kidney cancer patients.

Fig. 6 shows the scatter plot for *R448/360 (Plasma)* for the two subject groups. All the classifications between the two study groups were also well separated, indicating an average of 0.83 for the normal controls and 0.34 for the kidney cancer patients.

Hence, it was concluded that the three fluorescent biomarkers tryptophan, NADH, and FAD were out of proportion in normal controls as compared with kidney cancer patients, as pointed out by their ratios *R290/360 (Plasma)* and *R448/360 (Plasma)*. The scatter plots for *R290/360 (Plasma)* (Fig. 5) and *R448/360 (Plasma)* (Fig. 6) indicate a clear and visible difference between the two sets (Fig. 7).

To achieve the objective in discriminating the spectral features of plasma between normal controls and kidney cancer patient's samples, receiver operating characteristic curves (ROC) were plotted for *R290/360 (Plasma)* and *R448/360 (Plasma)*. The area under the curve was 0.849 for *R290/360 (Plasma)* and 0.978 for *R448/360 (Plasma)*, representing a significant level of separation. All the ratio parameters (*R290/360 (Plasma)*, *R448/360 (Plasma)*, and *R630/585 (cellular component)* are shown in Table 1.

Area Under the Curve

Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
R290/360 (Plasma)blue linein ROC curve	.849	.083	.001	.687	1.000
R448/360 (Plasma)Green line in ROC curve	.978	.024	.000	.930	1.000

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

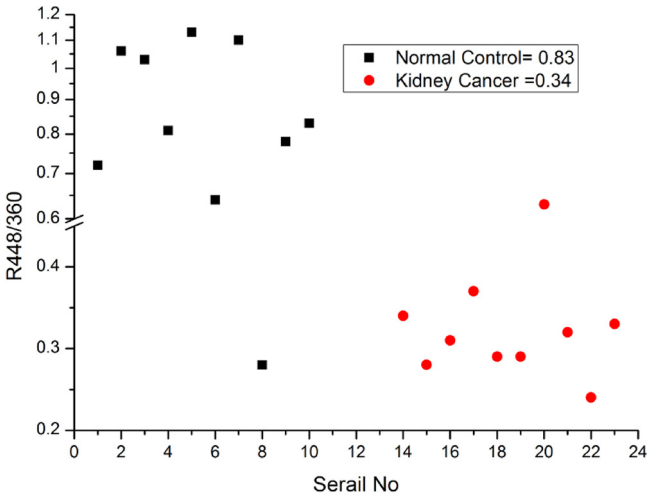

Fig. 6. Scatter plot of the ratio *R448/360 (Plasma)* as obtained from Fig. 1. a-Normal controls b- Kidney cancer patients.

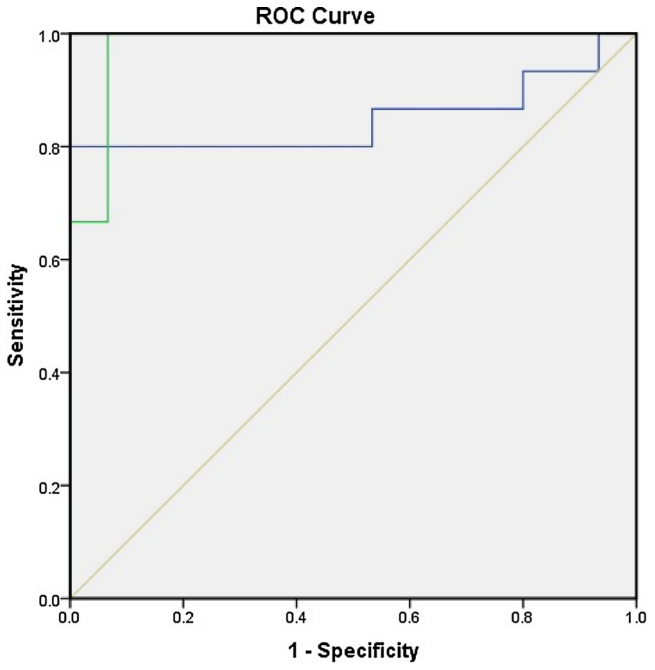

Fig. 7. Receiver operator curve (ROC) acquired from the statistical calculation of the data for R290/360 (*Plasma*) and *R448/360 (Plasma)*.

4. Discussion

The tenth most common type of cancer among men and women worldwide is kidney cancer. The major causes for the development of this disease are alcohol consumption, hypertension, low physical activity, and obesity. The current study introduces a simple fluorescence spectroscopy technique that offers good prospective for inexpensive kidney cancer detection. Different types of cancer biomarkers have been measured and detected using FES and SFXS. The recorded spectra for cancer patients were compared with those of age-adjusted normal controls, showing different spectral features. The basis for this technique is to identify and differentiate kidney cancer patients from normal controls by employing a set of ratio parameters acquired by SFXS and FES. The intensity ratio of two bands like tryptophan and NADH (*R290/360 (Plasma)*) in the SFXS of kidney plasma gives a clear differentiation between kidney cancer patients and normal controls. Similarly, the ratio between FAD and NADH (*R448/360 (Plasma)*) specifies different spectral signatures between kidney cancer patients and normal controls. This reflects a clear –distinction in the concentration of the three cancer biomarkers (i.e., tryptophan, NADH, and FAD) when malignancy sets in. It is also important to know that the spectral ratio *R290/360 (Plasma)* of normal controls is 2.60 against the 1.30 of kidney cancer patients. Similarly, the spectral *R448/360 (Plasma)* value of normal controls is 0.83, whereas that of the kidney cancer patients is 0.34. The FES of the acetone extract of blood components of normal and kidney cancer patients also point out good differentiation in their ratio R630/585 (cellular component) between the basic porphyrin peak at 585 nm (a protein found in hemoglobin) and the acidic porphyrin peak at 630 nm (neutral form that bind metals like Fe). This ratio was 2.2 times higher in kidney cancer patients than in the normal controls, confirming that porphyrin accumulation in the tumor site is significantly high owing to biochemical variation. This would also create an imbalance in the microbial activity of tissue or on their necrotic sites, which could lead to a high percentage of porphyrin in the circulating blood, another reason for the porphyrin elevation. The likely cause for the increase of porphyrin in the blood is enzyme release by the malignant cells, which may alter hemoglobin biochemistry [19]. This fact was in good agreement with our previous studies [13–17].

It is important to draw attention to the difference in the urine SFXS between kidney cancer patients and normal controls. For the normal controls, the first peak was at 365 nm (due to NADH) and the secondary peak at 450 nm (due to FAD), and the ratio between these closely related coenzymes was about 2. In contrast, for kidney cancer patients, there was a peak at 330 nm that could be due to some form of tryptophan or closely related amino acid. Normally, this amino acid is recycled into the plasma reservoir, since it is an essential bioprotein. However, in kidney cancer patients, the organ could have lost the capacity for this particular recycling process. It is again important to point out that this specific spectral biomarker was present in only 60% of

kidney cancers tested by us. This may be because the site of the cancer was not the same for all the patients. The FES of urine of normal controls and kidney cancer patients showed peaks at 475 nm (due to NADH) and 525 nm (due to FAD). Both these biomarkers in kidney cancer patients had almost 50% lower intensity than that in the normal controls.

It was also astonishing to observe that the cancer biomarkers (tryptophan, NADH, and FAD) shown in Fig. 1, which are well known amino acids involved in many cellular redox activities, were misregulated in kidney cancer patients, as reported in the previous findings [13–18]. Essential statistical evaluation was also performed on the ratio parameters *R290/360 (Plasma)* and *R448/360 (Plasma)* for the normal controls and kidney cancer patients. The specificity and sensitivity was 90% for accurate classification between the two study groups. This calculation was based on ten samples of normal control and ten samples of kidney cancer patients where one sample was observed during screening protocol as false negative/ false positive leading to a value of 90% specificity and sensitivity.

5. Conclusions

The current findings of this research specify that amino acids like tryptophan, NADH, and FAD are misregulated in kidney cancer patients as compared with that in healthy individuals. The ratio of the peak intensities between basic porphyrin (at 585 nm) and acidic porphyrin (at 630 nm) was elevated more than 2 fold in kidney cancer patients relative to normal controls. In SFXS of the urine of kidney cancer patients, there was a peak at 330 nm due to some form of tryptophan or closely related amino acid, which is a clear indication of kidney malfunction, compared with the normal controls. This peak at 330 nm was not present in the normal control. The present study provides excellent discrimination between the spectral features of kidney cancer patients and of normal controls, with a specificity and sensitivity of 90%. Our method would therefore be a good non-invasive and inexpensive protocol for the early diagnosis of kidney cancer, especially in small clinics and hospitals in countries that do not have easy access to MRI and CT facilities.

Author contributions

M. Atif and S. Devanesan conceived, designed, performed the experiments and wrote the paper; M. S. AlSalhi and V. Masilamani designed the technique and analyzed the data; K. Farhat and D. Rabah contributed samples.

Conflicts of interest

The authors declare no conflict of interests.

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