Optical detection of human urinary bladder carcinoma utilising tissue autofluorescence and protoporphyrin IX-induced fluorescence following low-dose ALA instillation

Rokahr, I; Andersson-Engels, Stefan; Svanberg, Sune; d'Hallewin, MA; Baert, L; Wang, I; Svanberg, Katarina

Published in:
OPTICAL BIOPSIES, PROCEEDINGS OF

DOI:
10.1117/12.228894

1995

Optical detection of human urinary bladder carcinoma utilising tissue autofluorescence and protoporphyrin IX-induced fluorescence following low-dose ALA instillation

Ingrid Rokahr[1], *Stefan Andersson-Engels*[1], *Sune Svanberg*[1]
Mari-Ange D'Hallewin[2], *Luc Baert*[2]
Ingrid Wang[3], *Katarina Svanberg*[3]

[1]Atomic Physics Division, Lund Institute of Technology, P.O. Box 118, S-221 00 Lund, Sweden
[2]Department of Urology, St. Pieter University Hospital, Leuven University, B-3000 Leuven, Belgium
[3]Department of Oncology, Lund University Hospital, S-221 85 Lund, Sweden

Abstract

Laser-induced fluorescence spectra were recorded in patients undergoing urinary bladder cystoscopy. The measurements were performed *in vivo* and the spectra were collected from normal and diseased tissue. The patients were divided into two groups. An instillation of a 1% δ-amino-levulinic acid (ALA) solution was performed 2-4 hours *prior* to the investigation of one group of patients. A second group of patients was investigated without any tumour marking substance. The fluorescence was detected following laser excitation at 405 and 337 nm.

Fluorescence emission related to ALA-induced protoporphyrin IX (PpIX) was detected in the ALA group for 405 nm excitation. The data were evaluated at the PpIX emission peak at 635 nm and at 490 nm, which approximately corresponds to the peak of the tissue autofluorescence. The data obtained with 337 nm excitation were evaluated at 400 and 460 nm as well as at 390 and 431 nm. The ratios of the respective wavelength pairs were formed in order to investigate the demarcation between tumour and normal tissue.

The tumour demarcation results were better and more consistent utilising the autofluorescence signal following excitation at 337 nm than the PpIX-related signal excited at 405 nm.

1. Introduction

Laser-induced fluorescence (LIF) has been used as an investigational tool for tissue characterisation in several clinical specialities [1 - 4]. In particular, the localisation and identification of malignant tumours have attracted increasing interest. LIF is often used in combination with photosensitising agents. With sensitive equipment, low-dose drug administration can be used for tumour detection, but also the fluorescence from endogenous fluorophores (the autofluorescence) gives information about the tissue and its pathological condition [2, 4].

A new way of tissue sensibilisation is the use of topical or local application of the haem precursor δ-amino levulinic acid (ALA), which within hours is transformed to Protoporphyrin IX (PpIX) in the haem cycle in the cells. ALA-induced protoporphyrin has been shown to accumulate selectively in tumours. The best demarcation has been achieved 2-4 hours after the administration [5].

0-8194-1991-5/95/$6.00

Although the administration procedure of ALA is uncomplicated and no side effects have been reported, it would obviously be an advantage, if a discrimination criterion between malignant and normal tissue could be obtained, based entirely on tissue autofluorescence.

In this study we investigated the tissue fluorescence demarcation for normal and malignant tumour tissue in the urinary bladder, using both the tissue autofluorescence and the ALA-induced PpIX-related signal. A comparison of both methods is presented. The work employing instillation of an ALA solution into the bladder [6] extends our previous study using Photofrin [4].

2. Method

In vivo fluorescence spectra were recorded from the urinary bladder wall in patients undergoing cystoscopy. A N_2 laser or a dye laser, pumped by a N_2 laser, was used as an excitation source. The laser pulses had a duration of 3 ns. The excitation wavelength was 337 nm for the N_2 laser and 405 nm for the dye laser. The excitation light was guided to the tissue through a 600 μm optical fused silica fibre with the distal end in direct contact with the tissue probed. The fluorescence light was collected and guided back through the same fibre. The fluorescence light was coupled out from the excitation path via a dichroic mirror and focused onto the entrance slit of a spectrometer equipped with an image intensified CCD camera. In front of the entrance slit of the spectrometer cut-off filters at 375 nm or 435 nm, respectively, were used to suppress scattered light for the two excitation wavelengths used. The spectra obtained were stored and processed by a personal computer. The fluorescence spectra were recorded in gating mode, triggered by the laser pulses. The CCD system we used was either a Peltier-cooled 2D CCD array or a linear diode array.

The spectra were obtained by adding 100 background corrected samples with an accumulation time of 0.05 s per sample. Fluorescence spectra were collected between 380 nm and 685 nm with a 10 nm full width at half maximum (FWHM) resolution.

3. Patient Groups and Measurement

3.1 Patients

Twenty-two patients undergoing investigation with a cystoscope were included in the study. Table 1 shows a patient list with the pathological diagnosis and the examination wavelengths. Fluorescence spectra of malignant urinary bladder tumour were recorded in 13 of these patients; 12 patients with invasive transitional cell carcinoma (TCC) and one with a carcinoma *in situ* (CIS). In addition, one patient with suspicious lesions was diagnosed with a moderate dysplasia (Pat. 8); another patient (Pat. 19) had a colon adenocarcinoma with invasion through the bladder wall. The patients were divided into two groups: ALA patients (Group I) and non-ALA patients (Group II). ALA was instilled into the bladder in 10 patients, while the other patients were investigated only detecting the tissue autofluorescence. The measurements were performed first at 405 nm excitation followed by corresponding recordings using 337 nm excitation.

3.2 ALA instillation

The ALA solution for instillation was prepared in the following way:

ALA was dissolved in saline (500 mg of ALA in 50 ml saline (1%)) and buffered to pH 5.0-5.5, in order to ensure stability of the ALA and to obtain a non-irritating solution. The solution was instilled into the

		405 nm excitation		337 nm excitation			
		ALA patients		ALA patients		non-ALA patients	
	Diagnosis	normal	lesion	normal	lesion	normal	lesion
Patient 1	CIS	X	X	X	X		
Patient 2	TCC	X	X	X	X		
Patient 3	TCC					X	X
Patient 4	TCC					X	X
Patient 5	TCC	X	X	X	X		
Patient 6	Normal					X	
Patient 7	TCC	X	X	X	X		
Patient 8	Moderate Dysplasia	X		X	X		
Patient 9	TCC					X	
Patient 10	TCC	X	X	X	X		
Patient 11	TCC	X	X	X	X		
Patient 12	Normal					X	
Patient 13	Normal					X	
Patient 14	Normal	X					
Patient 15	TCC	X	X	X	X		
Patient 16	TCC					X	X
Patient 17	Normal					X	
Patient 18	Normal					X	
Patient 19	Colon Cancer	X	X	X	X		
Patient 20	TCC					X	X
Patient 21	TCC					X	X
Patient 22	Cystitis						X

Table 1: List over the patients included in the study. An "X" marks the type of spectra that has been recorded on the patients.

bladder 2-4 hours before the investigation utilising a temporarily inserted catheter. After the instillation the catheter was withdrawn in the ALA-instilled patients among the first 12 patients (Pat. 1,2,5,7,8,10, and 11). The ALA-instilled patients among the remaining 10 patients (Pat. 14,15, and 19) had the catheter kept in place during the whole period in order to avoid uncertainties about the time of ALA exposure.

The patients were instructed to turn around every 10 minutes in the bed to ensure that the entire bladder wall was exposed to ALA. The bladder was emptied before the investigation started.

Fluorescence data were obtained from macroscopically visible TCC lesions and apparently normal bladder mucosa. As it is difficult to differentiate between normal mucosa, cystitis, dysplasia or carcinoma *in situ* (CIS) utilising endoscopic light, the suspicious bladder areas were investigated. Biopsies (up to 12 per patient) were taken from macroscopically normal spots, TCC lesions and suspicious areas.

4. Evaluation

Before evaluation of the data, the spectra were corrected for the spectral sensitivity of the entire detection system. The emission from a calibrated black-body radiator was recorded and all fluorescence spectra obtained were corrected with the experimental correction curve. In the analysis of the fluorescence signals, the intensities were evaluated at several wavelengths. The intensities were derived from the spectra using a computer program developed for this purpose [7]. Intensity ratios were formed from the intensity values measured in 3 nm broad wavelength intervals.

Mean values and standard deviations for several intensity ratios were calculated for both normal and tumour spectra (in case that the patient had a tumour) for each patient and for all normal and all tumour spectra within each group. The results were then compared for normal spectra with the ones for tumour spectra. A demarcation function D was calculated as $D=(m_n-m_t)/(\sigma_n^2+\sigma_t^2)^{1/2}$ [8], where m_n and m_t are the mean values of the ratios for tumour and normal tissue, respectively, and σ_n and σ_t are the corresponding standard deviations. The demarcation function was regarded to be a quantitative measure for the demarcation between malignant tumour and normal tissue. The demarcation is lower in cases of larger variations in the spectra and thus, larger standard deviations.

4.1 Excitation at 405 nm

With laser light at 405 nm the ALA-induced protoporphyrin was efficiently excited. The fluorescence spectra for the patients with ALA instillation were evaluated by forming the ratio I(635 nm)/I(490 nm). I(635 nm) corresponds to the peak of the PpIX signal, with the superimposed autofluorescence contribution at 635 nm subtracted. I(490 nm) corresponds approximately to the maximum tissue autofluorescence for 405 nm excitation.

The data from the tumour spectra for Pat. 19, who had an invading colon adenocarcinoma, were not included into the average over the tumour spectra of all patients.

4.2 Excitation at 337 nm

The ALA-induced protoporphyrin exhibits fluorescence in the red wavelength region also for 337 nm excitation. Anyhow, evaluating the blue-green autofluorescence signal, we regarded the ALA patients and the non-ALA patients as two separated groups in order to investigate, whether the autofluorescence signal was influenced by the administration of ALA or not. The average of all the data for the patients was formed.

A change in the maximum of the autofluorescence signal from about 400 nm for normal tissue to about 460 nm for tumour tissue was seen (provided that the spectra were not too much influenced by the reabsorption by blood). We evaluated the ratios I(460 nm)/I(400 nm) and I(431 nm)/I(390 nm). The latter ratio was included, because these wavelengths are equally influenced by light absorption by blood [9], making the ratio mainly independent on the presence of blood in the tissue.

5. Results

Examples of spectra recorded from a patient (Pat. 2) with TCC lesions are presented in Figs. 1 and 2. The spectra were recorded in a scan through the tumours of the patient, who had received an instillation of an ALA solution 4 hours before the investigation. The recorded fluorescence spectra and the

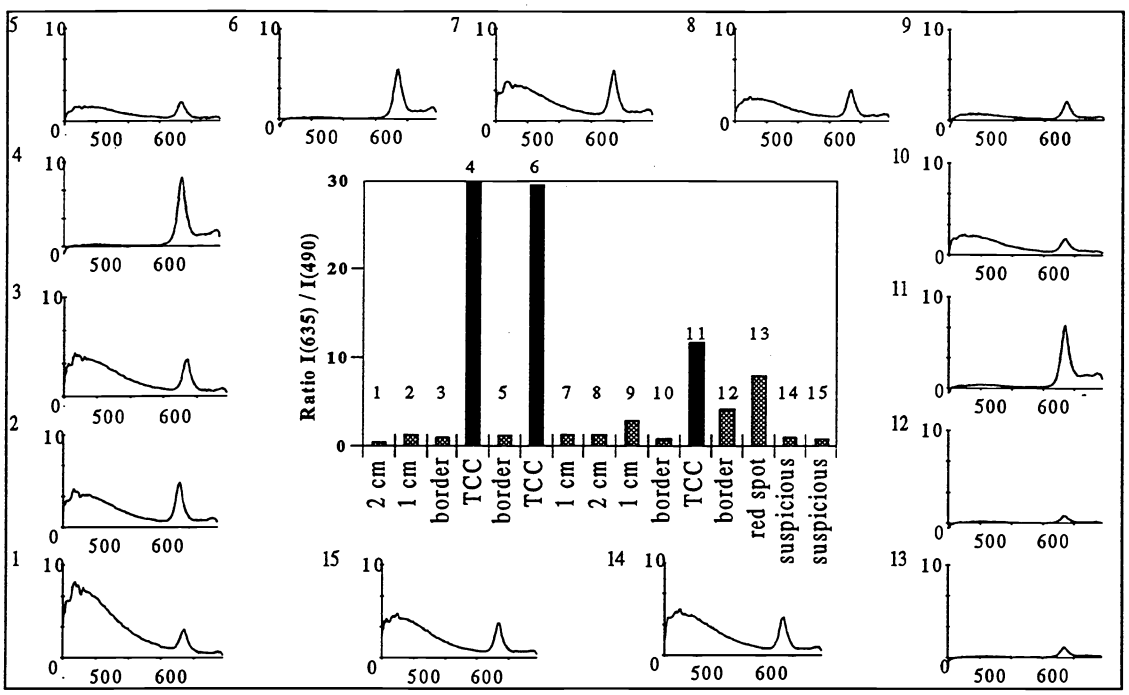

Figure 1. *Histogram of the intensity ratio I(635nm)/I(490nm) with corresponding spectra recorded from various areas in the urinary bladder wall, including transitional cell carcinoma tumours and normal areas. The patient (Pat. 2) had received 1% ALA solution as an instillation 4 hours before the investigation. The excitation wavelength was 405 nm.*

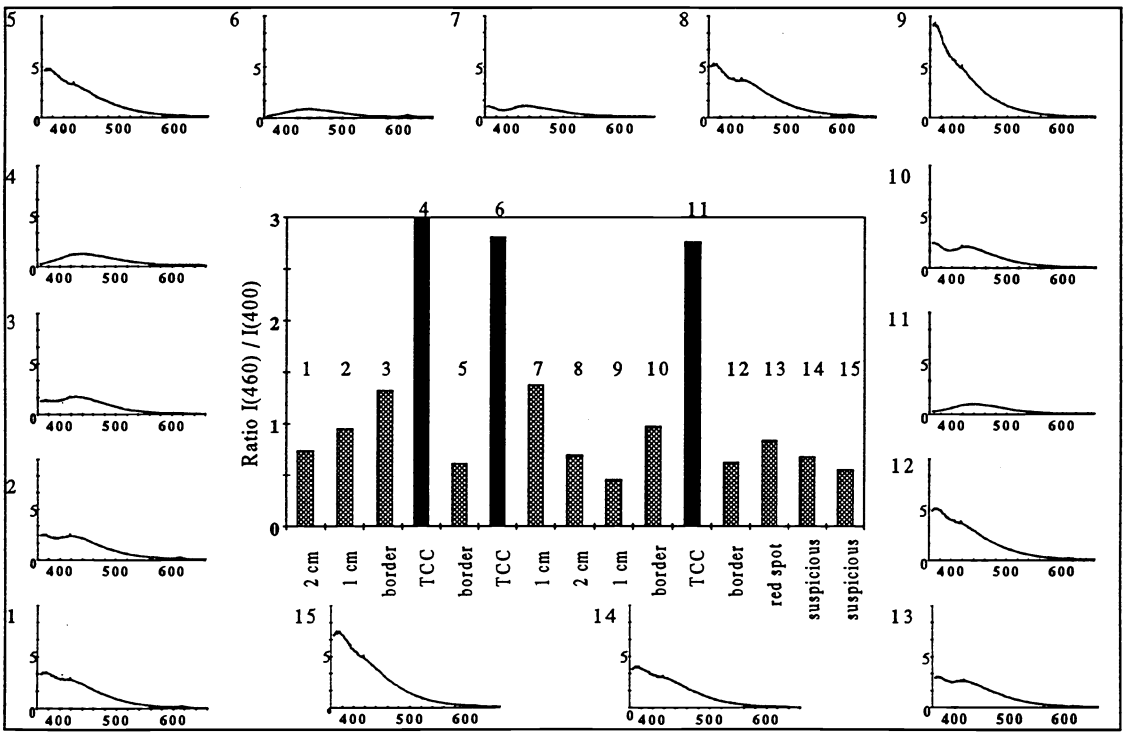

Figure 2. *Histogram of the intensity ratio I(460 nm)/I(400 nm) with corresponding spectra from the same patient as in Figure 1, but with the excitation wavelength 337 nm.*

corresponding evaluated intensity ratios are marked with the same identification numbers. Fig. 1 shows the spectra for 405 nm excitation, while the spectra for 337 nm excitation are given in Fig. 2. The ratios presented in the figures are I(635nm)/I(490nm) and I(460 nm)/I(400 nm), respectively. The spectra for the two excitation wavelengths were recorded from approximately the same location.

One patient with TCC was investigated, but not included in the study, due to of a strange shape in all fluorescence spectra obtained with 337 nm excitation. A fluorescence contribution from some molecules fluorescing at about 440 nm was overpresented and the evaluation at the chosen wavelengths could not be done due to the influence from these chromophores. One explanation for the high fluorescence at 440 nm could be that the fibre tip had got in contact with some non-biological material, such as desinfection fluid or had been contaminated with cotton fibres from the gauze used for cleaning the fibre. The data were excluded in the study.

The fluorescence spectra for the ALA-instilled patients obtained with 405 nm excitation did not result in any reliable demarcation between tumour and normal tissue. The mean values of the evaluated ratios are shown in Fig. 3a, and the corresponding demarcation functions are given in Fig. 3b. Two of the patients (Pat. 5 and Pat. 11) (in the group where the catheter was withdrawn) did not seem to have any uptake of ALA in tumours, as they did not show any PpIX-related signal. For some other patients the PpIX

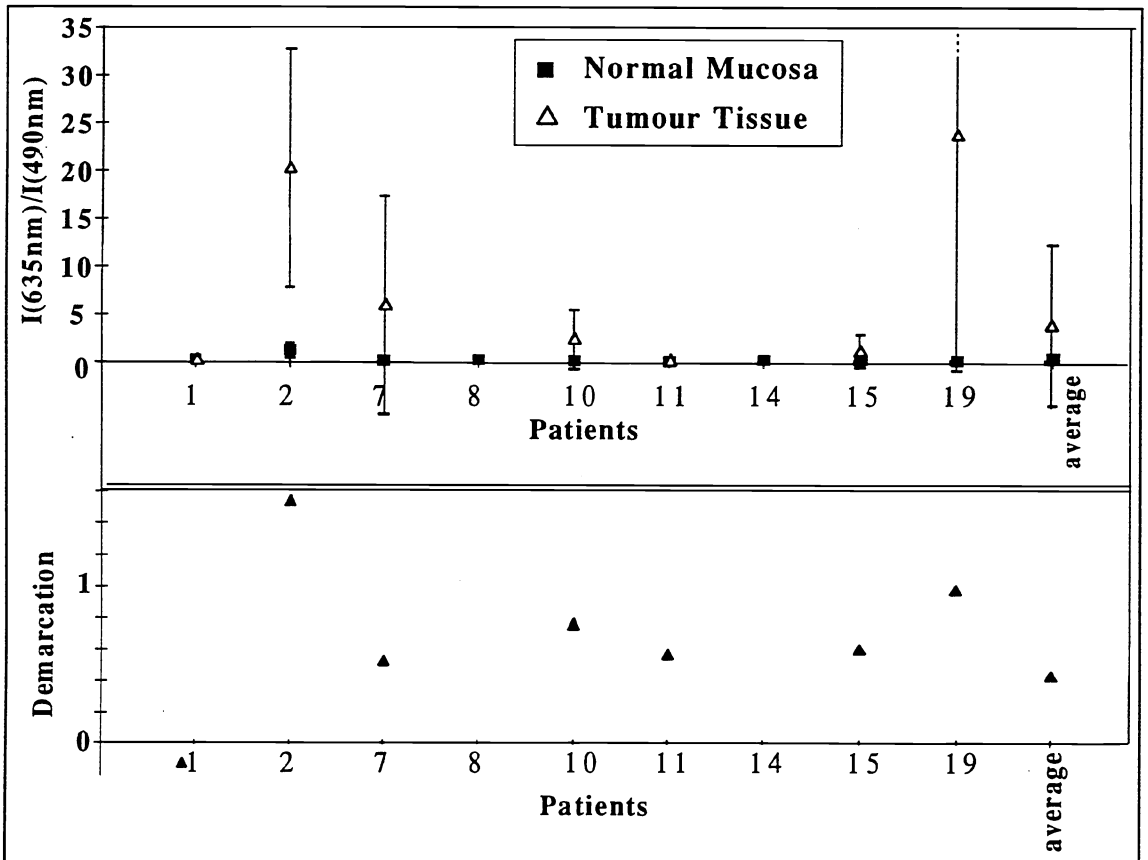

Figure 3. a) *Mean values and standard deviations for normal and tumour spectra recorded for each ALA-instilled patient, evaluated using the ratio I(635nm)/I(490nm). Excitation wavelength: 405 nm.*

b) *Tumour demarcation function D evaluated with the data obtained in Fig. 3a.*

fluorescence intensity varied over the tumour area. Especially necrotic tumour regions exhibited very low PpIX fluorescence, but also spectra from viable tumour areas failed in some cases in tumour detection. Variations result in relatively low ratio values and large standard deviations for tumour tissues. Therefore, the resulting demarcation function was lower than one for all patients with the exception of one. Spectra from the patient with CIS did not result in any demarcation between cancerous and normal tissue for 405 nm excitation.

In the evaluation of the spectra recorded with 337 nm excitation, we found that in general the two evaluated ratios were higher for tumour spectra than the ones for normal tissue spectra. The I(460 nm)/I(400 nm) ratios are presented in Fig. 4a for each patient, and the corresponding demarcation functions in Fig. 4b. The data I(431 nm)/I(390 nm) are shown in Figs. 5a and 5b, respectively. The results presented in the figures may indicate that the administration of ALA influences the autofluorescence signal of the tumour tissue.

Not all tumour spectra could be distinguished from the normal spectra. Regarding the ratio

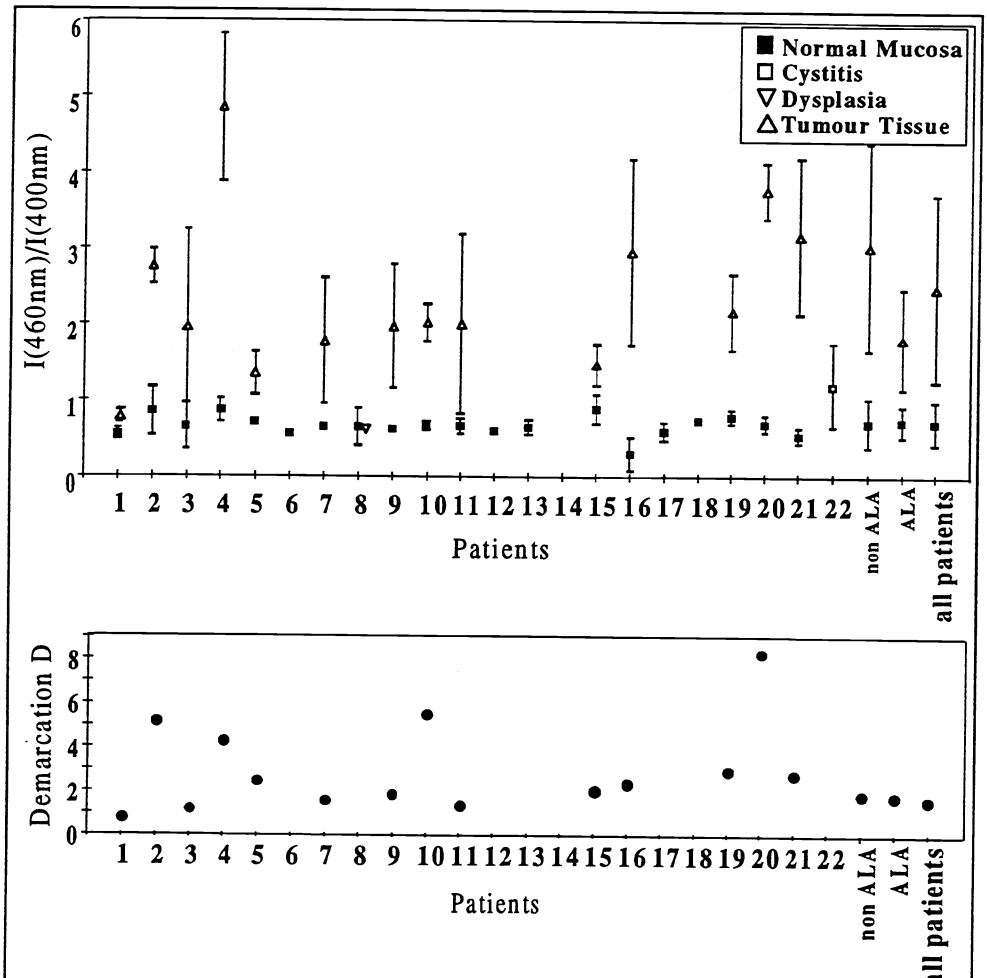

Figure 4. a) *Mean values and standard deviations for normal and tumour spectra recorded for each patient, evaluated using the ratio I(460 nm)/I(400 nm). Excitation wavelength: 337 nm.*
b) *Demarcation function evaluated with the data obtained in Fig. 4a.*

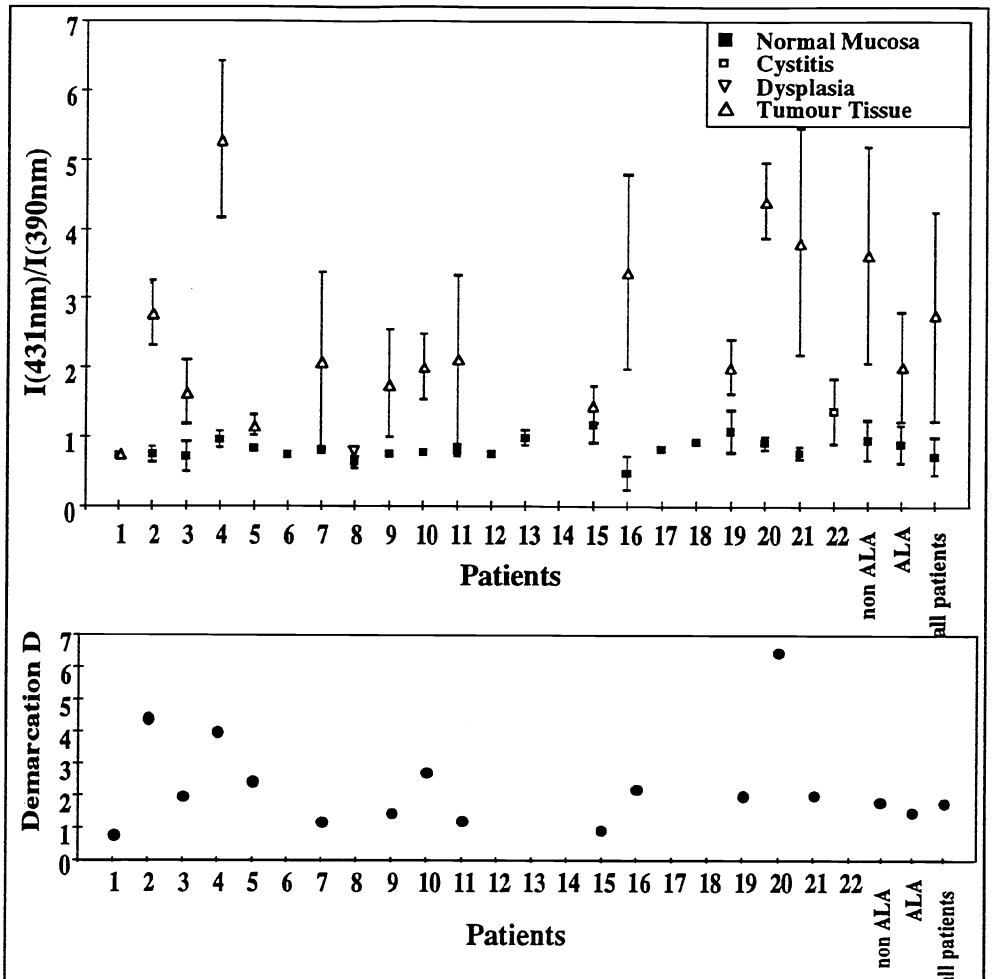

Figure 5. a) *Mean values and standard deviations for normal and tumour spectra recorded for each patient, evaluated using the ratio I(431 nm)/I(390 nm). Excitation wavelength: 337 nm.*
b) *Demarcation function evaluated with the data obtained in Fig. 5a.*

I(431 nm)/I(390 nm), we found that 8 out of 68 tumour spectra showed a lower ratio than the ratio for normal tissue from the same patient. Regarding the ratio I(460 nm)/I(400 nm) 3 out of 68 tumour spectra were lower than the respective ratios for normal tissue.

Figure 6 shows a two-dimensional plot of the ratios I(431 nm)/I(390 nm) and I(460 nm)/I(400 nm) for different tissue types. A region in the lower left quadrant shows a dominance of spectra from normal tissue, while the tumour spectra show higher ratios. Some spectra from cancerous tissue fall among the spectra for normal tissue.

The spectra from the patient with cystitis (Pat. 22) showed low ratios but could not clearly be distinguished from some tumour ratios. Fluorescence spectra recorded from spots that were pathologically classified as moderate dysplasia (Pat. 8) were sited between the normal spectra.

The examination of the patient with CIS (Pat. 1) was performed with measurements at 405 nm excitation and biopsies at the three points measured. Spectra using 337 nm excitation were then recorded at about the

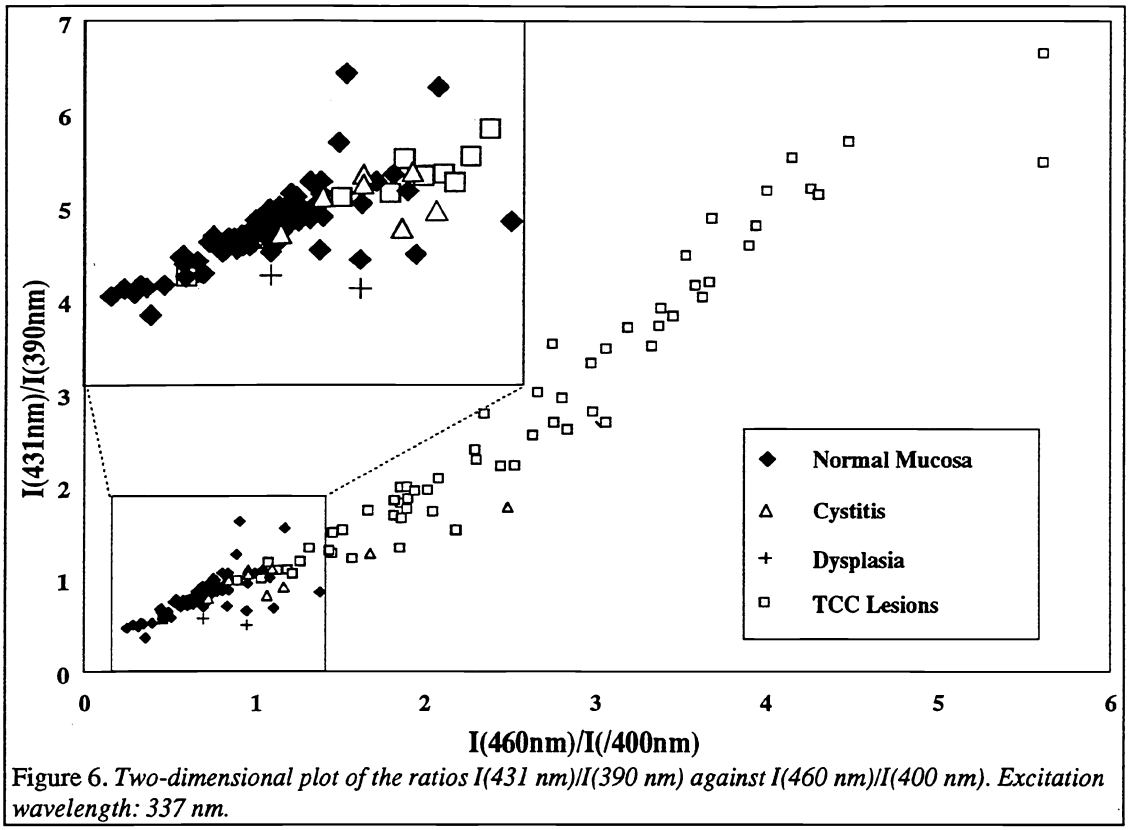

Figure 6. *Two-dimensional plot of the ratios I(431 nm)/I(390 nm) against I(460 nm)/I(400 nm). Excitation wavelength: 337 nm.*

same measurement points. Spectra were also taken very close to the biopsy points. The CIS spectra did not show higher porphyrin fluorescence following 405 nm excitation, than the normal spectra. By comparison, for 337 nm excitation the ratio I(460 nm)/I(400 nm) of CIS spectra was slightly higher than the ratio of normal spectra. No demarcation for the CIS spectra was obtained with I(431 nm)/I(390 nm).

6. Discussion and Conclusions

The autofluorescence signal has been shown to result in better tumour demarcation than the use of the ALA-induced PpIX signal for the detection of urinary bladder carcinoma.

Regarding the autofluorescence measurements with excitation at 337 nm, we found a demarcation between tumour and normal tissue for both fluorescence ratios evaluated (I(431 nm)/I(390 nm) and I(460 nm)/I(400 nm)).

Tumour detection by investigation of the ALA-induced PpIX fluorescence worked very well for some patients, but did not work out for all patients (spoiling the over all results for the protoporphyrin-based identification concept). In particular, two patients showed almost no PpIX signal from normal nor from tumour regions.

One of these patients (Pat. 5) did not seem to have retained any ALA. This patient belonged to the group, where the catheter had been withdrawn immediately after the instillation. We suspect that he might have got rid of the ALA soon after the instillation. To meet this problem on the uncertainty of the ALA

exposure time, we changed the procedure in the middle of the study. The patients had to keep the catheter until the investigation.

The other patient (Pat. 11) did not show any remarkable demarcation between tumour and normal tissue, but all regions showed low PpIX signal. This phenomenon might be explained by the location of the tumour. It was sited at the bladder neck and might not have been exposed to ALA for the entire instillation time.

Also in some other patients we found tumour areas with low or no PpIX signal. The areas included necrotic tumour tissue, but also to some extent tumours that were identified in the normal cystoscopic investigation.

Even though the investigation of tumours by the use of ALA-induced fluorescence worked well in some patients, the results from autofluorescence data are more encouraging.

The shift of the autofluorescence maximum from 400 nm for normal tissue to 460 nm for tumour tissue is distinctive and forming the ratio of the two fluorescence intensities enhances the demarcation [4]. Also the tumour detection using the ratio I(431 nm)/I(390 nm) worked out, but the blood independent criterion yielded no additional benefits in this study - although the blood interference is clearly visible in some spectra. (See for instance the dip at 400 nm in some spectra in Fig. 2). All tumours in this study that were correctly identified with the ratio I(431 nm)/I(390 nm) were also detected with I(460 nm)/I(400 nm).

The fact that ALA-induced PpIX may also influence the autofluorescence signal is interesting. It might be explained by shifts in the relative concentrations of fluorophores. As the demarcation seems to be better without ALA, the average results will even become better in a study only including autofluorescence.

It would be desirable to be able to distinguish suspicious areas like cystitis or moderate dysplasia from tumour tissue by a fluorescence examination. While TCC can easily be seen and diagnosed with a normal cystoscopic examination, biopsies from other suspicious lesions are often taken randomly. Here a fluorescence-based detection method could be a help guiding the biopsy procedure.

The single CIS in this study could not be detected, neither with the use of ALA nor by investigation of the autofluorescence. Further studies with a special emphasis on CIS are required to test the potential of LIF to identify these early tumours.

Even though ALA did not yield any reliable tumour identification in this study, other fluorescent substances have shown to be powerful as fluorescent markers for other tumours. Some of these substances might be useful in the detection of urinary bladder carcinoma.

In conclusion, the results from this study suggest that tissue autofluoresence might be a powerful tool to identify urinary bladder carcinoma, while PpIX fluorescence induced by ALA-instillation probably yields too unreliable results with the low dose used in this study. We succeeded best in tumour detection by investigating the autofluorescence with the ratio I(460 nm)/I(400 nm) at 337 nm excitation. More than 95% of all tumour spectra had a higher ratio than the corresponding spectra for normal tissue.

7. Acknowledgements

This work has financially been supported by the Swedish Cancer Society and by Xillix Technologies Corp., Richmond, B.C. Canada. Support from Dick Killander is highly appreciated.

8. References

1. A. E. Profio and O. J. Balchum, "Fluorescence diagnosis of cancer", in *Methods in Porphyrin Photosensitization,* D. Kessel, ed., Plenum (New York, 1985), 43.
2. S. Lam, B. Palcic, D. McLean, J. Hung, M. Korbelik, and A. E. Profio, "Detection of early lung cancer using low dose Photofrin II", *Chest* **97,** 333-337 (1990).
3. S. Andersson-Engels, Å. Elner, J. Johansson, S.-E. Karlsson, L.G. Salford, L.-G. Strömblad, K. Svanberg and S. Svanberg, "Clinical recording of laser-induced fluorescence spectra for evaluation of tumour demarcation feasibility in selected clinical specialities", *Lasers Med. Sci.* **6**, 415-424 (1991).
4. L. Baert, R. Berg, B. van Damme, M. A. D'Hallewin, J. Johansson, K. Svanberg and S. Svanberg, "Clinical fluorescence diagnosis of human bladder carcinoma following low-dose Photofrin-injection", *Urology* **41**, 322-330 (1993).
5. W. E. Grant, C. Hopper, A. J. MacRobert, P. M. Speight, and S. G. Bown, "Photodynamic therapy of oral cancer: photosensitisation with systemic aminolevulinic acid", *Lancet* **142,** 147-148 (1993).
6. M. Kriegmayr, R. Baumgartner, R. Knüchel, H. Stepp, F. Hofstädter, A. Hofstetter, "Detction of early bladder cancer by 5-aminolevulinic acid induced porphyrin fluorescence" accepted for publication in *J. Urology.*
7. R. Berg, "Spectrum Evaluator - a program for evaluating fluorescence spectra", Lund Institute of Technology, Lund Reports on Atomic Physics **LRAP-177** (1994).
8. P.S. Andersson, A. Gustavsson, U. Stenram, K. Svanberg, S. Svanberg, "Diagnosis of arterial artherosclerosis using laser-induced fluorescence", *Lasers Med. Sci.* **2**, 261-266 (1987).
9. S. Andersson-Engels, J. Johansson, U. Stenram, K. Svanberg, S. Svanberg, "Malignant tumor and atherosclerotic plaque diagnosis using laser-induced fluorescence", *IEEE Journal of Quantum Electronics* **26**, 2207-2217 (1990).