Protoporphyrin IX Occurs Naturally in Colorectal Cancers and Their Metastases

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ABSTRACT

Colorectal cancers exhibit a red fluorescence. The nature of the responsible fluorophore and its eventual diagnostic potential were investigated. Thirty-three consecutive colorectal resection specimen, 32 of which with histologically confirmed cancer, and a total of 1053 palpable mesenteric nodes were fluorometrically characterized ex vivo. Furthermore, frozen material from 28 patients was analyzed, selected for the availability of primary tumor material and metastatic tissue, e.g., lymphatic and liver metastases from the same patient. Biochemical characterization was carried out through chemical extraction and reversed phase high-performance liquid chromatography. The fluorescence spectra of tissues, tissue extracts, and standard solutions of porphyrins were determined using a pulsed solid-state laser system for excitation and an imaging polychromator, together with an intensified CCD camera for time-delayed observation. Protoporphyrin IX (PpIX) was identified as the predominant fluorophore in primary tumors and their metastases. The fluorescence occurred in the absence of necrosis and in sterile locations. In untreated cases (n = 24), PpIX fluorescence discriminates metastatically involved lymph nodes from all other palpable nodes with a sensitivity of 62% at a specificity of 78% (P < 0.0001). After neoadjuvant treatment of rectal cancer, the PpIX fluorescence level of the primary tumors was reduced and a discrimination of lymph nodes based on PpIX-fluorescence was impossible. We conclude that colorectal cancer metastases accumulate diagnostic levels of endogenous PpIX as a result of a tumor-specific metabolic alteration.

INTRODUCTION

The detection and visualization of macroscopically indiscernible malignancy by fluorescence detection of fluorophores, preferentially accumulated in or synthesized by tumor tissue, is tempting because of the potential ease of use and the lack of biological hazard to patient and investigator (1, 2). Autofluorescence features of cancer were investigated long before. The original observation of a red autofluorescence emitted under blue light from experimental tumors dates back to the French scientist Policard (3) in 1924. Others gave rather little attention to the phenomenon (4) because of its seemingly restriction to larger, ulcerated tumors easily visible by eye. Ghadially and Neish (5) reported in 1960 a series of experiments relating the presence of porphyrins to the colonization of the ulcerated tumors by bacteria, a finding that was supported by Harris and Werkhaven (6) in 1987 for cancers of the oral cavity in which a strong porphyrin fluorescence could be “wiped off” from the surface.

More recently, the fluorescence from PpIX formed endogenously after exogenous application of 5-ALA was demonstrated to be diagnostic in many superficial tumor diseases (7–10). The success of 5-ALA-based fluorescence detection systems in superficial applications led us to develop a similar approach for macroscopically indiscernible residual disease like lymphatic micrometastases. For this purpose, the recognition of a specific fluorescence signal in considerable tissue depth (several millimeters) was necessary. Exploiting the longer fluorescence lifetimes of porphyrins, a system of unprecedented sensitivity for porphyrins, at least so far in the clinical context, was developed. However, since the regular autofluorescence from normal and diseased human tissues, which contain considerable levels of porphyrins, may interfere with this stimulated autofluorescence from PpIX, the native autofluorescence was to be investigated in the first place.

Thus, initially as a prerequisite to detect micrometastases by PpIX fluorescence after 5-ALA administration, we studied time-dependent laser-induced autofluorescence of colorectal tumors and their metastases. We then discovered and reported the detectability and the fluorimetric characteristics of an endogenous porphyrin-like fluorophore in colorectal lymph node metastases (11). Our present investigation aims at the identification of the fluorophore and at the description of its potential diagnostic usefulness.

PATIENTS AND METHODS

Patients and Tissue Samples

The lymph node study comprises 33 patients who underwent primary surgical care for suspected or proven colorectal cancer at our institution, without any preselection. Five of those patients were pretreated by HRCT and three by RCT under an independent clinical study protocol (12). Information on patients, tumor location, tumor stage, and tumor grading, as well as pretreatment by RCT or HRCT, are reported in Table 1. Fluorescence spectra of the primary tumor and surrounding normal mucosa were investigated immediately after excision of the surgical specimen. Subsequently, all of the palpable nodules in the adjacent mesentery of the tumor were prepared and kept moist at room temperature. All of the specimens of primary cancers were carefully washed prior to fluorescence measurements. Laser-induced fluorescence of nodules was recorded before pathological evaluation. Table 1 includes the total number of nodules investigated by fluorescence spectroscopy. Care was taken that each nodule could be kept track of during the entire procedure of characterization. The time between cessation of blood supply and the last fluorescence measurements did not exceed 4 h. Sometimes, not all of the nodules resected from a particular patient could be investigated within that time. Furthermore, a nodule investigated by fluorescence spectroscopy sometimes turned out during pathological examination to consist of several distinct nodes. As additional information, given in parentheses, Table 1 specifies for each patient the number of noninvolved and involved lymph nodes, and connective tissue nodes, examined histologically.

Apart from the tissue specimen of 33 patients (primary tumors, lymph nodes and connective tissue nodes) investigated immediately after excision, a second subset of tissue samples not included in Table 1 was selected from a tissue specimen collection established since 1992 at the Department of Surgery and Surgical Oncology, Charité, Humboldt University, Berlin. All of the samples in this tissue collection were stored in the dark at ~80°C immediately after surgery. On the basis of the availability of primary tumor, normal tissue, lymph node metastases, or liver metastases from the same patient, tissue samples from 28 patients (20 with colonic and 8 with rectal cancer) were identified to determine the content of different porphyrins semiquantitatively by fluorescence spectroscopy and biochemical analysis.

Received 3/17/00; accepted 11/20/00.

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Support of Grant Schl #391-1 from the Deutsche Forschungsgemeinschaft.

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The abbreviations used are: PpIX, protoporphyrin; 5-ALA, 5-aminolevulinic acid; RCT, radiochemotherapy; HRCT, hyperthermic RCT; HPLC, high-performance liquid chromatography; OPO, optical parametric oscillator.
The highest purity commercially available.

Porphyric III dihydrochloride from Porphyrin Products (Logan, UT) were of nol and 2% sodium hydroxide in water. PpIX was purchased from Sigma.

Porphyrin Extraction and HPLC Analysis

for each patient and in Table 2 for all patients, takes the results of the

The total number of noninvolved and involved lymph nodes, given in Table 1 nodes together with the number of involved lymph nodes detected in this way.

were read after H&E staining. Table 1 gives the number of reevaluated lymph

10 sections). One equatorial section was stained by pancytokeratin antibody

10 cycles of microwave hyperthermia added.

n

solvable from Packard Instruments Co. (Meriden, CT) con-

Chemicals.

PROTOPORPHYRIN IX IN COLORECTAL Cancers

Pathology

When communicated, tumor stages were determined according to the re-

vised edition of the UICC’s TNM classification of malignant tumors (13). Routine pathological evaluation of the lymph nodes was based on a single equatorial section of each palpable node, stained by H&E. Palpable nodes containing neither lymph node structures nor metastatic tissue were classified as connective tissue nodes. Their palpable density was generally explained by

Numbers on involved lymph nodes include the results of reevaluation. Numbers given in parentheses represent the number of prepared nodes as determined

as connective tissue nodes. Their palpable density was generally explained by

The flow rate was 1.5 ml/min; injection volume was 20

methanol, 10% 2 mM sodium phosphate buffer (pH 7.5)] over the next 30 min.

et al.

with a Spectraflow 757 absorbance detector (Kratos Analytical Instru-

m particle size). Porphyrines

14): 100% solvent A [60% methanol, 40% 10 mM sodium phosphate buffer

were kept for 12 h at 50°C. The resulting dilutions were filtered (45

(115) 272 (277)

connective tissue nodes. Numbers on involved lymph nodes include the results of reevaluation. Numbers given in parentheses represent the number of prepared nodes as determined by pathology. Because of logistic reasons, not all of the nodes could be investigated by fluorescence spectroscopy.

18-year-old patient, submitted to radiation therapy after surgery to enable continence preserving resection.

Results of reevaluation included.

were kept for 12 h at 50°C. The resulting dilutions were filtered (45

the clear, slightly colored filtrates were stored at room temperature in the dark.

HPLC Analysis. The HPLC analysis was performed using a Spectra Phys-

soly solvent delivery system (Mod. SP9700, San Jose, CA, USA) and pumped with a Spectraflow 757 absorbance detector (Kratos Analytical Instru-

ments, Ramsey, NJ). The column used (Merck, Darmstadt, Germany) was a LiChrospher 100RP-H (4 mm × 250 mm; 5-μm particle size). Porphyrines were eluted according to the scheme described previously by Bellnier et al. (14): 100% solvent A [60% methanol, 40% 10 mM sodium phosphate buffer

(14): 100% solvent A [60% methanol, 40% 10 mM sodium phosphate buffer

The patient presented a symptomatic stenosis of the sigmoid and was submitted to urgent radical surgery without prior biopptic proof of malignancy.

Eight patients received neoadjuvant therapy under clinical trial conditions; RCT: radiochemotherapy, 5-Fu, 42 Gy radiation; HRCT, hyperthermic radiochemotherapy, RCT as with

m

were kept for 12 h at 50°C. The resulting dilutions were filtered (45

(115) 272 (277)

connective tissue nodes. Their palpable density was generally explained by

as connective tissue nodes. Their palpable density was generally explained by

m

five reevaluated lymph nodes detected in this way. The total number of noninvolved and involved lymph nodes, given in Table 1 for each patient and in Table 2 for all patients, takes the results of the reevaluation into account.

Porphyrin Extraction and HPLC Analysis

Chemicals. Solvable from Packard Instruments Co. (Meriden, CT) con-

contains 3% N,N-dimethyl lauryl amine oxide, 3% allyloxypropylenoxyethy- nol and 2% sodium hydroxide in water. PpIX was purchased from Sigma

Chemical Co. (St. Louis, MO), uroporphyrin III dihydrochloride and copro-

porphyrine III dihydrochloride from Porphyrin Products (Logan, UT) were of the highest purity commercially available.

Processing. Tissue (25–50 mg) were covered with 1 ml of Solvable, and were kept for 12 h at 50°C. The resulting dilutions were filtered (45 μm), and the clear, slightly colored filtrates were stored at room temperature in the dark.

Table 1 Patient characteristics and tumor staging for 33 investigated colorectal surgical specimens

All of the patients were operated on under the suspicion of colorectal cancer. In one case, the definitive histology was adenoma.

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Lymph nodes</th>
<th>Connective tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No treatment</td>
<td>RCT/HRCT</td>
</tr>
<tr>
<td></td>
<td>29  30</td>
<td>8  9</td>
</tr>
<tr>
<td></td>
<td>976 (974)</td>
<td>379 (310)</td>
</tr>
<tr>
<td></td>
<td>434 (582)</td>
<td>94 (116)</td>
</tr>
<tr>
<td></td>
<td>126 (182)</td>
<td>36 (67)</td>
</tr>
<tr>
<td></td>
<td>404 (404)</td>
<td></td>
</tr>
</tbody>
</table>

* Patient no. 13 (see Table 1) with adenoma included.

* Results of reevaluation included.

Table 2 Total numbers of palpable nodes and of histologically evaluated nodes in the lymph node study

Numbers in parentheses correspond to prepared nodes as determined by histology, numbers without parentheses to nodes investigated by fluorescence spectroscopy.

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in Solvable were neutralized with 1 N HCl to pH 7.5 prior to analysis. Standards were established using commercially available solutions of PpIX, uroporphyrin III, dihydrochlortrope, and coproporphyrin III dihydrochloride.

**Fluorescence Instrumentation and Spectroscopy**

The experimental set-up used for recording time-delayed fluorescence spectra is shown in Fig. 1. An OPO (Model A-1, GWU-LaserTechnik, Germany) pumped by the third harmonic (λ = 355 nm, \(E_{\text{pulse}} \approx 100–140 \text{ mJ}\)) of a Q-switched Nd:YAG laser (GCR 230-50; Spectra-Physics), provided pulsed laser radiation at a rate of 50 pulses per s, tunable between 410 nm and 2.2 μm. The energy and the duration of the OPO output pulses amounted to 15 mJ and 20 ns. Subsequently, the ramp laser pulse (λ = 355 nm) was removed from the OPO output by means of two dichroic beam splitters (HR 355, Laseroptik GmbH, Germany), the idler wave (720 nm–2.2 μm) from the OPO output by means of two dichroic beam splitters (HR 355, Laseroptik GmbH, Germany), the idler wave (720 nm–2.2 μm) by a corresponding short wave pass filter. The energy of the OPO output pulses was reduced to about 300 μJ using neutral density filters. The laser beam was coupled into a 600-μm hard-clad silica fiber. The fiber tip was placed at close proximity (<2 mm distance) to the surface of the intact nodule. The laser-induced fluorescence of the tissue was collected by the same fiber and guided to the entrance slit of an optical multichannel analyzer (see Fig. 1), consisting of an imaging polychromator (Spectra Pro-150; Acton Research Corp.) and a cooled, intensified CCD camera (Princeton Instruments Inc.). A dichroic beam splitter (550 DRLPO2; Omega Optical Inc.) served to decouple excitation and backscattered excitation light further. The intensifier of the diode array detector was gated by an electrical pulse (~180 V) of about 20 ns duration delivered by a high-voltage pulse generator (Model 6040, Berkeley Nucleonics Corp.) synchronized and delayed to the laser pulse. For this purpose, the high-voltage pulse generator was triggered by an electrical pulse provided by the power supply of the Nd:YAG laser.

The fluorescence spectra of tissue specimen were recorded at zero delay (\(t_d = 0 \text{ ns}\)) and at a delay of \(t_d = 20 \text{ ns}\). Subsequently, the spectra were corrected for the transmittance of the polychromator and for the spectral sensitivity of the photocathode of the intensified CCD camera but not for the transmittance of the long wave pass filter (λ_{550} = 550 nm) that was used to block off backscattered laser light. In addition, electronic background of the detector was subtracted from the raw data. Subsequently, the prompt \(I(λ, 0 \text{ ns})\) and delayed \(I(λ, 20 \text{ ns})\) spectra were normalized to the maximum intensity \(I(633 \text{ nm, 0 ns})\) of the corresponding prompt fluorescence spectrum. Because geometrical factors were unchanged when recording prompt and delayed fluorescence spectra, and fluorescence spectra were corrected for the number of laser pulses applied and for their pulse energy, normalized prompt (\(t_d = 0 \text{ ns}\)) and delayed (\(t_d = 20 \text{ ns}\)) fluorescence spectra \(I_\text{n}(λ, t_d) = I(λ, t_d)/I(633 \text{ nm, 0 ns})\) can be compared quantitatively. By normalization the strong dependence of the recorded fluorescence intensity on geometrical factors (e.g., distance between fiber tip and tissue surface) is mostly eliminated.

**Data Analysis**

The autofluorescence spectra exhibit well-defined bands (λ = 633 nm, 700 nm) denoted as specific autofluorescence bands and a broad unstructured background termed nonspecific autofluorescence. For quantification, we take the normalized fluorescence spectra \(I_\text{n}(λ, t_d)\) to be the sum of the normalized specific tissue autofluorescence \(I_\text{sp}(λ, t_d)\) and the nonspecific autofluorescence background \(I_\text{nsp}(λ, t_d)\):

\[
I_\text{n}(λ, t_d) = I_\text{sp}(λ, t_d) + I_\text{nsp}(λ, t_d)
\]

(A)

To correct for the nonspecific autofluorescence background in delayed \(I(λ, 20 \text{ ns})\) fluorescence spectra, we assume that (a) the prompt normalized fluorescence spectrum \(I_\text{n}(λ, 0 \text{ ns})\) is dominated by the nonspecific fluorescence background; and (b) the decay rate of the nonspecific fluorescence background during the delay time \(t_d\) is independent of wavelength. It follows that the nonspecific fluorescence background in delayed \(I(λ, 20 \text{ ns})\) fluorescence spectra is given by

\[
I_\text{nsp}(λ, 20 \text{ ns}) = \frac{I(595 \text{ nm, 20 ns})}{I(595 \text{ nm, 0 ns})}I(λ, 0 \text{ ns})
\]

(B)

The first factor on the right hand side of Eq. (B) takes the decay of the nonspecific fluorescence background into account. The wavelength λ = 595 nm was chosen because it lies outside the fluorescence bands of the specific tissue autofluorescence and the absorption band (λ = 570 nm) of hemoglobin (15).

It follows from Eqs. (A) and (B) for the intensity of the specific fluorescence bands in delayed spectra, i.e., for the normalized specific tissue autofluorescence:

\[
I_\text{sp}(λ, 20 \text{ ns}) = \frac{I(λ, 20 \text{ ns})}{I(595 \text{ nm, 20 ns})} - \frac{I(λ, 0 \text{ ns})}{I(595 \text{ nm, 0 ns})}
\]

(C)

The assumptions made above are met if the specific tissue autofluorescence bands in the prompt spectrum are small compared with the nonspecific fluorescence background, generally observed for normal surrounding mucosa and lymph nodes, but may be questionable in the case of tumors with strong specific tissue autofluorescence bands appearing in the prompt spectrum. However, in that case, nonspecific tissue autofluorescence background is virtually absent in delayed spectra and Eq. (C) amounts to \(I_\text{sp}(λ, 20 \text{ ns}) \approx I(λ, 20 \text{ ns})\).

Besides normalized specific tissue autofluorescence \(I_\text{sp}(λ, 20 \text{ ns})\) taken at the maximum of the main specific fluorescence band, the ratio

\[
R = \frac{I(633 \text{ nm, 20 ns})}{I(595 \text{ nm, 20 ns})}
\]

(D)

may be used for quantification of the spectra recorded (11). Both of the methods are strongly correlated leading essentially to the same conclusions. This is not too surprising, because the ratio \(I(633 \text{ nm, 20 ns})/I(595 \text{ nm, 20 ns})\) appears within the parentheses of Eq. (C). Therefore, when peak values rather than fluorescence spectra are to be compared, we use the ratio \(R\) of delayed fluorescence intensities rather than the normalized specific autofluorescence \(I_\text{sp}(λ, 20 \text{ ns})\) for quantification.
RESULTS

Fluorescence Spectra of Primary Tumors. The normalized autofluorescence spectra of a particular primary colonic tumor recorded at zero delay and a delay of 20 ns after pulsed laser excitation at $\lambda_{ex} = 505$ nm are illustrated in Fig. 2, a and b, respectively. To facilitate comparison of spectral shapes, the delayed spectrum of the tumor was multiplied by a factor of 4.

A well-defined fluorescence band with a maximum at $\lambda \approx 633$ nm and a second minor band at $\lambda \approx 700$ nm appear in the prompt and delayed fluorescence spectra of the tumor. In contrast, the surrounding mucosa exhibits a broad fluorescence spectrum essentially without any spectral signatures, apart from a minimum at $\lambda \approx 570$ nm, most likely caused by the absorption of hemoglobin (11, 15). This minimum can also be discerned in the delayed spectrum of the mucosa and in the prompt spectrum of the primary tumor. The delayed fluorescence spectrum of the primary tumor exhibits the specific autofluorescence essentially free of background. It follows that the fluorescence decay time of the specific autofluorescence is long, compared with the average decay time of the background fluorescence (nonspecific autofluorescence). Although in the case under study, neither the prompt nor the delayed fluorescence spectra of the surrounding normal mucosa show any specific autofluorescence bands, we would like to point out that in some cases these bands do appear in the fluorescence spectra of normal mucosa surrounding a primary tumor. Indeed, in 2 of 33 cases, the specific autofluorescence bands were even stronger in the spectra of the surrounding normal mucosa compared with the primary tumor itself.

The Nature of the Specific Autofluorescence in Colonic Primary Tumors. The delayed fluorescence (emission) spectrum (Fig. 2b) of the colonic primary tumor is strikingly similar to the fluorescence emission spectra of porphyrins. For comparison, Fig. 2c shows fluorescence spectra of PpIX, uroporphyrin III (Uro III), and coproporphyrin (Copro III), dissolved in methanol. Fluorescence was excited at $\lambda_{ex} = 505$ nm, the emission spectra, recorded at zero delay, were normalized to the maximum of the main fluorescence band. In particular, the fluorescence emission spectrum of PpIX closely resembles the delayed emission spectrum of the tumor shown in Fig. 2b. Our hypothesis that the specific autofluorescence observed in tumors originates from endogenous porphyrins, in particular PpIX, is further corroborated by the fluorescence excitation spectrum of a primary colonic tumor, illustrated in Fig. 3a, presented together with the excitation spectrum of one of its involved lymph nodes (Fig. 3b). To this end, delayed ($t_d = 20$ ns) fluorescence emission spectra of a primary colonic tumor were recorded at several selected excitation wavelengths ranging from $\lambda_{ex} \approx 480$ nm up to $\lambda_{ex} \approx 570$ nm. As can be seen from Fig. 3a, two excitation bands appear in the fluorescence excitation spectrum of the primary colonic tumor centered at $\lambda_{ex} = 510$ nm and $\lambda_{ex} = 550$ nm. These bands are shifted to slightly longer wavelengths compared to the maxima ($\lambda_{ex} = 505$ nm, $\lambda_{ex} = 540$ nm) of the Q-bands of PpIX in methanol (Fig. 2c). The fluorescence excitation and emission spectra recorded and the fluorescence decay times ($\tau_d \approx 14$ ns) that are observed strongly support the hypothesis that the specific autofluorescence observed in primary tumors originates from endogenous porphyrins, in particular PpIX.

Tumor Localizing Property of Specific Tissue Autofluorescence. Fig. 4 summarizes the results on the specific tissue autofluorescence of colorectal primary tumors in the lymph node study. Normalized fluorescence intensities $R$ of the primary tumor ($R_{tumor}$) and (for comparison) of the surrounding mucosa ($R_{mucosa}$) are given for 32 patients. One patient (no. 13), with no cancer but with an adenoma, is included in Fig. 6 as well as adenomatous polyps, identified in seven cancer specimens (patient numbers 4, 6, 8, 24, 26, 29, and 32).
28, and 32). In 31 of 32 cancer specimens, the autofluorescence ratio $R$ exceeded 1 ($R_{\text{cancer}} > 1$), clearly indicating the presence of the fluorophore. The one negative case (patient no. 25) had been treated effectively by HRCT leading to a downstaging from $uT_3$ to ypT$_2$. In 26 of 33 patients, normal mucosa showed normalized fluorescence intensities of $R_{\text{mucosa}} < 1$. In 30 cancer cases, contrast between tumor and surrounding mucosa was positive, i.e., $R_{\text{tumor}} > R_{\text{mucosa}}$. In one of the remaining cases (patient no. 25), contrast was negative possibly because of the low fluorescence signal of the tumor treated by HRCT. In the other case (patient no. 17), normalized fluorescence intensity was large both of the tumor ($R_{\text{tumor}} = 1$) and the surrounding mucosa ($R_{\text{mucosa}} = 15.5$) as well. No special medical or alimentary condition was identified in the latter patient to account for this atypical result. Normalized fluorescence intensities of adenomatous polyps were found to lie between the values for normal mucosa and malignant tumors in five of seven cases, above $R_{\text{mucosa}}$ and below $R_{\text{tumor}}$ in one case each. In the single case presenting a stenosis because colonic adenoma, the normalized fluorescence intensity at the surface of the adenoma did not exceed substantially the normalized fluorescence intensities at the normal mucosal surface.

We conclude this section by noting that Fig. 4 provides evidence of the tumor localizing properties of the endogenous porphyrin, probably PpIX, which causes the specific tissue autofluorescence.

**Specific Autofluorescence in Lymph Nodes.** The autofluorescence spectra of two particular lymph nodes of a patient without pretreatment, one involved and one noninvolved node, are shown in Fig. 5, a and b and Fig. 5, c and d, respectively. Apart from the absorption by hemoglobin at $\lambda = 570$ nm, no distinct spectral features appear in both prompt fluorescence spectra. However, the fluorescence spectrum of the involved lymph node taken at a delay of $t_d = 20$ ns exhibits two fluorescence bands centered at about $\lambda = 630$ nm and $\lambda = 700$ nm. In contrast, no such bands can be discerned in the delayed spectrum of the noninvolved lymph node. We attribute the specific autofluorescence of the involved lymph node to the same fluorophore present in primary tumors. This result is supported by Fig. 6, which compares the normalized specific tissue autofluorescence $I_{\text{sp}}^\text{n}(\lambda, 20 \text{ ns})$ of a primary tumor and one of its regional involved lymph nodes of one particular patient (no. 6). The normalized specific fluorescence $I_{\text{sp}}^\text{n}(\lambda, 20 \text{ ns})$ was calculated subtracting the nonspecific background from the delayed spectrum. Apart from an artifact centered at $\lambda \approx 560$ nm, the normalized specific fluorescence spectra of the involved lymph node and of the corresponding primary tumor are strikingly similar. Furthermore, the fluorescence excitation spectra observed for both fluorescence bands ($\lambda_{\text{obs}} = 635$ nm, 690 nm), illustrated in Fig. 3b for an involved lymph node further support this result. The fluorescence excitation spectra are similar to the excitation spectra of the corresponding primary tumor (see Fig. 3a) and of the excitation spectrum ($\lambda_{\text{obs}} = 635$ nm) of PpIX in methanol. It follows that specific tissue autofluorescence, most likely originating from PpIX, occurs not only in primary tumors but also in involved regional lymph nodes, although at a much reduced intensity. However, specific tissue autofluorescence may be observed in pathologically noninvolved lymph nodes as well.

**Patients without Pretreatment: Autofluorescence of Lymph Nodes versus Pathology.** We investigated (Tables 1 and 2) a total of 1053 nodes by fluorescence spectroscopy, consisting of 528 nonin-
volved lymph nodes, 126 involved lymph nodes and 399 connective tissue nodes, according to pathology. During the course of the present investigation it became apparent that pretreatment by RCT and HRCT has considerable influence on the autofluorescence spectra of lymph nodes. Therefore, we present the results on patients without pretreatment and those undergoing RCT or HRCT separately.

A total of 796 nodules from patients without pretreatment were investigated by fluorescence spectroscopy consisting of 434 noninvolved lymph nodes, 90 involved lymph nodes, and 272 connective tissue nodes. In Fig. 7, we have plotted corresponding normalized cumulative frequencies versus the ratio $R$ of fluorescence intensities in delayed fluorescence spectra of all involved, all noninvolved lymph nodes, and all connective tissue nodes investigated. The involved nodes show a broader distribution of $R$ values, shifted toward higher fluorescence ratios ($R_{\text{median}} = 1.11$) as compared with the noninvolved lymph nodes ($R_{\text{median}} = 0.82$) and connecting tissue nodes ($R_{\text{median}} = 0.73$). All of the distributions overlap considerably, the overlap being strongest between connective tissue nodes and noninvolved lymph nodes.

If, as in our previous work (11,16), a discriminator value $R = 1$ is chosen, a sensitivity of 62% at a specificity of 78% results for the detection of a metastatic node within all nodes. These numbers compare rather well with those obtained in our preliminary study (Table 3).

Figure 8 compares the distribution of the parameter size (volume as determined from two diameters) in a way that is similar to Fig. 7. While there is a difference between the distributions of the involved nodes ($V_{\text{median}} = 0.26 \text{ cm}^3$; 68% interval: 0.04–0.95 cm$^3$) and the uninvolved nodes ($V_{\text{median}} = 0.14 \text{ cm}^3$; 68% interval: 0.02–0.29 cm$^3$), the discrimination by volume is worse than by specific autofluorescence.

Table 3 Diagnostic value of specific autofluorescence detection, based on a discriminatory value of $R = 1$

<table>
<thead>
<tr>
<th>Preliminary study</th>
<th>Present study without pretreatment (RCT, HRCT)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>lymph nodes</td>
</tr>
<tr>
<td>$n$</td>
<td>171</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>65%</td>
</tr>
<tr>
<td>Specificity</td>
<td>85%</td>
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<tr>
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<td>51%</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>91%</td>
</tr>
</tbody>
</table>

The data from the preliminary investigation (11) are incorporated for comparison. At that time, only histologically confirmed lymph nodes were evaluated. Numbers refer to nodes investigated by fluorescence spectroscopy.

The maximum sum of sensitivity and specificity for discrimination by volume is about 120% compared with 140% for a discrimination based on fluorescence.

However, in view of a direct clinical application, i.e., as a fluorescence-guided lymph node biopsy, a casewise analysis is possible. As demonstrated for patient no. 16 (Fig. 9), the overlap in fluorescence readings between metastatically involved and noninvolved nodes at or around a $R$-value of 1 is unimportant, as long as the nodes with the highest fluorescence readings would correctly reflect the true nodal status of the patient. If all of the patients are analyzed in this way, a meaningful conclusion can be drawn only for the node-positive patients, because any biopsy of node-negative patients would always reveal the correct nodal status. Of 13 patients with lymph node metastases, the sampling of the one node, associated with the highest fluorescence signal, would correctly predict the nodal status of 10 patients. A sample of the two nodes with highest fluorescence readings would yield a correct result in 12 patients (Table 4).

Pathological reevaluation was carried out on 50 lymph nodes in 10 patients, presenting high absolute fluorescence readings and classified as normal by routine pathology. Eight of 50 reevaluated lymph nodes were reclassified as metastatic, and the nodal status of 1 patient was converted from negative to positive. In this case, the positive nodes showed $R$-values of about 2.
Influence of Pretreatment on Specific Tissue Autofluorescence.

Primary tumors pretreated by HRCT or RCT presented reduced \( R \)-values. Without pretreatment, \( pT_2 \) cancers showed higher fluorescence values than \( pT_3 \) or \( pT_4 \) cancers (\( R_{\text{median}} = 18.0, 10.5, \) and 5.9, respectively). After pretreatment, the responders (yp\( T_2 \)) did show lower \( R \) values (\( R_{\text{median}} = 1.3 \)) than nonresponders (yp\( T_3 \); \( R_{\text{median}} = 4.8 \); see Table 1 and Figure 4). For patients after pretreatment by RCT or HRCT, the fluorescence characteristics of connective tissue nodes and of uninvolved and metastatically involved lymph nodes did not present any statistically significant differences (Fig. 10).

Biochemical Verification. Using the tissue samples from the tissue collection of the Robert Roesle Hospital, reversed phase HPLC runs (Fig. 11) were characterized by an early eluting broad peak at 2 min, which was enhanced by the addition of uroporphyrin III or coproporphyrin III standards. This early peak was constant with respect to the tissue type in each patient. A second peak occurred at 24 min, which could be increased by the addition of PpIX standard solution. The second peak is enhanced in primary tumors and lymph node metastases as compared with normal tissue. Fig. 12 demonstrates the fluorescence emission spectra of eluates corresponding to the late HPLC peak for all three of the tissue types. The fluorescence spectra closely resemble the spectra of the PpIX standard and the fluorescence characteristics observed in the colorectal primary tumors and metastases.

Fig. 13 compares the occurrence of the specific autofluorescence signal in colorectal primary tumors, and their respective lymph node and liver metastases. The normalized fluorescence intensities, \( R \), of the primary tumors, of the lymphatic metastases, and of the liver metastases were significantly \( (P < 0.05) \) higher than \( R \) in normal mucosa.

DISCUSSION

Although some bacterial hemoglobin degradation at the luminal surface of the primary tumors may contribute to their autofluorescence signal (5, 6), the specific autofluorescence observed in our study occurred whether or not the tumor cells were exposed to bacterial colonization and persisted after careful washing of endoluminal tumor specimen, thus representing with high probability a specific metabolic abnormality of the tumor cells. Furthermore, in preliminary experiments, anaerobic bacterial colonization leading to bacterial PpIX production could be demonstrated by culture from primary tumor biopsies but not from samples of lymphatic, liver, and peritoneal metastases, all positive for PpIX fluorescence.

The growing knowledge about the mechanisms of 5-ALA stimulated PpIX accumulation further supports this assumption. The exact mechanisms are, however, unclear. One theory suggests an unchanged enzymatic conversion from the precursor 5-ALA to PpIX and a reduced incorporation of iron into the PpIX to heme. The latter might be a consequence of a reduced activity of the ferrochelatase, which is discussed for several malignancies (17–21) and was reported to be reduced by factors ranging from 50 in hepatomas as compared with normal liver (22) to a factor of 3 in colonic cancer with respect to liver.
surgery to patients who would not profit from it (39, 40). However, this technique still suffers a number of problems that range from the development of human to mouse antibodies (41) over the restrictions associated with the use of radioactive material to the lack of an ideal tumor-associated antigen (38). In our series, a sensitivity of 62% at a specificity of 78% may seem rather low; however, it is still far better than any conventional method of intraoperative staging, because it is mainly done by palpation. Our finding that the size of a lymph node is a bad predictor of metastatic status is well in accordance with the literature (42–44). If one further assumes that the simulation of a fluorescence-guided biopsy protocol, which would correctly predict the lymph node status in 30 of 32 patients in this series, is transposable from the ex-vivo to the in-vivo situation, such procedure may have immediate clinical significance. However, in vivo fluorescence measurements are rather limited in depth (probably 5–8 mm). An in vivo clinical study is, therefore, necessary to describe the feasibility of the approach and the reproducibility of the results in an intraoperative situation.

Furthermore, the refinement of pathological lymph node staging by immunohistochemical (45–47) and molecular techniques (48, 49) offers a substantial improvement in sensitivity, eventually delivering the key for an understanding of the prognostic variability in Dukes B patients. However, to carry out such extensive evaluation on any of the median 32 lymph nodes sampled in this study from each patients would be unjustifiable economically. Therefore, a fluorescence-guided and budget-priced technique to preselect regional lymph nodes that are at high risk of harboring metastatic tumor cells for in-depth pathological evaluation may represent the key for a more general use of such extended pathological lymph node staging.

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