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REVIEW

Colonic and gastric cancer metastatic lymph nodes: applications of autofluorescence-based techniques

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Abstract Lymphadenectomy and extended lymphadenectomy are still controversial issues in surgical oncology. The detection methods for metastatic lymph nodes include lymphoscintigraphy and radiolabelled antibody, but immunohistochemical sentinel lymph node (SLN) identification is commonly used. The potential diagnostic use of cell and tissue autofluorescence (AF) is well known. Here we review our studies on the application of AF-based techniques for diagnosing metastatic lymph nodes. We had previously demonstrated that AF imaging allows discrimination between hyperplastic and primary neoplastic lymph nodes. Our more recent studies show that the combination of autofluorescence microspectroscopy and multispectral imaging autofluorescence microscopy can be applied to the diagnosis of secondary neoplastic lymph nodes in gastric and colorectal cancer. These techniques have been validated by histochemical and immunohistochemical methods. Studies are in progress to select the best dye tracer for SLN identification, to improve image quality and to develop a software for automatic analysis of tissue AF to help the decision-making process during surgery.

Keywords Gastric cancer · Colorectal cancer · Sentinel lymph node · Autofluorescence microscopy multispectral imaging · Autofluorescence microscopy

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Introduction

The accurate evaluation of the lymph node status during the management of gastrointestinal (GI) cancer is one of the most important factors in determining several issues, such as the surgical approach, the extension of lymphadenectomy and the clinical outcome of patients. The methods available for the detection of metastatic lymph nodes are numerous, including lymphoscintigraphy and radiolabelled antibody detection. However, in most types of cancers the technique currently used is sentinel lymph node (SLN) identification. In the recent years this procedure has opened a new window in the clinical practice for GI cancer surgery. Individualized selective lymph node dissection for GI cancer based on SLN status would seem to be a reasonable surgical approach, although some

differences between colorectal and gastric cancer need to be examined.

The lymphatic drainage of the GI tract is much more complex than other sites and skip metastasis are rather frequent.

Colorectal cancer

An adequate lymphadenectomy during colectomy is an essential component of accurate staging. Both the AJCC and UICC recommend the examination of at least twelve lymph nodes for specimen [1, 2] although more nodes may be needed for better predictive value. However, even when a "suitable" number of lymph nodes are removed, aberrant lymph node drainage is still possible, so that lymph nodes harbouring metastasis may be present outside the field covered by a standard lymphadenectomy.

A focussed analysis of SLN may reveal a malignancy that would otherwise have remained undetected by conventional surgical and pathological methods. Those patients which are consequently upstaged will benefit from adjuvant chemotherapy. In present clinical practice, between 20 and 30% of node-negative patients will develop locoregional or systemic disease and adjuvant chemotherapy can significantly improve their 5-year survival rate [3].

Several techniques have been reported to improve LN detection, including LN revealing solution, fat-clearing methods [4], RT-PCR and immunohistochemistry. Unfortunately, these techniques are costly and labour-intensive, making them unsuitable for large-scale application. The stringency of performing a more detailed histopathological examination only on suspicious or enlarged lymph nodes, selected during surgery, is also not useful, since 69% of metastatic lymph nodes are smaller than 5 mm in size [5].

As a general concept, SLN mapping is useful not only to identify nodal micrometastases but also to avoid the use of very sensitive histopathological techniques on a large scale. SLN can also increase the number of tumour-draining lymph nodes identified by the pathologist, thus leading to a more accurate tumour staging and a better selection of candidates for chemotherapy. Adjuvant chemotherapy can significantly improve the 5-years survival of patients with node positive colorectal cancer [6].

Published studies on sentinel lymph nodes biopsy (SLNB) in colorectal cancer showed considerable heterogeneity with regard to the detection techniques used, the practical definition of the SLN, the time interval chosen between dye injection and SLN-detection, the histopathological techniques used, and the composition of the patients' groups. Consequently, the detection rate, sensitivity and rate of false negative LNs vary considerably.

In most trials the intraoperative subserosal injections of blue dye method have been used. Several authors have reported that the ex vivo SLN detection rate and sensitivity are comparable to the in vivo technique [7–9] however, aberrant lymphatic drainage, outside the field of the planned resection, cannot be detected with this method, and it has been reported in 2–8% of the patients affected by colon cancer [10, 11]. This may be due to the partial obstruction of the lymphatic channels caused by tumour cells, with consequent blockage of the tracer migration. In general, the rate of false—negative SLNB increases with the size and depth of infiltration (pT) of the primary tumour, but no correlation between tumour infiltration and the rate of false—negative have been reported [12]. The improved nodal staging by SLN mapping and the subsequent application of very sensitive histopathological techniques, result in the upstaging of up to 46% for patients with micrometastases [13, 14]. Nevertheless, it is still unclear whether the presence of micrometastasis is a prognostic indicator for disease-free and overall-survival in patients staged node-negative by routine HE-staining [15–19]. The growing use of laparoscopic colectomy for colorectal cancer has raised the question of whether SLNB could be applied during laparoscopic procedures. Although the number of studies on laparoscopic SLNB is still limited, the published data show an acceptable detection rate and sensitivity [20].

Sentinel lymph nodes biopsy has been shown to be less reliable in rectal cancer when compared to colon cancer [21] with a false negative rate of 56% [22]. This was thought to be due to the close vicinity of the primary tumour (shine-trough phenomenon) as well as the effects of neoadjuvant radio-chemotherapy.

Gastric cancer

The optimal extent of lymphadenectomy in gastric cancer is still under discussion. The individual characteristics of the tumour, such as location, depth of invasion, maximal diameter, macroscopical and histological type can all affect the procedure. The surgical approach is more conservative only for patients with mucosal lesions, whereas if submucosal lesions are present, the same treatment as more advanced gastric cancer would be required, unless the clinical usefulness of SLNB is established [23].

The lymphatic drainage of the stomach is more complex than that of the breast and skip metastasis have a high rate of 15–20% [22]. Since many gastric tumours spread also through the lymphatic system, the histological assessment of the first draining lymph nodes has both prognostic and therapeutic significance. All the regional LNs are routinely removed en bloc with the resected segment, and the value of SLNB is still controversial.

During the past 2 years, a growing number of clinical trials evaluating the feasibility and accuracy of SLNB in gastric cancer have been published [22–30].

Most authors consider the blue dye and radiolabelled techniques as complementary and recommend their combined use [31]. The data available at present do not justify a reduced extent of lymphadenectomy on the basis of SLNB in gastric carcinoma, but provide strong evidence for an improvement in tumour staging using this procedure [22]. The SLNB can be useful not only for the improvement in tumour staging by detecting micrometastasis, but also for changing the GI cancer patient therapeutical approach to minimally invasive treatments, including a laparoscopic approach [32, 33]. In Japan, large-scale multi-centre trials are ongoing at present to evaluate the mapping by dye techniques using subserosal injection. Another group is studying the use of radiocolloid in SLNB in gastric cancer [24–33]. Feasibility studies of laparoscopic SLN mapping for gastric cancer will follow. The results of all these trials will provide useful direction for the future use of SLN navigation surgery in gastric cancer.

The autofluorescence

The potential use of cell and tissue autofluorescence (AF) for diagnostic purposes has already been recognized [34]. However, in the past, fluorescence microscopy was mostly oriented towards the use of exogenous markers because of the difficulties in detecting and interpreting the AF signals, due to their low intensity and spectral complexity respectively.

More recently, the availability of high sensitivity, low noise, charge-coupled device (CCD) cameras has allowed the detection of low-quantum yield AF signals at level comparable to those obtained with high-quantum yield exogenous markers [35]. Consequently, the possibility of utilizing AF-based techniques both in research and diagnostics on single living cells or on tissue samples is now being reconsidered.

Many studies have evaluated the use of AF-based techniques, particularly the spectroscopic ones, to discriminate normal tissues from neoplastic lesions of the skin, oesophagus, colon, lung, bronchi, brain and bladder, both in vitro and in vivo, to aid diagnosis or intraoperative delineation of tumour resection margins [36]. In a previous study by our group about the lymph nodes status, we demonstrated that AF imaging allows discrimination between hyperplastic and primary neoplastic lymph nodes [37].

Study on metastatic lymph nodes (colonic and gastric cancer)

Autofluorescence microspectroscopy (AMS) and multispectral imaging autofluorescence microscopy (MIAM) can be applied to the diagnosis of secondary neoplastic lymph nodes in gastric and colorectal cancers. The AF pattern as well as the emission spectrum can be used to distinguish between normal and metastatic lymph nodes.

In a previous study we selected lymph nodes from 30 patients, 15 affected by colorectal cancer and 15 by gastric cancer. We deliberately chose patients who were in the advanced stages of the disease and were candidates for adjuvant therapy. In these patients, the removal of suspected metastatic lymph nodes, independently from location, did not affect the individual patient's scheduled therapeutic procedure. None of the patients had received neo-adjuvant therapy. The lymph node biopsies were collected during surgical resection of the tumour.

Fresh lymph node biopsies were divided in two parts: one was fixed in 10% formalin, paraffin-embedded and was processed using standardized histochemical and immunohistochemical techniques for diagnostic purposes; another was frozen in liquid nitrogen and then stored at −80°C, until MIAM and AMS analyses were performed.

The results obtained with the different techniques were then compared.

Each patient signed a written consent before being enrolled in the study. A database was created to register patient information, preoperative and histopathological staging, adjuvant therapy and follow-up data.

The present study was initiated in November 2004, and it is still running. Three hundred and seventy-two patients affected by colon cancer and 118 patients affected by gastric cancer were operated on in our Institution (2004–2008). The selection criteria stated that only patients with clinical (preoperative stage) T1 N0 M0 and T2 N0 M0 according to the fifth edition of AJCC, entered the study. All the patients had biopsy-proven adenocarcinoma. All the patients were older than 18 years and capable to give their informed consent.

Immunohistochemistry

Haematoxylin–eosin stained sections from each fixed sample were assessed by the pathologists. A representative section of 4 μm from each lymph node was selected for the immunohistochemical analysis. All sections were dewaxed in Bio-Clear (Bio-Optica, Milano, Italy) and hydrated with grade ethanol decreasing concentrations in distilled water. The streptavidin–biotin peroxidase method was used for immunostaining. A cocktail of broad-spectrum anti-cytokeratin, clone AE1/AE3 (BioGenex, San Ramon, CA, USA) was used as primary antibody. Antigen retrieval was routinely performed by microwave pre-treatment (Microwave MicroMed T/T Mega, Milestone, Bergamo, Italy) in 10 mM sodium citrate buffer (pH 6.0) for 30 min. All tissue sections were placed in the Ventana Nexes automated stainer (Ventana Medical Systems Inc., Tucson, AZ, USA) and the iVIEW DAB Detection Kit (Ventana) was

used as revelation system. Samples were then incubated with pancytokeratin monoclonal antibody AE1/AE3. After the staining run was completed, the tissue sections were removed from the stainer, counterstained with Mayer's haematoxylin, dehydrated and mounted with Permount. Formalin-fixed, paraffin-embedded sections of GI carcinoma were used as positive controls. Negative control sections for immunostaining were stained without the primary antibody.

The same procedure was applied on contiguous sections of those analysed by AMS and MIAM. In this case, the antigen retrieval was not necessary since the tissue was frozen and not fixed.

Multispectral imaging autofluorescence microscopy and autofluorescence microspectroscopy

Lymph node sections, 5-μm thick, were obtained from frozen biopsies, mounted on slides and immediately analysed by AMS and MIAM.

Tissue AF was analysed by an inverted epifluorescence microscope (Nikon Eclipse TE 2000 E, Nikon, Firenze, Italy). Oil-immersion CFI S fluor 40 × (N.A. 1.3) and CFI S fluor 20 × (N.A. 0.75) objectives were used. The excitation light source was a high-pressure mercury lamp (HBO 100 W, Osram, Milano, Italy). The 365-nm excitation wavelength was obtained by filtering the mercury lamp emission with an interference filter (10-nm FWHM, 365FS10-25, Andover Corp. Salem, NH, USA), which excluded the light coming from nearby Hg lamp emission lines other than the one selected. The fluorescence signal was transmitted through a dichroic mirror at 400 nm (DM400, Nikon) and detected by a spectrophotometer, for spectral measurements, or by a Hires IV cooled digital CCD camera (DTA, Cascina, Italy), equipped with a Kodak KAF261E detector (20 μm, 512 × 512 pixels), for images acquisition. Therefore, the whole system allowed sequential measurements of spectra and images on the same sample.

The spectrophotometer was based on a polychromator (SpectraPro 500i ARC, MA, USA; 500-mm focal length), connected to the microscope through an optical fibre bundle (1-mm diameter) and comprising a 16-bit Hires III cooled digital CCD device (DTA) with a back-illuminated SITe sensor (24 μm, 330 × 1,100 pixels). Overall wavelength resolution was about 0.34 nm in the selected range (375–750 nm). The SpectraPro was equipped with a grating (150 lines/mm) blazed at 380 nm. The spectrum analyser was calibrated in wavelength using spectral lamps (Hg, Cd, Kr, Xe). The calibration of the intensity was carried out by using a calibrated halogen lamp (EG&G, Princeton, NJ, USA): the maximum error in wavelength measurement was ± 1 nm over the whole considered bandwidth. Each spectrum was calibrated for modifications induced by the detection optics. The correction function $C(\lambda)$ for the microscope optics was obtained by measuring the light spectrum of an halogen lamp directly through the fibre optic bundle of the spectrum analyser and dividing it by the spectrum of the same lamp passing through the microscope optics as well. Peak identification was performed on mean curves (15 acquisitions on different tissue areas, showing similar histological conditions as judged from AF images). Peak positions were identified by polynomial fits applied locally to mediated spectrum curves. Maximum differences in fluorescence peaks were calculated by subtracting peak positions.

All the calibration procedures were performed only once. Before every measurement session the efficacy of the calibration was checked by fluorescence standards.

Autofluorescence imaging was accomplished by using a motorized filter wheel, containing up to eight different interference filters, placed in front of the CCD detector. This allowed for multispectral sequential acquisition in different emission bands. The filter combination was chosen depending on the main spectral bands, as determined by preliminary analysis of the AF spectra. Both the CCD camera and the filter wheel were controlled by a modified routine running under Visa software (DTA).

AF images were digitized directly in the CCD controller with 16-bit dynamics and transferred to the storage computer on a digital interface. In order to determine the observation field dimensions, we measured the spatial calibration factor (μm/pixels) by using 6-μm diameter fluorescent microspheres (Molecular Probes, Leiden, The Netherlands) observed with a 100 × objective. The corresponding calibration factor was 0.13 μm pixel^{-1} resulting in field size with 40 × and 20 × objectives of about 172 μm × 172 μm and 345 μm × 345 μm, respectively.

Statistical analysis

The following definitions were used for calculations:

Detection rate (%): number of patients with successfully retrieved SLN × 100/number of patients enrolled; sensitivity (%): number of patients with involved SLN × 100/number of patients with macrometastases in any lymph nodes; accuracy (%): number of patients with correct prediction of the nodal status × 100/number of patients enrolled; false negative rate (%): 100% − sensitivity; negative predictive value (%): number of nodal negative patients × 100/(number of nodal negative patients + number of false negative patients).

In order to evaluate the potential diagnostic application of the proposed AF-based techniques, the concordance of different raters in interpreting images was tested by Kappa statistics on a group of 35 AF images (30 from neoplastic

lymph nodes and 5 from controls). Unweighted κ-values (degree of agreement) [38] were evaluated considering the three possible pairings among three different raters, who scored the images in a blind fashion, according to the three categories: metastatic (M), probably metastatic (P) and hyperplastic (H) (normal) lymph node sections.

Results

The detection rate (number of patients with successfully retrieved SLN × 100/number of patients enrolled) was 96.5%, the sensitivity (number of patients with involved SLN × 100/number of patients with macrometastase in any lymph nodes) was 100%, and the accuracy (number of patients with correct prediction of the nodal status × 100/number of patients enrolled) was 90.5%. Accordingly to the restricted selection criteria and as it is stated in literature, the expected metastatic lymph nodes identified by immunohistochemistry were very few ($n = 4$).

In our study, aimed at the characterization of AF emission of metastatic lymph nodes obtained from patients affected by GI cancer, both multicoloured imaging and spectroscopic techniques were utilized.

In order to evaluate the possibility to differentiate normal from metastatic lymph nodes, bioptic sections of the organs, prepared as described above, were sequentially processed by AMS and MIAM.

The AF spectra of metastatic lymph nodes revealed significant differences compared to controls (Fig. 1a). The former showed a wide broadening towards the green-red region of the electromagnetic spectrum and a red-shift of the maximum of about 10 nm. When blue dye (Patent Blue) was used as tracer, the spectra of both normal and metastatic lymph nodes shifted of about 20 nm, but the difference between them remained clearly visible (Fig. 1b).

The maximum intensity variation between the AF spectra of normal and metastatic tissue section was at 504 nm (dotted vertical line in Fig. 1a; no Patent Blue was introduced).

By applying the multicoloured imaging technique we monitored monochrome images of the specimens, related to the fluorescence of endogenous chromophores emitting in a narrow band of wavelengths, which were then recombined in a single multicoloured image. When a 365-nm excitation was used, the endogenous chromophores expected to substantially contribute to the AF emission in the lymph node tissues were collagen, elastin and nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) in the blue band and flavins in the green band. A contribution in the red, due to fluorophores, such as lipopigments and porphyrins, was also considered. The spectral bands for monochrome image acquisition (see "Multispectral imaging autofluorescence microscopy and autofluorescence microspectroscopy" section) were chosen considering the spectral characteristics, in terms of emission peaks, of these fluorophores.

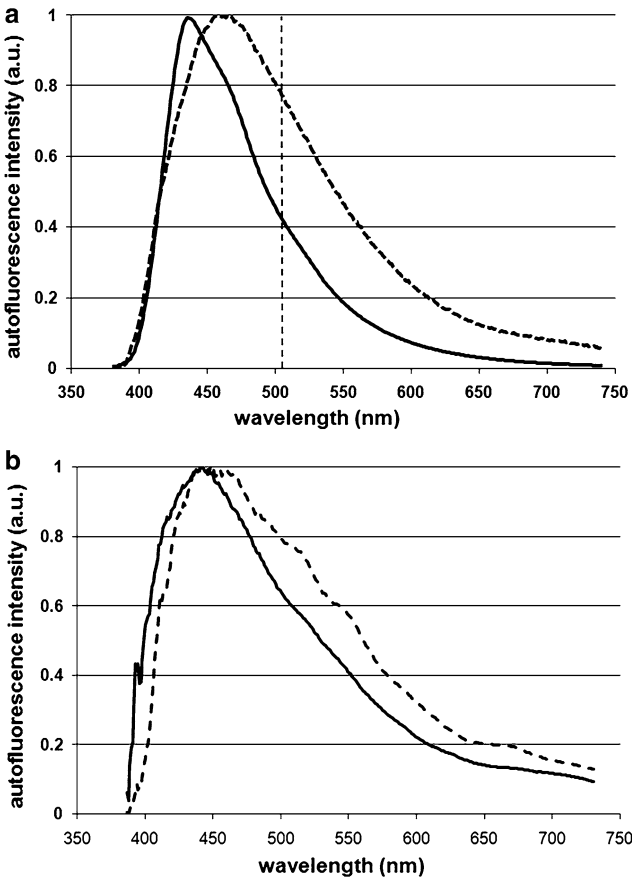

Fig. 1 Autofluorescence spectra: comparison between normal and metastatic lymph nodes. **a** Hyperplastic (*continuous line*) and metastatic (*dotted line*) lymph nodes. No exogenous dye is present. *Vertical dotted line* indicates the wavelength of maximum AF intensity variation (504 nm) between normal and metastatic samples **b** cystic non-metastatic (*continuous line*) and metastatic (*dotted line*) lymph nodes. Patent Blue dye is present

In Fig. 2, examples of AF multicolour images from non-neoplastic and neoplastic lymph node sections are reported.

The AF pattern of non-neoplastic lymph nodes (Fig. 2a), already described in details in previous studies [37], is characterized by strongly fluorescent connective trabeculae which separate faint fluorescent cortical follicles. At low magnification, the typical structural organization of the lymph node can be easily recognized: the connective trabeculae originate from the capsule, in itself very fluorescent, and penetrate into the medullar part of the organ. Collagen and elastin are the main responsible for the AF of capsula and trabeculae, while the emission from the lymphocytes in the germinative follicles is due to NAD(P)H and flavins.

As expected, a very faint fluorescence characterized the follicles, due to the low AF of the lymphocytes [39], the

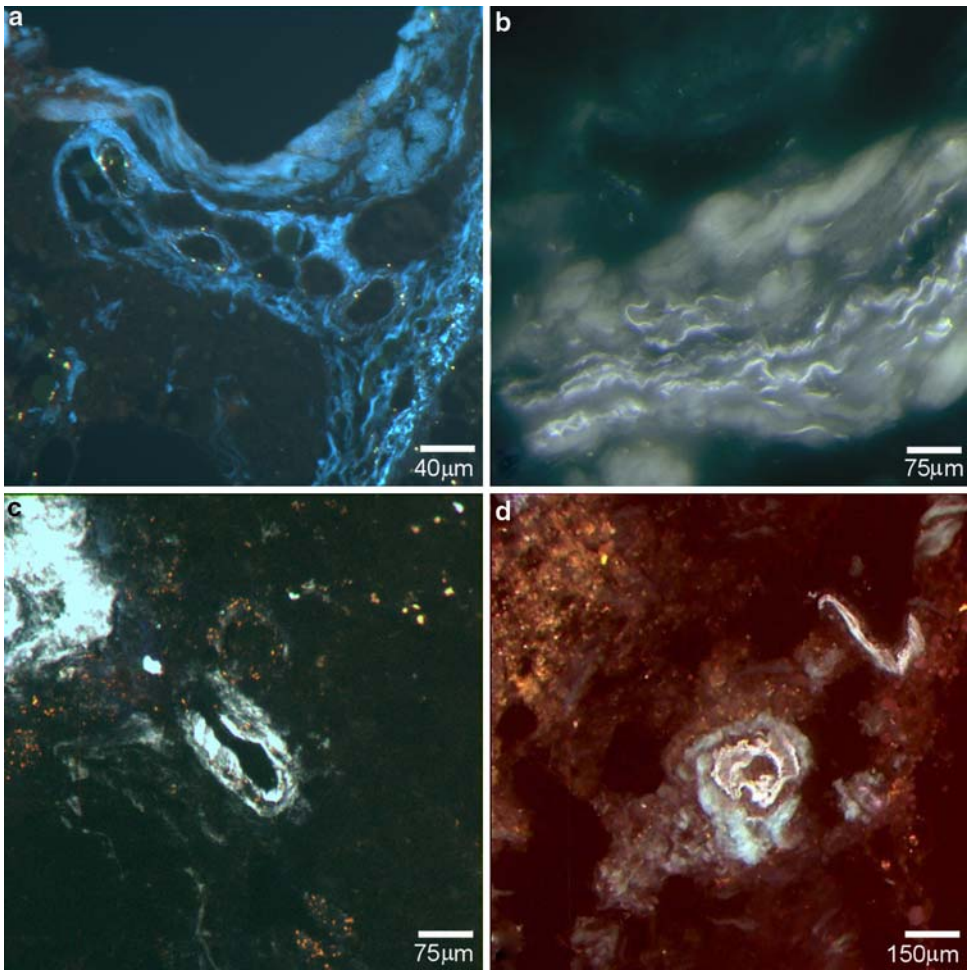

Fig. 2 Autofluorescence images of bioptic sections from normal (**a**) and metastatic lymph nodes from GI tumours (**b**, **c**, **d**). No Patent Blue is present. For each sample, three 40-nm wide (full width at half maximum) spectral bands, peaking at about 450, 550 and 650 nm were used in order to sequentially acquire three AF images with the same integration time. The overall acquisition was no longer than 12 s. Monochrome images were then combined in a single multicoloured image using the RGB technique. The multicoloured images were obtained by the Image Combine Channels algorithm of Corel PHOTO-PAINT v 6.0 software (Corel Corporation, Ottawa, ON, Canada), following identification of the three grey scale images (acquired at 650, 550 and 450 nm, respectively) with the RGB components. Non-neoplastic lymph nodes (**a**, **b**) are collected during colecistectomy for lithiasis, while images from neoplastic lymph nodes (**c**, **d**) are taken during colonic resection for cancer

most represented cell population in lymph nodes. When excited at 365 nm, a wavelength not suitable to induce nuclear AF, the lymphocytes show a fluorescence emission lower than other cell types, due to the low ratio between the cytoplasmic and the nucleic volumes.

The most important characteristic revealed by AF imaging of samples from patients with metastasis from GI tumours, is the complete loss of structural organization in lymph node tissues (Fig. 2b, c, d). The connective stroma is strongly altered because the radial organization of the connective trabeculae is generally substituted by a disordered network of fibres (Fig. 2b). Moreover, sections from pathological biopsies often present formations completely unrelated to lymph node tissues, such as tubular shaped structures surrounded by cubical or cylindrical cells, clearly resembling a glandular organization (Fig. 2c, d). These cells have different morphology, greater volume and higher AF emission when compared to lymphocytes. Another characteristic revealed by the AF pattern of metastatic sections is the presence of numerous orange-red, strongly fluorescent granules (Fig. 2c, d) which are clearly responsible for the broadening of the AF spectra towards the red wavelengths observed in the metastatic lymph nodes. In all the samples examined, the metastatic infiltration revealed by AMS and MIAM was confirmed by immunohistochemical diagnosis (data not shown).

Discussion

In the last years a growing attention has been paid to the SLNB. Although the routinely application in colon cancer is still under debate because of reports of varying accuracy and concerns regarding reliability and reproducibility, the identification of lymph node metastases is one of the most important prognostic factors in most solid cancers. The identification of nodal metastases is important also for treatment because significant survival benefit have been shown with adjuvant chemotherapy for node-positive patients.

It has been suggested that node-negative patients to conventional histopathology may harbour occult lymph node metastases. Such patients, if identified, could potentially benefit from adjuvant chemotherapy.

A detailed process of every lymph node removed in a standard colon resection would be far too labour intensive and costly; SLN mapping has the potential to obviate this obstacle by limiting the number of lymph nodes to be evaluated. The blue die is the identification tracer most used. The simplicity and flexibility of the blue dye techniques has helped establishing its role as the marker of choice for SLN identification. To date no perspective study has been performed on early stage colon carcinoma cancer and very few on gastric carcinoma. Furthermore, the rate of positive nodes in such patients who are expected to be node negative, is very low. A significant number of samples is very difficult to reach. The aim of our study, nevertheless is to evaluate the feasibility of the AF techniques in presence of lymph nodes micrometastases and/or lymph nodes harbouring few neoplastic cells. The suspected lymph node must be sliced in a large number of thin sections to evaluate even a small number of neoplastic cells. The study evaluates the feasibility and accuracy of the autofluorescence techniques. In the last two decades, very significant technological improvements have been achieved in the fields of excitation sources, light delivery systems and sensors for the detection of low-intensity signals. At the same time, the knowledge on the photo-physical characteristics of endogenous cellular and tissue fluorophores has increased. These advancements have renewed the interest on AF analysis and opened new perspectives for the application of AF-based techniques both in research and diagnostics.

We have reported here, the characterization of AF emission in lymph node tissues and the possible application of AF analysis to distinguish between secondary lymphoadenopathies, derived from tumours of the GI tract, and normal lymph nodes.

Normal and metastatic lymph node sections are characterized by different AF emissions, as shown in Fig. 1a, b where the respective spectra are compared. The altered spectral shape found in the metastatic specimens is the expression of changes in concentration and distribution of endogenous fluorophores, both intra- and extracellularly. It has been observed that in neoplastic cells the equilibrium between oxidized and reduced forms of flavins and pyridinic coenzymes, as well as the ratio between free and bound forms of NAD(P)H, change due to alterations in the cells' energy metabolism [40].

Studies comparing neoplastic tissues from the cervix [41], larynx [42] and GI tract [43] with the relative controls, have shown a decrease in AF emission in the pathologic samples, due to the alteration of the connective stroma, in particular of collagen and elastic fibres.

In spite of the low-quantum yield, collagen gives the greatest contribution to the tissue AF emission because of its high concentration [44]. Conversely, the contribution of flavins is less than 5%, notwithstanding the higher quantum yield [45]. NAD(P)H and oxidized flavins, the major intracellular fluorophores, have a lower concentration compared to the extracellular fluorophores that are therefore the main cause of the differences in AF emission between normal and affected tissues, as proved by studies on colon tissues [45].

Previous findings on primary adenopathies [46] as well as those reported here on metastatic lymph nodes from GI tumours, are consistent with a decreased contribution of collagen to the AF emission, due to the disorganization of the connective stroma.

In fact, a disorderly and not well evident network of fibres, which substituted the organized trabecular structures that normally separate the germinative follicles is often revealed by AF imaging when the tissue is infiltrated by the tumour (Fig. 2b, c).

Both spectra and images of metastatic specimens clearly demonstrated a significant increase in AF emission in the range 470–700 nm.

The image analysis revealed the presence of granules with intense orange-red fluorescence and areas characterized by brown-reddish diffuse fluorescence in the pathologic tissue (Fig. 2b, c, d).

These findings indicate that lipopigments have accumulated in the tissue. These are a class of fluorescent compounds, with emission peaks in the yellow-red band [47], derived from conjugation of oxidized lipids and degraded pigments.

Lipopigment accumulation in neoplastic tissues other than metastatic lymph nodes had been described by others [48–50].

In conclusion, comparing by spectroscopy and imaging the AF emission of metastatic lymph nodes from GI tumours with controls, three main differences have been observed:

(a) the disorganization of the connective stroma;
(b) the accumulation of lipopigments;
(c) the presence of tubular shaped structures with glandular-like organization.

These three points could represent key information for the diagnosis of metastatic lymph nodes from GI tumours by AF techniques, on condition that a suitable model is developed.

The loss of well organized connective stroma, which seems recurrent in tumours, has been found in both primary and secondary neoplastic lymph nodes. Conversely, the accumulation of lipopigments, deriving from the oxidative metabolism, often observed in metastatic lymph nodes of the GI tract was not found in primary neoplastic lymph nodes.

Therefore, the present study proves that it is possible to distinguish metastatic from normal lymph nodes by

analysing the AF emission of the tissues with AMS and MIAM techniques. When an excitation at 365 nm was used, we found that the maximum spectral differences between normal and metastatic lymph nodes were at about 515 and 504 nm with and without Patent Blue respectively. By limiting our analysis to the ability of AF spectral measurements in discriminating metastatic from normal specimens, monitoring AF emission in correspondence with these wavelengths ensures the greatest sensitivity.

However, further improvements could be achieved by studying the dependence from the excitation wavelength of the fluorescence difference spectrum, in order to favour the emission from connective stroma and lipopigments.

One of the major problems presented by AF analysis of complex biological samples, such as tissues sections, is the discrimination power of spectral measurements. This is because biological tissues contain many spontaneously fluorescent molecules whose spectra are partially overlapped. Therefore, the diagnostic effectiveness of spectroscopic measures alone is not enough when border-line cases are examined. Previous studies have proven that the association of spectroscopical analysis with multispectral AF imaging may greatly improve tissue diagnosis by combining spectral and spatial resolution. This would give information both on the spectral characteristics of endogenous fluorophores and on their distribution in the tissue.

The combined application of AMS and MIAM techniques can represent a powerful tool for tissue diagnostics and, in particular, for the detection of metastatic lymph nodes.

Comparison of AMS and MIAM diagnosis with histochemical and immunohistochemical methods provided a satisfactory validation of the proposed techniques.

Studies are in progress in order to improve the quality of the images from specimens collected utilizing the Patent Blue. In fact, these images have a diffuse background fluorescence probably due to the permanence of dye molecules in the tissue. This fluorescence is an impediment to obtain the highest quality, which is in our opinion needed to achieve the clinical diagnostic target: the detection of micrometastases and/or few metastatic cells in the lymph nodes. The results shown in this paper suggest the possibility of improving the current diagnostic procedures for malignant lymph node alterations. In fact, the application of AF-based techniques could have the following advantages:

1. Absence of any chemical treatment of the specimens.
2. Reduction in time consuming procedures.
3. Reduction of manipulation artefacts.
4. Cost reductions since reagents, specific antibodies, markers are no longer needed whereas the cost for instrumentation upgrade can be easily amortized.
5. The sample preparation could be performed directly in the surgical rooms and a computer controlled microscope could allow the remote analysis of the specimen by the pathologist.
6. The future perspective of possible in vivo diagnostics.

Studies are currently in progress aimed at the development of a software for an automated analysis of tissue AF by scanning all the specimen sections in order to help the decision-making process during surgery.

Conflict of interest statement The authors declare no existing conflicts of interest.

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