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Confocal laser endomicroscopy for diagnosing lung cancer *in vivo*

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ABSTRACT: Confocal laser endomicroscopy is a novel endoscopic technique that may allow imaging of living cells in lung tissue *in vivo*. We assessed the potential of this technique for the detection of histology during screening bronchoscopy for lung cancer.

32 patients with suspected malignancies underwent bronchoscopy with endomicroscopy using acriflavine hydrochloride. Standardised areas and localised lesions were analysed by *in vivo* confocal imaging during bronchoscopy and biopsies were taken. Confocal images were graded and correlated prospectively with conventional histology from biopsies.

Acriflavine hydrochloride yielded high-quality confocal images and strongly labelled airway epithelial cells. No side-effects were noted. 75 522 confocal images from 56 different locations were compared prospectively with histological data from biopsy specimens. Endomicroscopy allowed subsurface imaging with detailed analysis of cellular and subcellular structures. Neoplastic changes could be predicted with high accuracy (sensitivity 96.0%, specificity 87.1%, accuracy 91.0%).

Confocal laser endomicroscopy with acriflavine is a novel diagnostic tool for the analysis of living cells during bronchoscopy and permits virtual histology of neoplastic changes in the airways with high accuracy. This technique may enable the rapid diagnosis of neoplasia during ongoing endoscopy in patients with suspected lung cancer.

KEYWORDS: Bronchoscopy, endomicroscopy, *in vivo* imaging, lung cancer

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Confocal laser endomicroscopy (CLE) is a novel technique for imaging live cells during endoscopy [1–8]. Upon systemic administration of the dye fluorescein, CLE can be performed *in vivo* during ongoing endoscopy and permits real-time subsurface imaging of cellular and subcellular structures in the mucosa. Initially, this technique was applied to various inflammatory diseases of the gastrointestinal tract such as inflammatory bowel diseases, Barrett's oesophagus and coeliac disease. It was found that high-quality *in vivo* imaging could be performed and CLE was utilised for rapid diagnosis and for the targeting of biopsies. In addition, CLE was shown to predict neoplasia in the stomach, oesophagus and colon with high accuracy [1–8].

In contrast to gastrointestinal endoscopy, there are only a few studies of CLE in pulmonary disease. Recently, THIBERVILLE *et al.* [9] published images of alveoli by CLE in the absence of contrast agents. They described alveolar structures *in vivo* and identified elastin as the main endogenous fluorophore. In smokers, alveolar macrophages carrying autofluorescent pigments were the only cellular structures that could be visualised. Furthermore, CLE allowed imaging of the central airways, but such imaging yielded information about elastin fibres only [10]. Thus, CLE without contrast agents could not be used for the assessment of airway epithelial cells and lung cancer. In order to circumvent the problems of absent staining in the central airways, FUCHS *et al.* [11] used the dye fluorescein for CLE imaging in the lung in analogy to the field of gastroenterology, where this dye yielded excellent staining of colonic epithelial cells. However, fluorescein-aided CLE did not allow staining of epithelial cells in the central airways, but resulted only in staining of the lung interstitium and alveolar space. Thus, fluorescein-based CLE cannot analyse the cellular structures of epithelial cells in patients with suspected lung cancer *in vivo*.

In gastrointestinal endoscopy, acriflavine has been recently used to supplement intravenous or topically applied fluorescein in order to visualise cellular structures during CLE [9]. Using this approach CLE has facilitated the identification of cellular structures (*e.g.* nuclei) and bacteria in the gastric and colonic mucosa.

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TABLE 1 Histological results

Neoplastic lesions	
SCLC	7
NSCLC	
Adenocarcinoma	5
Squamous cell carcinoma	8
Metastasis	
Renal cell carcinoma	1
Colorectal carcinoma	1
Large cell neuroendocrine carcinoma	1
Non-neoplastic lesions	
Chronic bronchitis	5
Inflammatory pseudotumour	1
Granulation tissue	1
Sarcoidosis	1
Papillomatosis	1

Data are presented as n. SCLC: small cell lung cancer; NSCLC: nonsmall cell lung cancer.

However, there are no data available regarding acriflavine-aided CLE of the central airways. The aim of the current study was to evaluate imaging of central airway lesions with CLE using acriflavine as a contrast agent. We demonstrate that acriflavine-aided CLE permits analysis of airway epithelial cells and can be used for *in vivo* diagnosis of lung cancer with high accuracy.

METHODS

Bronchoscopy

This study was approved by the ethical committee of the University Hospital Erlangen (Erlangen, Germany). All patients gave informed consent.

Bronchoscopy and CLE were performed in 32 consecutively recruited patients (nine females, 23 males; mean±SD age 64.5±11.3 years) with nodular lesions of the central airways. Final diagnosis of patients was based on the histology of biopsies (table 1). Bronchoscopy was performed under *i.v.* conscious sedation (midazolam 3–7 mg and pethidine 50–100 mg) with a video-chip endoscope (Olympus BF 1T180 or BF Q180; Olympus, Tokyo, Japan). The patients were monitored *via* ECG, pulse oximetry and intermittent noninvasive measurement of blood pressure. Oxygen ($\geqslant$2 L·min^{-1}) was given *via* nasal probe.

CLE

CLE was performed with the Cellvizio system (Mauna Kea Technologies, Paris, France) working at a wavelength of 488 nm. A 1.4-mm diameter confocal miniprobe (AlveoFlex; Mauna Kea Technologies) was used. The technical details of the confocal probe were as follows. The probe has a lateral resolution of 3.5 μm, a field of view of 600×500 μm and a penetration depth of 0–50 μm. CLE was performed 2 min after dye administration in macroscopically normal, standardised areas and within exophytic nodular lesions. Images were stored as digital files. Biopsies were taken after completion of the CLE analysis. Sections were made and stained using standard haematoxylin and eosin methods.

TABLE 2 Confocal pattern classification

Confocal diagnosis	Cell structure	General tissue architecture	Example
Normal mucosa	Bright nuclei Overlapping of cells	Homogeneous	Fig. 1a
Inflammation/ regeneration	No overlapping Moderately enlarged cytoplasm	Heterogeneous	Fig. 1b
Neoplastic lesion	No overlapping Chaotic cell distribution Heterogeneous size of nuclei Variable distances between nuclei	Heterogeneous Dark areas with weak or no acriflavine uptake	Fig. 1c

Image analysis

The video sequences were analysed with the software included (Cellvizio Viewer, version 1.4.1; Mauna Kea Technologies) after bronchoscopy. The automatic greyscale feature for optimal analyses of the recorded sequences was used. Findings from CLE analysis were compared with histopathological analysis of two experienced cytopathologists (J. Schubert and M. Vieth) in a prospective and blinded fashion.

Staining agent

In the present study, we used acriflavine for imaging, as CLE without dyes and fluorescein-aided CLE did not permit the imaging of airway epithelial cells [11]. To our knowledge this is the first report on topical usage of acriflavine for CLE of the central airways. Although acriflavine has been discussed as a potentially carcinogenic and mutagenic substance due to its nuclear staining properties, no evidence for this has been found in epidemiological studies [12]. Therefore, acriflavine was described as a group 3 (''not classifiable as to its carcinogenicity to humans'') carcinogen by International Agency for Research on Cancer reports in 1987 and 1991 [13, 14] and has been classified as ''probably not carcinogenic to humans'' [14]. Furthermore, data on the long-term use of orally administered acriflavine for HIV therapy did not show an increase in tumour rate in this population at high risk for neoplastic diseases [15–17]. The follow-up time in these studies was $\leqslant$38 months [16]. However, this observation period does not exclude potential carcinogenic effects at later time points. Finally, acriflavine showed anticancer activity *in vitro* in recently published studies [18, 19]. Thus, acriflavine might be an interesting substance for various diagnostic and therapeutic purposes in humans. Consistently, acriflavine has been used for CLE in patients with gastrointestinal diseases without reported side-effects [4]. Furthermore, a pilot study on acriflavine use in the central airways suggested that acriflavine administration is a safe procedure. In this study, 10 patients with subglottic stenosis received topical administration of a 2% acriflavine solution and no side-effects were noted over a 5-month observation period [20]. It thus appeared unlikely to us that a further 40-fold diluted 0.05% acriflavine solution would

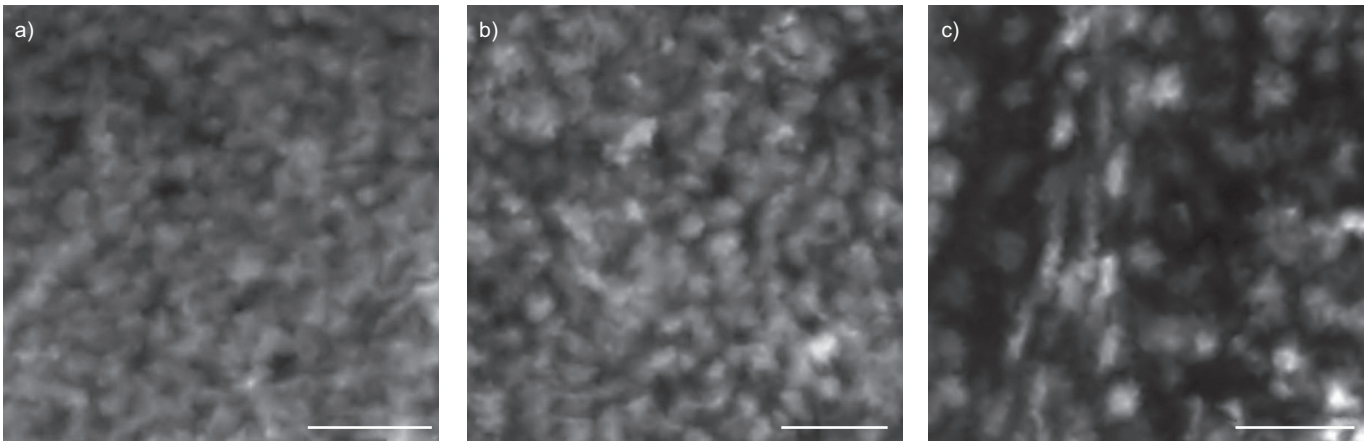

FIGURE 1. Examples are shown for a) normal mucosa, b) inflammation regeneration and c) neoplastic lesion. Scale bars=50 μm. Please refer to table 2.

result in relevant side-effects, due to the low dosage and the topical application of acriflavine. However, in agreement with the ethical approval, acriflavine was only used in patients with suspected neoplasias in this study.

In the present pilot study, 10 mL of a 0.05% acriflavine solution (5 mg acriflavine per patient) was applied *via* the working channel of the bronchoscope into the distal trachea and both main bronchi. Application was followed by a waiting period of 2 min to allow distribution and cellular uptake of acriflavine. After 2 min a homogeneous breathing-dependent distribution of the staining was noted. CLE was subsequently performed with the Cellvizio system, as detailed above, and the endomicroscope was placed *via* the working channel of the bronchoscope on top of localised lesions in the central airways. This approach was possible by the use of a 1.4-mm diameter confocal miniprobe. In addition to localised lesions, areas of the central airways that were macroscopically normal in appearance were examined by CLE in all patients.

Confocal pattern classification

A confocal pattern classification was developed in close collaboration between endoscopists (F.S. Fuchs and S. Zirlik) and pathologists (J. Schubert and M. Vieth) to allow confocal image analysis. Accordingly, 12 931 confocal images of the airways of 12 patients were analysed in comparison with the histological findings of the same locations, to identify criteria for the presence of normal mucosa and also neoplastic or non-neoplastic tissue. Important criteria included the characteristic structure of the cytoplasm and nucleus of airway epithelial cells and tissue architecture in CLE analysis (table 2).

RESULTS

In the present pilot study on the use of acriflavine for CLE, acriflavine was applied *via* the working channel of the bronchoscope into the distal trachea and both main bronchi. Subsequently, a probe-based endomicroscope was used to analyse macroscopically normal areas and exophytic, localised lesions. Topical application of acriflavine for CLE could be

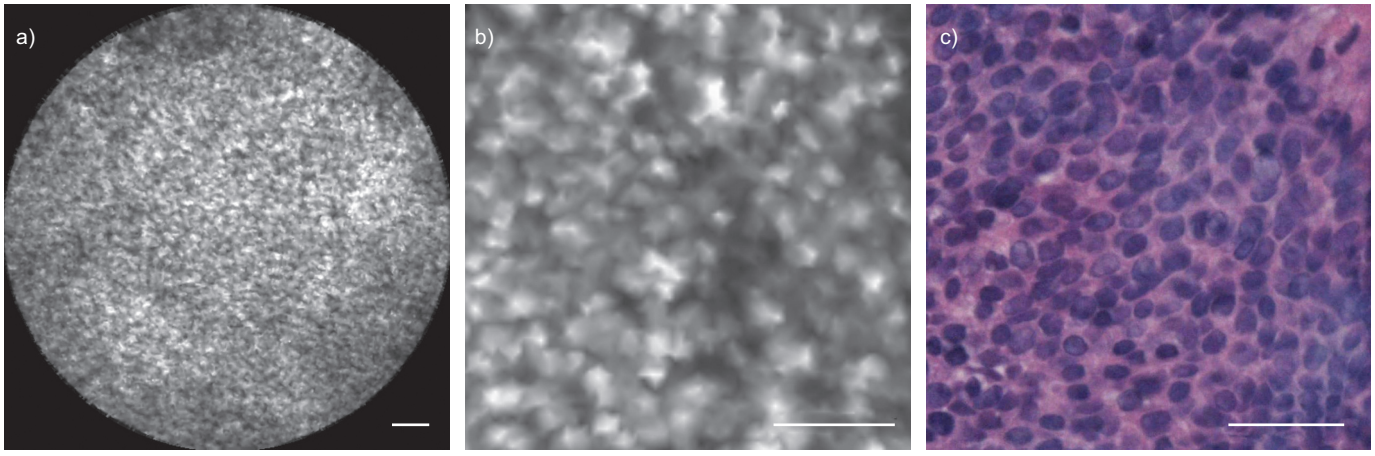

FIGURE 2. *In vivo* confocal laser endomicroscopy (CLE) imaging of normal airway mucosa after topical administration of acriflavine during bronchoscopy. The confocal probe was positioned in the central airways after staining with acriflavine. Confocal images were recorded and compared to conventional histology. A homogeneous distribution of nuclei corresponding to small, light grey, densely packed and mostly overlapping spots was noted during endomicroscopy: a) overview; b) and detailed view. c) haematoxylin and eosin-stained sections from lung biopsies in this area are shown for comparison. It should be noted that sections were cut in a horizontal orientation to facilitate comparative analysis with CLE images. Scale bars=50 μm.

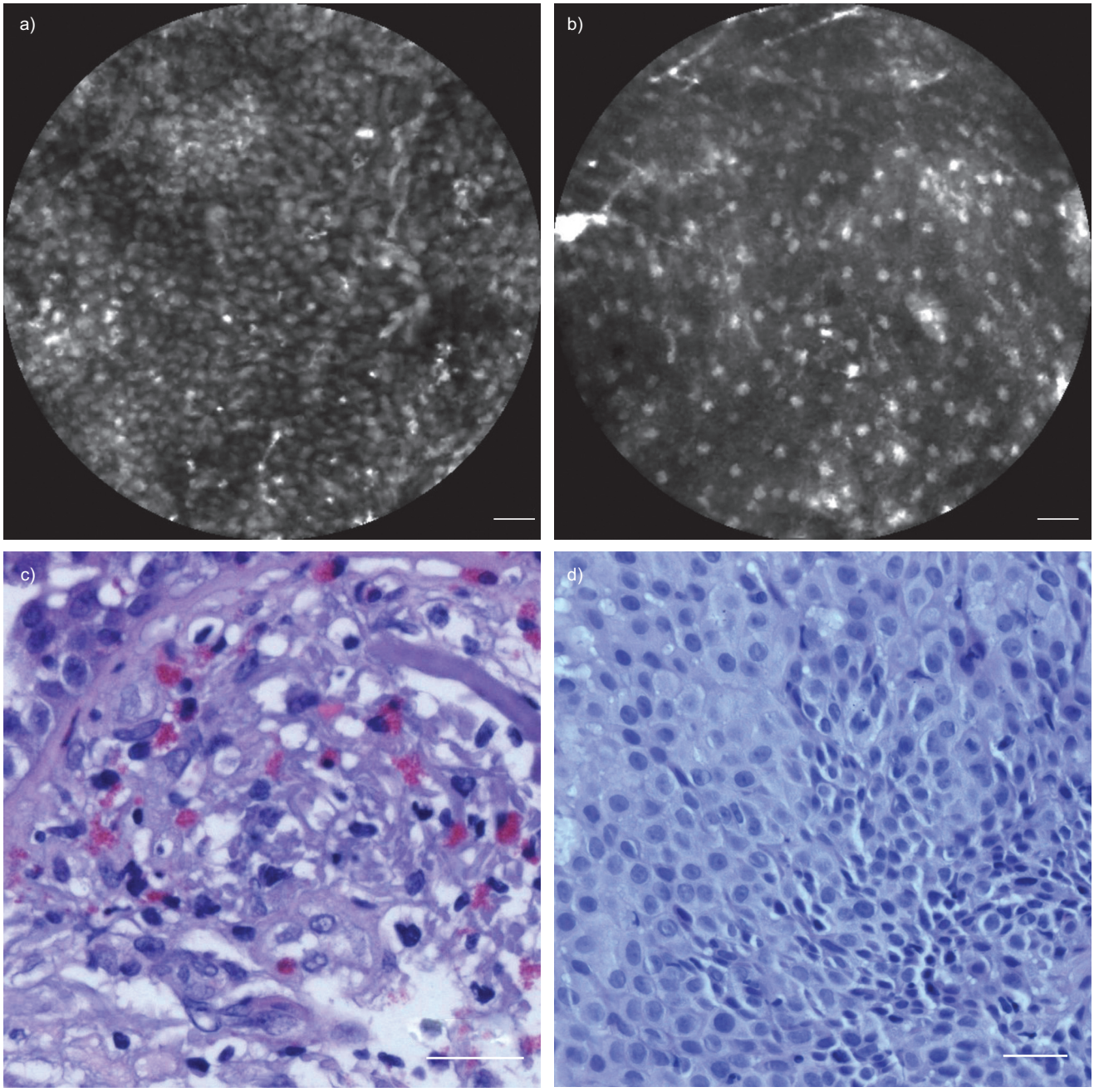

FIGURE 3. *In vivo* imaging during bronchoscopy. Assessment of a) inflammatory and b) metaplastic changes in the central airways by acriflavine-aided confocal laser endomicroscopy. Inflammatory and metaplastic lesions led to an increased distance between nuclei, and little or no overlapping of nuclei was noted. Cells had an enlarged cytoplasm, particularly in metaplastic disease. Histology from bronchial biopsies confirmed the presence of c) inflammation and d) squamous cell metaplasia. Scale bars=50 μm.

performed in all 32 patients during bronchoscopy. Staining with acriflavine was performed in the central airways and distal trachea followed by CLE in these areas. CLE was well tolerated and no marked side-effects were noted. In all patients, an excellent staining of epithelial cells in exophytic lesions was achieved, and normal mucosa of the central airways could be visualised using acriflavine-aided CLE.

Normal mucosa

In all areas with macroscopically normal bronchial mucosa, a unique homogeneous staining pattern was identified (fig. 2a). There was intense fluorescent staining of the nuclei of airway epithelial cells by acriflavine, resulting in the detection of small, light grey, densely packed and mostly overlapping spots during endomicroscopy *in vivo*. This staining pattern correlated well with that of nuclei detected in histological samples from the same regions (fig. 2b and c) further suggesting that endomicroscopy is capable of analysis of airway epithelial cells.

Non-neoplastic lesions

In exophytic and infiltrative lesions with local inflammation, a loss of cellular homogeneity with large prominent airway

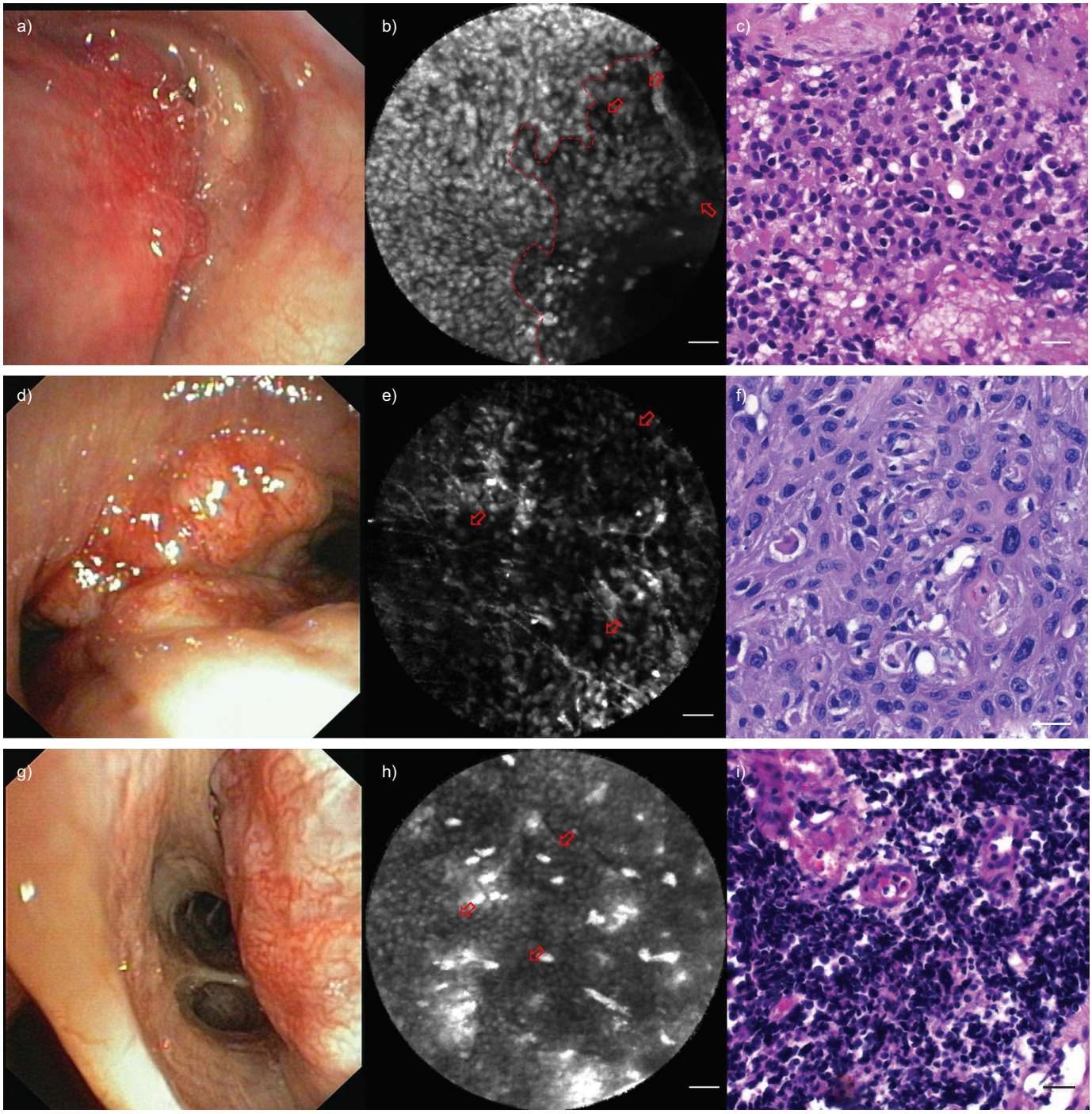

FIGURE 4. Adenomatous carcinoma of the lung: a) bronchoscopic view of the bronchus intermedius, which was almost occluded by an exophytic lesion; b) confocal *in vivo* imaging of the lesion upon topical staining with acriflavine showed dark areas with little or no acriflavine uptake, indicative of neoplasia. Some tumour cells are highlighted (arrows). Furthermore, inflamed areas with increased distance between nuclei were noted surrounding the tumour. The borderline between inflamed (left side) and neoplastic tissue (right side) was clearly visible (red dashed line). c) histological biopsies from the neoplastic area identified by confocal laser endoscopy (CLE) were stained with haematoxylin and eosin (H&E) and confirmed the presence of neoplasia (adenomatous carcinoma). Squamous cell carcinoma of the lung: d) bronchoscopic view of the main carina, showing a large nodular lesion. The confocal probe was placed on top of the lesion for *in vivo* imaging. e) confocal imaging during ongoing bronchoscopy after staining with acriflavine showed large dark areas with heterogeneous cell populations indicating the presence of neoplasia. Several tumour cells are highlighted (arrows). f) biopsies were taken and sections were stained with H&E. Histological analysis confirmed the presence of lung cancer (squamous cell carcinoma of the lung). Small cell carcinoma of the lung: g) bronchoscopic view of the right lower lobe in a patient with small cell lung cancer (SCLC). h) CLE after staining with acriflavine identified numerous small, roundish tumour cells *in vivo*. These cells had a dark appearance with little or no acriflavine uptake (arrows). i) histological analysis of biopsies in this area confirmed the presence of numerous tumour cells characteristic of SCLC. Scale bars=50 μm.

TABLE 3 Correlation between confocal imaging and histology

Confocal diagnosis	Sites	Histology			
		Normal mucosa	Inflammation/regeneration#	Neoplastic lesion	
				Lung cancer¶	Metastasis
Normal mucosa	16	16	0	0	0
Inflammation/regeneration	12	5	6	1	0
Neoplastic lesion	28	2	2	22	2

Data are presented as n. #: inflammatory pseudotumour, granulation tissue, sarcoidosis, papillomatosis; ¶: including small cell lung cancer (n=7), nonsmall cell lung cancer (n=13) and large cell neuroendocrine cancer (n=1).

epithelial cells was detected. The distance between the cells was expanded and airway epithelial cells displayed a prominent bright nucleus and a dark cytoplasm (fig. 3a and c). In patients with squamous cell metaplasia, confocal endomicroscopy detected numerous large cells. Whereas their nuclei were relatively small, their cytoplasm was extremely enlarged resulting in a ''fried egg''-like structure of cells (fig. 3b and d).

Neoplastic lesions

In nonsmall cell lung cancer (NSCLC) and exophytic metastasis of extrapulmonary carcinomas, dark neoplastic cells were identified. These cells showed large nuclei, heterogeneous in size. Particularly, nuclei showed weaker acriflavine uptake and were less densely arranged than in normal mucosa (fig. 4). Furthermore, a wide and dark cytoplasm surrounding the heterogeneous nuclei was noted, allowing discrimination between normal and dark, neoplastic airway epithelial cells. Thus, acriflavine-aided confocal imaging could be used for the precise mapping of tumour cell spreading in the airway epithelium.

In patients with small cell lung cancer (SCLC), a homogenous pattern with weak acriflavine uptake by small dark round cells was observed. Cells were much smaller compared to cells in patients with NSCLC (fig. 4g–i). Furthermore, in contrast to inflammatory lesions, cells had a very small cytoplasm. Thus, acriflavine-aided CLE led to a characteristic staining pattern in SCLC that could be used for the *in vivo* diagnosis of this disease.

Diagnostic yield of CLE

The confocal images of the airways were analysed for the presence of neoplasia using a newly designed confocal pattern classification. Accordingly, initial data from 12 patients were used together with pathologic criteria for the design of a confocal pattern classification (table 2). Based on the combined analysis of the images by endoscopists and pathologists it was found that cell structure and the general tissue architecture were important for discrimination between neoplastic and non-neoplastic tissue in the lung. Accordingly, the confocal pattern classification stratified neoplastic and non-neoplastic tissue into three different types (normal tissue, inflammation/regeneration and neoplasia) based on the characteristic structure of the cytoplasm and nucleus of airway epithelial cells and the tissue architecture in CLE analysis. Whereas bright, partially overlapping nuclei in a homogeneous tissue architecture were noted in normal tissue, cells without overlapping nuclei but an expanded cytoplasm were seen in inflamed/regenerative tissue with heterogeneous tissue architecture. Furthermore, a chaotic distribution of dark cells with heterogeneous nuclei was indicative of neoplasia.

The confocal pattern classification was used prospectively to predict histopathology. Accordingly, 96 video sequences with 75 522 confocal images (nine frames·s^{-1}, 2.5 min per location on average) from 56 different locations, including 19 biopsies from macroscopically normal mucosa and 37 biopsies from exophytic or infiltrative lesions, were analysed in a blinded fashion. All sites were evaluated with CLE before taking biopsies, and histological results were compared with CLE findings.

Results from confocal imaging and histology are shown in table 3. CLE showed a sensitivity of 96.0% and a specificity of 87.1% for the detection of malignant lesions. The positive predictive value was 85.7% and the negative predictive value was 96.4%. Overall, 91.0% of all sites were correctly classified by CLE.

DISCUSSION

CLE is a novel technique for *in vivo* imaging of the mucosa. In the present pilot study, we have used the contrast agent acriflavine for confocal imaging of the bronchial mucosa in patients with suspected lung cancer. We observed that this technique facilitates the detection of airway pathology. By directly comparing data from CLE with histopathological findings from biopsies we demonstrate that such acriflavine-aided CLE detects neoplastic changes in localised lesions in the central airways with high accuracy.

Bronchoscopy has witnessed many improvements in recent years, including the development of video and high-resolution endoscopes. In addition, autofluorescence bronchoscopy (AFB) was introduced as a red flag technique to identify localised lesions, but unfortunately yielded a high rate of false positive results [21]. Furthermore, filter techniques such as narrow band imaging (NBI) have been used to facilitate the detection of localised lesions in the airways [22]. However, none of these techniques permitted *in vivo* imaging of the central airways at cellular resolution.

CLE has been previously used to detect mucosal changes in the gastrointestinal tract [1–8]. In addition, recent studies have used this approach for imaging of the lung [9–11]. It was found that autofluorescence and fluorescein-aided CLE can be used for imaging of alveoli and the lung interstitium. In contrast to the gastrointestinal tract, however, fluorescein was not able to

penetrate into epithelial cells in the airways. Thus, CLE using fluorescein could not be used for the analysis of lung cancer.

Here, we have used acriflavine-aided CLE as a novel approach for imaging of airway epithelial cells *in vivo*. In a pilot study using a probe-based CLE system and topical staining, we could show that topical application of acriflavine in the airways was safe and could be done easily. Aside from minimal oozing of blood in some cancer patients upon placement of the confocal probe, we could not see any adverse events of CLE during bronchoscopy. Limitations of the technique were related to the fact that the probe-based system could not be introduced into smaller airways in a targeted fashion, thereby restricting the usage of CLE to the central airways. In addition, costs might be another limitation for implementation of CLE during bronchoscopy. It should be noted that the costs for a functional confocal unit are currently ~€120 000. Furthermore, at least in our clinical setting, the costs for a single procedure were ~€250 for the consumables. The time necessary to perform each bronchoscopy was 5–10 min.

The image quality of acriflavine-aided CLE was remarkably high. In fact, acriflavine-based imaging permitted cellular analysis *in vivo* and could be used to discriminate between normal airway epithelial cells and altered epithelial cells in inflammatory and neoplastic areas. Assessment of mucosal inflammation could be performed in macroscopically inflamed areas but also in the boundary area of exophytic tumour growth. This technique thus might be a valuable tool for future research on peritumoural inflammation. As CLE could identify tumour cells in the boundary areas of neoplastic lesions where the mucosa was macroscopically normal, evaluation of tumour spread prior to curative surgery of lung cancer might be another potential application of CLE. Furthermore, it may be helpful to characterise airway inflammation in chronic diseases such as asthma and chronic obstructive pulmonary disease, with a possible influence on therapy strategies. Finally, CLE may open the door to molecular imaging of airway epithelial cells *in vivo*, as this technique has been recently used beyond acriflavine staining for vascular endothelial growth factor receptor and CD44v6 staining of colonic epithelial cells [23–27].

Another point that should be considered when introducing confocal endomicroscopy relates to image analysis. To achieve the goal of this exploratory pilot study we had to analyse a very large number of single images. This was necessary to develop an image classification which enables tissue evaluation. In order to develop this classification, it was essential to carefully review all images and video sequences. In future studies, however, we believe that it will be sufficient to review the video sequences for *in vivo* confocal analysis. As the average length of the videos was 2.5 min, we feel that 2.5 min will be sufficient for final analysis in the future. This additional time may be justified by the immediate information obtained about the localised lesion and the potential reduction in the number of necessary biopsies. In this regard, it should be noted that confocal endomicroscopy has been shown to significantly reduce the number of necessary biopsies in the field of gastroenterology [27].

Acriflavine-aided CLE could distinguish between neoplastic and non-neoplastic lesions. In particular, a direct comparison to histological results showed that this technique has a high sensitivity, specificity and accuracy for the detection of neoplastic lesions during ongoing bronchoscopy. Based on our present pilot study, acriflavine-aided CLE emerges as a promising new approach for *in vivo* imaging of lung cancer and may be useful in the clarification of local visual appearances. However, we cannot conclude that CLE is superior to AFB or NBI. Thus, future studies should focus on the combined use of AFB, NBI and CLE during bronchoscopy. In any case, however, it is likely that CLE may reduce the rate of false positive results of AFB- or NBI-guided biopsies.

In conclusion, acriflavine-aided CLE of the central airways emerges as a feasible and safe method for the detection of neoplastic changes during bronchoscopy. Although some limitations of this method currently exist, this technique allows a unique look at cellular structures of airway epithelial cells and permits subsurface imaging. In fact, concerning the differentiation between neoplastic and non-neoplastic tissue, the diagnostic yield of acriflavine-aided CLE was comparable to histology of lung biopsies in the majority of cases. Thus, CLE may be helpful to reduce the number of biopsies and to optimise targeted biopsies in the lung. Furthermore, this technique allowed detailed assessment of cellular structures and lung cancer and predicted the presence of neoplastic changes with high accuracy. While acriflavine-aided CLE has been previously established in gastrointestinal disorders [4, 26–28], future research is needed to clarify the clinical significance of acriflavine-aided CLE in inflammatory and neoplastic lung diseases. It is unlikely, however, that acriflavine-aided CLE will be able to replace conventional biopsy for lung cancer, as histological analyses are currently essential to determine tumour subtypes and predictive molecular markers (*e.g.* epidermal growth factor receptor mutation status). However, CLE guided tissue sampling might improve the accuracy of conventional biopsies and therefore may provide a higher yield of tumour tissue for subsequent analyses. Furthermore, CLE may lead to an optimised rapid *in vivo* diagnosis of neoplastic changes in patients with suspected lung cancer.

STATEMENT OF INTEREST

None declared.

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