Fluorescence detection may help to demarcate skin cancer from normal skin, thus to reduce the potential of incomplete treatment resulting from unawareness of tumour extension in surrounding skin. In this study we evaluated the difference between autofluorescence of basal cell carcinomas ($n = 21$) and the normal-appearing skin surrounding them. Referring to the difference found, a point-by-point measurement was taken from the tumour lesions outwards to the surrounding skin to locate the differentiation point of autofluorescence on the skin. Protoporphyrin IX fluorescence was measured from the same spots using the same procedure, after the tumours and the surrounding skin had been treated with topical 5-aminolevulinic acid methyl ester cream. The point-by-point measurement enabled us to locate the vanishing point of the protoporphyrin IX peak, which was compared with the differentiation point of autofluorescence to assess the utility of autofluorescence in tumour demarcation. Illuminated by 370nm light, both the tumour and surrounding skin emitted a fluorescence with peak intensity at $455 \pm 3$nm. The peak intensity was 53% ($18–84\%$) (median, range) lower in the tumours than in normal skin ($p < 0.001$). In 78% of the measurements, the differentiation point of the autofluorescence was within $3$mm of the vanishing point of the protoporphyrin IX peak. Autofluorescence may be used in BCC demarcation. Key words: tumour border; fluorescence differentiation; in vivo.

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Basal cell carcinoma (BCC) of the skin is the most common cancer in Caucasians. It is estimated that nearly 2 million new cases are diagnosed worldwide, annually, and the incidence is rising (1–4). BCCs are slow-growing, locally invasive tumours that rarely metastasize but can result in extensive morbidity through local recurrence and tissue destruction. Recurrence is often the consequence of incomplete removal of the cancer tissue, part of which may be invisible to the naked eye. In surgical excision, the recurrence rate is reported to be 30% to 67% for the lesions with incompletely removed margins (margin positive) (5–8). This is significant compared to the low recurrence rate (1–10%) in cases where the tumours were cleared sufficiently (8–10).

To accomplish complete removal of the tumour and maximum preservation of normal skin, different techniques such as Mohs’ micrographic surgery, laser Doppler velocimetry and fluorescence imaging have been studied for their application in skin cancer demarcation (11–15). Mohs’ surgery involves the sectioning of entire margins of excised tissue, microscopic identification and mapping of remaining tumour, and repeated excisions until a tumour-free margin is ensured. The technique is invasive and costly in time and manpower (16). Laser Doppler velocimetry was used to measure the blood circulation point-by-point to locate the BCC border in one study (12).

Fluorescence detection as a non-invasive and fast approach has gained increasing interest for its application in diagnosis and demarcation of various cancers. Numerous studies have been carried out focusing on detecting the selective concentration of exogenous highly fluorescent substances in cancers, or changes in endogenous fluorophores in cancer tissues (17–21). Application of 5-aminolevulinic acid (ALA)-induced protoporphyrin IX (PpIX) fluorescence in BCC demarcation has shown success (13–15). The major disadvantages of this approach are that 3 to 4h are needed to generate detectable PpIX fluorescence after the ALA application, and the PpIX may cause skin photosensitization (20). Though the selective concentration of PpIX may benefit patients with photodynamic therapy, it is not, however, a practical approach for more demarcation. Very limited information is available on the role of skin autofluorescence in the detection of BCCs, with the exception of one study (21) showing no difference between the autofluorescence of BCC lesions and normal skin.

However, it has been reported that autofluorescence detected from melanoma of the skin and cancers of other organs such as bladder, lung and gastrointestinal tract is useful in discriminating malignancies from normal tissues (19, 22–26). The theoretical background for distinctive autofluorescence changes in cancer tissues has also been investigated (26–28). The altered metabolic state of cancer cells and the resulting abnormal concentration of nicotinamide dinucleotide (NAD) have been suggested to be the cause of the changes in autofluorescence of cancers (19, 27). Other factors such as morphological alterations of tissues were also suggested to be contributors (26).

The aims of this study were to evaluate the difference between the autofluorescence of BCC lesions and the normal skin surrounding them, and to assess the feasibility of autofluorescence in the demarcation of BCCs. ALA-induced PpIX fluorescence, the broadly studied approach for BCCs demarcation, was used as a control.

SUBJECTS AND METHODS

Patients

The local ethics committee approved the research protocol (Ref: KF02-128/95, Copenhagen County Ethics Committee). The study included a total of 13 patients (6 males, 7 females) with 21 BCCs; median age was 69 years, range 40 to 80 years. Ten of the tumours were primary BCCs and 11 were recurrent; 9 were superficial BCCs, 9 nodular and 3 intermediates. All of the tumours were verified histopathologically.
Fluorescence measurements

The autofluorescence spectra were recorded from BCC lesions and the surrounding skin before biopsy and treatment. Three measurements were taken from different spots within the lesion area. Ten measurements were taken from different spots of the surrounding normal-appearing skin 2–4 cm away from the visible border of the tumour. Each measurement spot was 1 mm in diameter. After noticing the difference between the autofluorescence of tumour and normal skin in the first 5 patients, we decided to take an extra set of measurement for the rest of the tumours. This set of point-by-point measurements was taken from the tumour centre outwards to surrounding normal skin with 1-mm interval. In 4 tumours, this measurement was done twice, each in a different direction (Table I).

After the autofluorescence measurements, the tumours with a margin of 1–4 cm normal-appearing skin were treated with 5-aminolevulinic acid methyl ester (ALAME) cream (Metvix®, 160 mg/g, PhotoCure AS, Oslo, Norway) and occluded with plastic film for 4 hours. Following the removal of the plastic film and wiping the treated area, the skin fluorescence was recorded with the same procedure as in the autofluorescence measurements.

The fluorescence spectra were measured with a fluorescence spectrometer system (Fl3095, J&M Analytische Mess-und Regeltechnik GMBH, Aalen, Germany). The light source of the system is a high-pressure xenon lamp (175 W) with a monochromator (band width 8 nm). The detector is a MMS photo diode array system (256 elements; pixel resolution: 3.2 nm/pixel, Zeiss, Germany). A quartz Y-fibre (Volpi AG, Zurich, Switzerland) was used to conduct the excitation light to the skin and collect the fluorescence to the detector. The detector output was displayed and stored in a personal computer that controlled the operation of the monochromator.

The fibre probe was placed gently and perpendicularly on the skin during the measurements. A 370 nm excitation wavelength was used to induce both the autofluorescence and PPIX fluorescence. A filter (Schott GG420) was inserted between the receiving fibre and the detector to diminish the reflected excitation light from entering the detector. All measurements were performed in a dark room at room temperature. The fluorescence spectrometer system was calibrated daily by measuring a fluorescence standard.

Data analysis

The means of the spectra were taken to represent the autofluorescence spectra of BCC and normal skin, respectively. The intensity maxima and its wavelength position were read out for each of the mean spectra. The difference in autofluorescence intensity between the tumour and normal surrounding skin was calculated as:

\[
\text{relative change} = \frac{I_{\text{normal}} - I_{\text{tumor}}}{I_{\text{normal}}} \times 100\%
\]

in which \( I \) is fluorescence intensity.

To assess whether it exceeds normal variation, this change was compared to the within-subject variation, which was calculated for the 10 measurements from surrounding normal-appearing skin:

\[
\text{relative variation} = \frac{2I_{\text{std}}}{I_{\text{mean}}} \times 100\%
\]

in which \( I \) is the fluorescence intensity.

The PPIX fluorescence intensity was read out at 630 nm for the ALAME-treated area, and the intensity of this wavelength was also read out for normal skin. The relative change and relative variation were calculated and compared for this wavelength in the same way as for the autofluorescence.

The autofluorescence intensity of each point in the point-by-point measurements was compared with the intensity of the tumour lesion and normal skin obtained in the preceding measurements. The differentiation point of autofluorescence, where the fluorescence intensity turns to normal skin level, was found on the skin, and its distance from the visible border of the tumour was measured. The same procedure repeated after the ALAME treatment enabled us to find the vanishing point of the PPIX peak, where the characteristic PPIX peak became invisible and the spectral line returned the same level as that in non-ALAME-treated normal skin. Its distance from the visible border was measured. The differentiation point of autofluorescence was compared with the vanishing point of the PPIX peak. The match between the two points was judged to be “yes” or “no” according to the distance between them, e.g., ≤3 mm: yes; >3 mm: no (14).

The Wilcoxon matched pairs test was used to examine the difference between the relative change of fluorescence in tumour and the relative variation of fluorescence in normal skin. The Mann–Whitney test was used to calculate the significance of the difference between the relative changes of fluorescence in primary and recurrent tumours, and between superficial and nodular tumours. \( P \) values less than 0.05 were considered significant.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Measurement</th>
<th>Extension of low-intensity autofluorescence (mm)</th>
<th>Extension of PPIX peak (mm)</th>
<th>Difference between the two extensions (mm)</th>
<th>Match (Y/N)</th>
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<tr>
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<td>3</td>
<td>1</td>
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</tr>
<tr>
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<td>5</td>
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</tr>
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<td>3</td>
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<tr>
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<td>1</td>
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<td>5</td>
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<td>0</td>
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<td>1</td>
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<td>7</td>
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</table>

\(^a\) The superscripts indicate different lesions of a patient.
\(^b\) In these lesions, the point-by-point measurement was carried out twice, each in a different direction.
\(^b\) Y = the match between the two borders ≤3 mm; N: >3 mm.

\( \text{Table I. The extension of low-intensity autofluorescence and protoporphyrin IX (PpIX) peak beyond visible tumour border} \)
RESULTS

The autofluorescence spectra of both the BCC lesion and the surrounding skin ranged from 420 nm to 700 nm with the intensity maxima at 455 ± 3 nm (Fig. 1). The PpIX fluorescence had dual peaks around 630 nm and 700 nm, of which the 630 nm was the dominating one (Fig. 2). This fluorescence was mainly detected from the tumour lesions, but rarely from skin that was more than 1 cm away from the visible border of the lesions.

The peak intensity of autofluorescence was 53% (18–84%) (median (range)) lower in BCC lesions than in normal surrounding skin \( (p < 0.001) \), while the relative variation in the normal skin was 14% (8–38%) (median (range)) \((p < 0.001)\). This difference occurred in all types of BCCs investigated and was identical between primary and recurrent BCCs, and between superficial and nodular BCCs \( (p > 0.05) \).

The PpIX intensity was 3.2 (0.8–11.2) (median (range)) times higher in BCC lesions than in normal surrounding skin \( (p < 0.001) \), while the relative variation of the fluorescence intensity at 630 nm was 0.2 (0.04–0.4) (median (range)) in normal skin (Fig. 2).

Point-by-point measurement across the tumour and surrounding skin showed that 56% of the differentiation point of autofluorescence and 83% of the vanishing point of the PpIX peak were located 3 mm or further beyond the visible tumour border. The difference between the two points was within 3 mm in 78% of the measurements (Table I).

DISCUSSION

We used a 370 nm excitation wavelength and observed a broad emission spectrum with peak position around 455 nm. This spectrum may correspond to several different fluorophores such as NADH, collagen and elastin, since all of these fluorophores can be found in skin and all have emissions at this wavelength \((19, 21, 26, 29)\).

In agreement with numerous studies reporting the decrease in autofluorescence intensity in various cancers \((19, 22–24)\), we found that the 455 nm skin fluorescence had lower intensity in BCC lesions than in normal skin. Stenerborg et al., however, used a 375 nm excitation wavelength and did not find any difference between the total fluorescence (area beneath the curve for wavelength region 400 to 700 nm) of skin cancer and normal skin; they did not compare the peak intensities in the study \((21)\).

A multiple-factor mechanism including changed metabolism and blood perfusion, and degraded connective tissue, is likely to explain the low autofluorescence found in BCCs \((19, 26, 27, 30, 31)\). In most of the tumours, the increase in blood perfusion was obvious, since the lesions were red, which means increased absorption of the fluorescence signal by the haemoglobin. In accordance with this, Kirsner et al. \((12)\) reported successful demarcations of BCC lesions using laser Doppler velocimetry to measure the changed blood perfusion. The contribution of collagen and elastin to the fluorescence changes in BCCs needs further elucidation, though Mitra & Marks \((28)\) noted a decreased concentration of procollagen in malignant lesions. It is well known that the NADH fluorescence indicates the metabolic state of tissues, a fact that is considered by some researchers to be the cause of the low fluorescence in cancers \((19, 27)\). It is speculated that when tumour cells proliferate quickly, the ratio between the oxidized \((\text{NAD}^+)\) and the reduced form \((\text{NADH})\) of NAD alters. The accumulation of less fluorescent \(\text{NAD}^+\) results in the decreased fluorescence in cancers \((32)\). Nevertheless, careful studies are needed to investigate the mechanisms of the changes found in cancer autofluorescence.

The selectivity of PpIX fluorescence in BCCs has been well documented \((13–15, 20)\). The contrast between the fluorescence intensity in tumour and the surrounding skin is important, since it might suggest whether an imaging system could be adapted to distinguish the tumour from normal skin. In the studies using the PpIX fluorescence imaging system, the contrasts were reported to be 2.1 ± 0.5 and 1.8 ± 0.3, which enabled successful image processing \((14, 15)\). In our study, the contrast between the autofluorescence intensity of normal skin and tumour was 2.1 (1.2–6.6) (median (range)), suggesting that autofluorescence-based image processing of BCCs may be possible.

A recent study by Wennberg et al. \((14)\) revealed a good correlation between tumour sizes evaluated by fluorescence imaging and histopathology. Therefore, we compared the extension of low-intensity autofluorescence in the surrounding skin of BCCs with the extension of the PpIX peak. A relatively good match was found between the two, suggesting that a further study is worthwhile, in which histopathology must be
involved to verify whether the extension of the low-intensity autofluorescence is due to the existence of cancer tissue. There is great advantage in using autofluorescence in cancer demarcation, since there is no need of introducing exogenous substances into tissue, which is time-consuming and may bring along potential hazards (13–15, 20). Autofluorescence image processing would overcome the slowness and inconvenience in the point-by-point measurement, as employed in the present study and the study using laser Doppler velocimetry (12).

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REFERENCES