Diagnosis of Basal Cell Carcinoma by Raman Spectroscopy

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Skin cancers are the most common form of malignant neoplasms in man. In this work, near-infrared Fourier transform (NIR-FT) Raman spectroscopy was used to study the molecular alterations in the most common skin cancer, basal cell carcinoma (BCC). Biopsies from 16 histopathologically verified BCC and 16 biopsies from normal skin were harvested and analysed by NIR-FT-Raman spectroscopy using a 1064 nm Nd: YAG laser as a radiation source. Differences in Raman spectra between BCC and normal skin indicated alterations in protein and lipid structure in skin cancer samples. Spectral changes were observed in protein bands, amide I (1640–1680 cm⁻¹), amide III (1220–1300 cm⁻¹) and ν(C–C) stretching (probably in the amino acids proline and valine, 928–940 cm⁻¹), and in bands characteristic of lipids, CH₂ scissoring vibration (1420–1450 cm⁻¹) and —(CH₂)ₙ— in-phase twist vibration around 1300 cm⁻¹. Moreover, possible changes in polysaccharide structure were found in the region 840–860 cm⁻¹. Analysis of the band intensities in the regions 1220–1360, 900–990 and 830–900 cm⁻¹ allowed for a complete separation between BCC and normal skin spectra. In addition to a direct assessment of spectral intensities, a neural network analysis was performed, which confirmed the differences in spectra between BCC and normal skin. In conclusion, Raman spectra from BCC differ considerably from those of normal skin. Hence, Raman spectroscopy can be viewed as a promising tool for the diagnosis of skin cancer. © 1997 by John Wiley & Sons, Ltd.

INTRODUCTION

The most common cancer in humans is skin cancer and its incidence is increasing dramatically.¹ Cutaneous tumors originate from different types of cells present in the skin, e.g. malignant melanoma from melanocytes (pigment cells), squamous cell carcinoma and basal cell carcinoma from epidermal cells (keratinocytes). Skin tumors are divided into benign and malignant, the most common malignant skin cancer being basal cell carcinoma (BCC).²,³ Clinically, BCC appears as a well circumscribed pearly or pigmented raised lesion, often located on the face or ears. Exposure to ultraviolet or ionizing radiation and fair skin complexion are major pathogenic factors in the development of BCC, and therefore BCC is particularly prevalent in Caucasians with a history of excessive sun exposure.⁴ A study from Minnesota gave annual incidence for males and females of 175 and 124 per 100,000, respectively.⁵ Clinical diagnostic accuracy for BCC is rather poor, being only 65% for practising dermatologists.⁶ BCC is often hard to distinguish from pigmented naevi, warts, seborrhoeic keratoses and skin changes associated with excessive sun exposure such as actinic keratoses and senile sebaceous hyperplasia. Histopathologic examination of skin biopsies by microscopy is the standard technique used for the confirmation of the diagnosis but in cases of multiple suspicious skin lesions excessive biopsying is impractical and often unacceptable for the patient. Therefore, there is a search for rapid non-invasive techniques which can be applied for initial screening and selection of lesions for further biopsying.

Spectroscopic techniques are now emerging as powerful methods in medical diagnosis.⁷–⁹ Raman spectroscopy, being non-destructive and providing information about the molecular structure of the tissue, is of particular interest in cancer diagnosis. This technique has recently been used for investigations of the skin in both animals and man.¹⁰–¹³ Raman spectroscopic studies of skin inflammatory diseases⁴ and evaluation of drug effects on epidermis¹⁴,¹⁵ indicate that molecular alterations in normal and diseased skin manifest themselves in Raman spectra.

In this work, we used Raman spectroscopy to investigate whether any alterations in molecular structure occur in BCC and whether Raman spectroscopy could be used for the diagnosis of this type of skin cancer. Raman studies of skin cancer have not been reported before, but it is known that Raman spectra of certain neoplastic tissues (brain, genitourinary tract, breast and colon) and their normal counterparts differ from each other.⁸,¹⁶–¹⁸ The idea of using Raman spectroscopy for cancer diagnosis has been forwarded and patented.¹⁹ However, studies on skin cancer have not yet been reported. Moreover, to date the major obstacle has been the lack of a reliable and objective method for analysis of Raman spectra. Therefore, we assessed if neural network modeling could be used to differentiate the spectra of BCC from those of healthy skin. A neural network is a computer program, resembling a chain of neural cells, which can be trained to capture even small...
changes in spectra not matching the standard spectrum. Analysis with neural networks has previously been attempted with success for Raman spectra. Work regarding the application of neural networks to transmittance, reflectance and Raman spectra show a superior performance compared with traditional linear models such as multiple linear regression, spectral library searching, partial least squares and cluster analysis.

EXPERIMENTAL

Skin sampling

Raman spectroscopy was performed on 16 '3 mm' punch biopsies from histologically verified BCC and 16 control biopsies from healthy skin. Biopsies were obtained upon informed consent and the protocol was approved by the Ethics Committee of Copenhagen. Before sampling, the skin was cleansed with 70% ethanol and anaesthetized with a 2% lidocaine solution without adrenalin. Samples were kept at 4 °C in a moist environment. Raman spectroscopy was performed within less than 30 min after collecting the biopsies. No sample pretreatment was performed.

Instrumental

We used an FRA 106 Raman module on a Bruker IFS 66 optics system (Bruker, Karlsruhe, Germany). Radiation of 1064 nm and 300 mW from an Nd:YAG laser was used for excitation. Biopsies were placed in stainless-steel cups and the laser beam was focused on the biopsy in a spot ca. 100 µm in diameter. For each biopsy 250 scans were accumulated with a resolution of 4–6 cm⁻¹. The total registration time was 10 min. To prevent the laser power or scanning time used from modifying spectral changes, for several samples Raman spectra were obtained with lower power (30–300 mW) and shorter scanning periods (2–10 min). OPUS software (Bruker) was used to evaluate spectral characteristics (peak wavenumber position and integrated characteristics) of the bands. Zero filling with a factor of two was applied. Background correction for instrument characteristics was performed. We assumed that an incandescent lamp is a black-body emitter at 3200 K and we obtained the Fourier transform (FT) Raman spectrum of the lamp. This spectrum was divided by a black-body radiation spectrum calculated at 3200 K using the following expressions:

\[
E_{\text{rad}}(\text{W m}^{-2} \text{ cm}^{-1}) = \frac{2 \times 10^7 \pi h c^2 \nu^3}{(\exp(100hc/k_B T) - 1)}
\]

All the measured near-infrared (NIR) FT-Raman spectra were divided by the resultant spectrum.

Data analysis

The original OPUS format was transferred to ASCII format and analyzed by the Matlab (S. Natick, MA, USA) program. No normalization of any particular spectral band was performed but all spectra were scaled by substraction of the mean and division by the standard deviation, which is a standard procedure to facilitate graphical presentation of data. In no case was smoothing procedures used.

Analysis of the spectra was performed with two methods. Initially, 16 spectra of BCC and 16 spectra of healthy skin were visually inspected and spectral differences identified. Then, the areas under the curves were calculated in the regions of most prominent differences between BCC and normal skin. Lower borders of the areas were lines connecting wavenumbers 1220–1360, 900–990 and 830–900 cm⁻¹ (Fig. 1).

Finally, the same 32 spectra were used to train and test the neural networks. Artificial neural networks (ANN) were employed for the classification of the spectra, since they offer the capability of learning nonlinear arithmetic operations based on a training set and

![Figure 1](https://example.com/figure1.png)

Figure 1. Raman spectra of healthy skin (upper row) and basal cell carcinoma (lower row). Regions of most pronounced spectral changes are shown. For quantitative analysis of spectral changes (see Table 1) the indicated areas under the curves were calculated in the regions 1220–1390, 900–990 and 830–900 cm⁻¹.
can generalize a compact model which can later be applied to unknown spectra of interest.\textsuperscript{20,22,25}

The primary objective was to see whether the ANN model was able to distinguish correctly between the two classes of spectra and predict correctly a class of unknown spectra. Second, it was important to see which features of the spectra the model were found to be significant for the problem at hand, and to compare them with the area calculation discussed earlier. For this reason, the ANN model was presented with the whole spectrum without principal component analysis (PCA) for preprocessing.

ANN models were trained to output $-1$ for control skin and $+1$ for BCC, using back-propagation.\textsuperscript{20,26} The performance of the model was evaluated during training by cross-validation, in which some of the spectra are left out and used for testing how well the model predicts unseen spectra. Owing to the limited number of spectra in this study, only one control and one BCC spectrum were left out. This process of ‘leaving one out’ was repeated for 16 different pairs of control and BCC spectra in such a way that each spectrum was left out for cross-validation exactly once. The method yielded 16 different networks, each trained on a set which was slightly different from the previous one.

A two-layer, feed-forward network was used. The Raman intensities at the 1607 different wavenumbers equally spread between 400 and 3500 cm$^{-1}$ were used as inputs. Figure 2 shows the structure of a network with only five inputs. The inputs were multiplied by the input weights ($v_i$) and summed at the hidden units ($h_j$). A transfer function mimicking the firing threshold of a biological neuron is applied and the result was then multiplied by the output weights ($w$) and summed at the output ($y$). A negative output is interpreted as $-1$ (control skin) and a positive as $+1$ (BCC).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{network_structure.png}
\caption{Structure of the feed-forward neural network used for classification, with only five inputs shown ($x_i$). The inputs and a bias 1 are multiplied by the input weights ($v_i$) and summed at the hidden units ($h_j$). A transfer function mimicking the firing threshold of a biological neuron is applied and the results (and a bias) are multiplied by the output weights ($w$) and summed at the output ($y$). A negative output is interpreted as $-1$ (control skin) and a positive as $+1$ (BCC).}
\end{figure}

RESULTS AND DISCUSSION

Raman spectra were obtained at Raman shifts from $-1000$ to $3500$ cm$^{-1}$. No spectral changes were observed on comparing spectra obtained from the same samples at different laser powers and time intervals excluding these factors as sources of spectral differences. Spectral regions showing the most significant spectral changes between normal skin and BCC skin samples are given in Fig. 1. Significant protein bands are found in the amide I region (1640–1680 cm$^{-1}$) and amide III region (1250–1300 cm$^{-1}$).\textsuperscript{31–34} Both regions reflect the secondary protein structure. In the amide I region the low-wavenumber part around 1650 cm$^{-1}$ is less intense in the BCC samples. This might indicate disturbances of $\alpha$-helix structure in this sample as compared with normal skin. An overall loss in total intensity seems to occur for both the amide I and amide III regions in the BCC samples. The alterations of $\alpha$-helix in BCC is supported by decreasing intensity of the band around 1270 cm$^{-1}$ in the amide III region. Although a change in the protein secondary structure is evident in the BCC samples, complete quantification of the observed spectral intensity changes is difficult to achieve. The spectral intensity changes in the amide III region (1220–1290 cm$^{-1}$) as compared with the intensities in the 1290–1360 cm$^{-1}$ region can be used to distinguish between Raman spectra of normal skin and BCC. Quantitatively, in normal skin the ratio of the area under the curve between 1290 and 1360 cm$^{-1}$ to that between 1220 to 1290 cm$^{-1}$ was 0.67 (95% confidence intervals 0.63–0.70) whereas in BCC it was significantly higher, 1.37(1.21–1.54) (Table 1). The lack of overlap between confidence intervals indicates that these values can potentially be used for distinguishing between spectra of BCC and normal skin.

Significant differences are also found in the region from 800 to 1000 cm$^{-1}$. Specific assignments are more difficult to perform in this region. In normal skin a strong broad band is observed with a maximum around 950 cm$^{-1}$ and another band with two maxima around 850 and 870 cm$^{-1}$. Most probably the bands are due to single bond stretching vibrations as reported for the amino acids proline and valine\textsuperscript{35,36} and polysaccharides.\textsuperscript{37} For these bands a marked loss of intensity was observed in BCC (Fig. 1). A complete separation of the normal skin and BCC spectra was
Table 1. Comparison of Raman intensities from normal skin and basal cell carcinoma (means with 95% confidence intervals in parentheses)

<table>
<thead>
<tr>
<th>Area under the curves in region</th>
<th>Normal skin</th>
<th>Basal cell carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1290–1360)/(1230–1290) cm(^{-1})</td>
<td>0.29 (0.22–0.36)</td>
<td>1.37 (1.21–1.54)</td>
</tr>
<tr>
<td>900–990 cm(^{-1})</td>
<td>0.20 (0.17–0.22)</td>
<td>0.10 (0.08–0.12)</td>
</tr>
<tr>
<td>830–900 cm(^{-1})</td>
<td>1.16 (0.14–0.18)</td>
<td>0.07 (0.04–0.10)</td>
</tr>
</tbody>
</table>

*The ratio between the areas is given.

achieved after comparison of areas under the peaks of 900–990 and 830–900 cm\(^{-1}\) [0.20(0.17–0.22) and 0.16(0.14–0.18) in normal skin vs. 0.10(0.08–0.12) and 0.07(0.05–0.10) in BCC] (Table 1).

Lipid structure is represented by the \(\text{CH}_2\) scissoring vibration between 1420 and 1450 cm\(^{-1}\) and the \((\text{CH}_2)\text{--}\) in-phase twist vibration around 1300–1310 cm\(^{-1}\). Changes in Raman spectra in BCC samples were observed in both regions (Fig. 1).

The visual inspection and analysis of spectra is subjective and as such prone to bias. To analyse the spectra objectively we trained neural networks to capture differences between spectra from BCC and normal skin. The neural network system was able to differentiate between BCC and healthy skin in all 16 trials. Wavenumbers chosen objectively by the neural networks for spectral recognition are shown in Fig. 3. A single network did not use all of the wavenumbers marked in Fig. 3, which indicates that the BCC spectra differ from control skin spectra in several areas. Additionally, the random initialization of the networks caused the nets to choose differently between neighboring wavenumbers (Fig. 3). All wavenumbers, with the exception of amide I and the \(\text{CH}_2\) scissoring vibration, were found in the areas previously chosen by us. Thus, neural networks confirmed the existence of changes in protein, lipid and polysaccharide conformation in BCC. Moreover, several new positions of difference between normal skin and BCC were identified by neural networks: 1682, 1599, 1354–1358, 984, 905 and 832–834 cm\(^{-1}\). Further training of neural networks is necessary to establish whether the additional regions of interest have any value for the spectral diagnosis of BCC.

CONCLUSIONS

This work demonstrates that Raman spectroscopy can be used to distinguish between basal cell carcinoma and normal skin. The spectra of basal cell carcinomas were remarkably different from those obtained from normal skin, with spectral changes indicating alterations in protein and lipid structure. Our results and the evidence that other types of cancer have Raman spectra different from those of normal tissue counterparts support the concept that in the future Raman spectroscopy can be used for cancer diagnosis. This idea is especially attractive in dermatology, since skin cancer is exposed and easily accessible for in situ examination, without the necessity for invasive sampling. The development of fast detectors is necessary for the development of clinical Raman instruments and some important progress has recently occurred in this field. Our data suggest that the problems associated with the analysis of Raman spectra can be partially solved by using neural network modeling. In our series, neural networks were capable of complete separation between normal and skin cancer spectra. It must be emphasized, however, that our neural network data are of a preliminary character. Since the spectra of BCC and normal skin are different and therefore easy for neural networks to classify, the nets choose only a few wavenumbers for recognition of the spectra. Neural network retraining with a greater number of spectra and spectra of different types of skin tumors will include more wavenumbers. This will enable the neural network model to explore fully information carried in Raman spectra. It should be possible in future to diagnose not only basal cell carcinoma but also other skin tumors. Establishment of a spectral data bank will ease this task and allow for international cooperation in the field of skin cancer detection.

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Figure 3. Neural network analysis of spectra from BCC. Analysis was performed as described in the Experimental section. Wavenumbers used by the system for recognition of the spectra are marked with crosses.
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