Discrimination of Basal Cell Carcinoma and Melanoma from Normal Skin Biopsies *in Vitro* Through Raman Spectroscopy and Principal Component Analysis

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Abstract

Objective: Raman spectroscopy has been employed to discriminate between malignant (basal cell carcinoma [BCC] and melanoma [MEL]) and normal (N) skin tissues *in vitro*, aimed at developing a method for cancer diagnosis. **Background data:** Raman spectroscopy is an analytical tool that could be used to diagnose skin cancer rapidly and noninvasively. **Methods:** Skin biopsy fragments of $\sim 2 \text{ mm}^2$ from excisional surgeries were scanned through a Raman spectrometer (830 nm excitation wavelength, 50 to 200 mW of power, and 20 sec exposure time) coupled to a fiber optic Raman probe. Principal component analysis (PCA) and Euclidean distance were employed to develop a discrimination model to classify samples according to histopathology. In this model, we used a set of 145 spectra from N (30 spectra), BCC (96 spectra), and MEL (19 spectra) skin tissues. **Results:** We demonstrated that principal components (PCs) 1 to 4 accounted for 95.4% of all spectral variation. These PCs have been spectrally correlated to the biochemicals present in tissues, such as proteins, lipids, and melanin. The scores of PC2 and PC3 revealed statistically significant differences among N, BCC, and MEL (ANOVA, $p < 0.05$) and were used in the discrimination model. A total of 28 out of 30 spectra were correctly diagnosed as N, 93 out of 96 as BCC, and 13 out of 19 as MEL, with an overall accuracy of 92.4%. **Conclusions:** This discrimination model based on PCA and Euclidean distance could differentiate N from malignant (BCC and MEL) with high sensitivity and specificity.

Introduction

THE INCIDENCE AND MORTALITY RATES OF SKIN CANCER have increased dramatically in the last decade, and it has become the most common form of cancer in the Brazilian population. In 2012, the estimated incidence of non-melanoma skin cancer in Brazil was $\sim 134,000$ new cases.¹ Despite a permanent government campaign for people to protect themselves from sunlight, which would ultimately help to reduce the incidence rate, only the early detection of skin neoplasias, principally the elevated lethality melanoma (MEL), could help to decrease mortality rates associated with this disease.²

The challenge of modern medicine is to develop techniques that instantly investigate the morphological and biochemical changes in biological tissues and fluids and give early and potentially useful diagnostic information non-

invasively and nondestructively *in situ*. Vibrational spectroscopy, particularly Raman spectroscopy, has potential in the diagnosis and study of the evolution of human diseases and has been employed in several bio-tissue studies, including prostate, gastric, breast, and lung cancer, among others, both *in vitro* and *in vivo*.^{3–12}

Abnormal tissues have differences in constitution compared with normal ones, reflected in the observed Raman bands related mainly to proteins, lipids, and nucleic acids, which are mainly caused by differences in the neoplasia metabolism or changes in the cellular and subcellular structures and function.^{3,13–15} Those changes could be readily detected by Raman spectroscopy and, therefore, this technique has been considered a promising tool for the discrimination of changes from benign to malignant tissues in different pathologies *in vivo*.¹³ The use of near-infrared excitation (typically 785 or 830 nm from diode lasers) has an

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important advantage of decreased sample autofluorescence in biological specimens.¹⁶ The use of a fiberoptic Raman probe would provide the capability for specific molecular fingerprinting and analysis *in vivo*,^{10,12,13,17} and for detecting skin tissue alterations using higher wave numbers.¹⁸ Automated Raman systems for a rapid *in vivo* biological tissue evaluation in <1 sec have been recently proposed.^{12,17}

The spectral differences of non-melanoma and MEL lesions in skin tissues compared with normal ones (N) have been studied with Raman spectroscopy.^{18–26} The discrimination of MEL and/or basal cell carcinoma (BCC) from N *in vitro* and *in vivo* has been achieved through algorithms implemented using statistical^{18,23,25,27} and biochemical^{22–24,27,28} methods. Statistical methods include principal component analysis (PCA), which is a data reduction technique that can be used to group spectra with selected features according to differences in the pathology, through a suitable discrimination technique (such as linear discriminant analysis and Euclidean or Mahalanobis distances) applied to the most relevant principal components.¹³ Discrimination of BCC from normal skin fragments *in vitro* has been achieved with high sensitivity and specificity using PCA.^{22,27} Biochemical spectral models are based on the calculated (or estimated) concentration of the most relevant biochemicals presented in a particular tissue sample by ordinary least-squares fitting of the basal biochemical compounds to be estimated and the original tissue spectrum.^{3,13} Then, the tissue discrimination and disease grading could be done by comparing the alterations in these compounds that occurred in each tissue type with a suitable discrimination technique. Several studies proposed the elucidation of skin biochemistry aimed at cancer diagnosis.^{3,6,8,10,14,15,27}

The purpose of this work is to implement a diagnostic model to spectrally discriminate BCC and MEL from N using PCA and Euclidean distance applied to the Raman spectra of selected biopsy fragments obtained from resectional surgeries. Also, it is intended to correlate the spectral information carried by the first principal components vectors (PCs) to the main biochemicals responsible for the observed spectra of skin (actin, collagen, triolein, and melanin), with the aim of investigating how those bands appear in the PCA vectors and whether a discriminating model based on PCA is as sensitive as a biochemical model presented previously,²⁹ which would thus validate the PCA model as equivalent to a biochemical model based on pure compounds.

Material and Methods

This study followed the Brazilian guidelines for research with humans/human materials and was approved by the Council on Ethics in Human Research: Unicaselo. Spectra were the same ones as presented elsewhere.²⁹ Skin tissue fragments of ~2 mm were withdrawn from the center of resected lesions obtained from excisional biopsies, snap frozen, and stored in liquid nitrogen (–196°C) for the spectroscopic study. Samples were warmed to room temperature and kept moist with 0.9% saline solution before collecting the Raman spectra. We found no interference of the saline in the spectra of tissues.

For Raman spectra collection, we used a portable, near-infrared, dispersive Raman system (P-1 Raman system, Lambda Solutions, Inc. MA), with 830 nm excitation, ad-

justable laser power up to 350 mW, and spectral resolution of ~2 cm^{–1} in the range of 400–1800 cm^{–1}. The spectrometer was connected to a Raman probe (Vector probe, Lambda Solutions, Inc. MA) of ~3 m long, with band pass and rejection pass filters. The 1320 × 100-pixel, back-thinned, deep-depletion CCD was cooled to –75°C to decrease thermal noise. The Raman signal was collected in 5 sec, and 10 scans for all samples. Laser power was set to 200 mW for N and BCC samples and reduced to 50 mW for MEL samples, because of the high absorption of melanocytes.

For data collection, each tissue fragment was placed in a sample holder made of aluminum, and then the probe was placed at a distance of 10 mm, perpendicular to the tissue surface. The signal scattered by the samples was then collected by the probe and coupled to the signal port of the P-1 spectrometer for dispersion (spectrograph with 1200 grooves/mm grating). Gross spectra were then stored for preprocessing. Samples were measured under the same experimental conditions at the same day.

The procedures for spectra calibration (pixel-to-Raman shift correlation and spectrometer-spectral response correction) were performed using the software Matlab (Mathworks, Inc., MA, version 5.2), following the procedure described elsewhere.³⁰ The fluorescence background was removed by baseline correction function of commercially available software such as OriginPro (OriginLab Corp., MA, version 8.0). In this approach, straight lines are drawn in selected positions of the spectrum to create a baseline, and then these lines are subtracted from the gross spectrum. Cosmic rays spikes were removed manually. After calibration and preprocessing, spectra were normalized by the area under the curve and plotted in the spectral range of 400–1800 cm^{–1}.

Two to five spectra were collected in each fragment, in a total of 47 samples with the following diagnostics confirmed by histopathology: 15 as N, 29 as BCC, and 4 as MEL. We also collected spectra of keratosis, poroma, and fibrosis, which were withdrawn because of the small number of samples. Some spectra presented low signal-to-noise ratios and were not considered. A total of 30 spectra of N, 96 spectra of BCC, and 19 spectra of MEL tissues were considered for the PCA model.

Using the Raman spectra from N, BCC, and MEL samples, we developed a discrimination model based on the multivariate statistics PCA and Euclidean distance to classify skin tissue samples according to the histopathology in a retrospective analysis, in which the model is constructed and validated using the same data set. PCA has been applied with great success in the analysis of biological samples, being an important part of the spectral models, which detect alterations that occur after changes in the biochemistry of biological tissues.^{22,27,31–34} Within a set of spectral data, many different variations (such as differences in constituents, instrument variations, and sample handling) typically make up a particular spectrum. With several changes occurring at the same time, however, only a few independent variables account for all spectral differences, normally attributed to differences in the constitution of the samples. PCA extracts the relevant information from the original data (*A*) and generates two new variables, PCs and scores (*S*): $A_{m \times n} = PC_{m \times n} \cdot S_{n \times n}$, where *m* is the number of data points in the Raman shift wave number and *n* is the number of spectra of the model. The first PCs are

related to the most important variation of all spectra; the last ones carry only noise, and S are related to the weight of each PC to reconstruct the original data.³⁵

Because S is the intensity of each PC to form the original spectrum, and the PC carries spectral information in the form of peaks and valleys in positions related to the main Raman bands of tissue biochemistry, PCA could be used to help reveal tissue constitution and to discriminate and classify samples into well-defined categories according to the histopathology.

The Euclidean distance was employed as a discriminator to separate the data set into classes (N, BCC, and MEL) according to the histopathology, by determining the least linear distance from a specific point to the center (mean) of the class that is thought to belong, and comparing this distance to the distance to the vicinity group. The Euclidean distance d follows the expression,³⁶ $d(x, u) = [(x - u)^T \cdot (x - u)]^{1/2}$, where x is the vector of sample intensities (in our case, it is the principal component score S) and μ is the mean of the group. To choose which principal component would give the best classification, we employed ANOVA with a 5% significance level and chose the PC s with lower significance level. Then, we calculated the Euclidean distance to discriminate a sample of one class with respect to the remaining classes. Sensitivity, specificity, and overall accuracy were calculated for each score combination.

PC and S were obtained from all spectra using a routine written in Matlab with the non-linear iterative partial least squares (NIPALS)-PCA algorithm. The Euclidean distance was calculated using a routine under Matlab written by Paulo R. Galhanone and adapted to our problem.

Results

Figure 1 shows the mean Raman spectra of N, BCC, and MEL skin tissues in the fingerprint region. The Raman bands of skin can be assignment to proteins/amino acids, lipids/phospholipids, and nucleic acids; a more detailed band assignment can be found elsewhere.²⁹ Important spectral differences were found in the region of 800–1000 and 1200–1400 cm^{-1} , corresponding to bands assigned to nucleic acids, melanin, lipids, and (primarily) proteins.

The spectra of a normal skin fragment have features of proteins present in the dermis (mainly actin, collagen, and elastin) that can be identified by bands at 857, 939, 1004, 1248, 1271, 1452, and 1658 cm^{-1} , and several other weaker vibrations in the range 400–1000 cm^{-1} . The bands at 1063, 1128, the range 1270–1300, and at 1452 cm^{-1} could be attributed to the saturated fatty acid of ceramides from the epidermis and phospholipids sphingomyelin and phosphatidylcholine from the cell membrane. The band at 718 cm^{-1} could also be attributed to phospholipids. Spectral features arising from unsaturated lipids (mainly triolein from adipocytes) in the skin appears mainly at 1092, 1271, 1301, 1452, and 1658 cm^{-1} .

BCC skin has several spectral features at the same positions as the N, indicating similar biochemical constitution, with remarkable differences in the intensities at the 800–1000 and 1200–1400 cm^{-1} . Also, small peaks in the range from 600 to 1000 cm^{-1} and the peaks 1004, 1092, and 1128 cm^{-1} are of higher intensity in BCC, which would indicate higher lipid content. MEL spectrum is characterized by spectral features more likely related to BCC and a strong fluorescence background (not shown) because of the melanin. For this reason, laser power was reduced to 50 mW to avoid tissue burning.

To discriminate malignant from normal skin tissues, we developed a classification model based on PCA by using the principal component scores extracted from the Raman spectra data set and calculating the Euclidean distance from each sample to each group mean ("center of mass"). Figure 2 (left) shows the principal component vectors $PC1$ to $PC4$. These PC s are responsible for >95% of all spectral variations and could spectrally discriminate each tissue fragment. Figure 2 (right) also shows, for comparison, the spectra of selected tissue biochemical compounds (actin, collagen, triolein, and melanin). These compounds present Raman bands that were closely related to the spectral information carried by each PC vector. For example, $PC1$ is closely related to the spectra of proteins (mainly the collagen fibers from the connective tissue of the dermis and to some extent the actin from the cell's cytoskeleton), with characteristic peaks at 800–1000 cm^{-1} (C-C backbone stretching of proteins; proline/hydroxyproline/tyrosine), 1004 cm^{-1} (C-C stretching of

FIG. 1. Mean Raman spectra of normal skin (N), basal cell carcinoma (BCC), and melanoma (MEL) with vertical dotted lines labeling the peaks described by Silveira et al.²⁹ Laser power: 150 mW for N and BCC and 50 mW for MEL, wavelength: 830 nm, spectral resolution: 2 cm^{-1} , accumulation time: 20 sec. Spectra were offset for clarity.

FIG. 2. Left: plot of the four principal component vectors (PC1 to PC4); right: Raman spectra of the most relevant biochemicals of skin tissues from Silveira et al.²⁹

aromatic ring breathing mode of phenylalanine), 1250–1350 cm⁻¹ (C-N and N-H of amide III), 1452 cm⁻¹ (CH₂ and CH₃ deformations), and 1658 cm⁻¹ (C=O stretching of amide I). PC2 presents remarkable spectral features of triolein, with negative double peaks at 1271 and 1301 cm⁻¹ (CH₂ groups: twisting and wagging of methylic and carboxylic sided chains) and a negative peak in the region of 1000–1200 cm⁻¹ and melanin, with positive bands at 1342 cm⁻¹ (C-C stretching of aromatic ring and C-H bending) and 1613 cm⁻¹ (in-plane stretching of the aromatic ring) and some remnant peaks from proteins (in the range 800–1000

and at 1004 cm⁻¹). PC3 is well characterized by the bands from proteins (in the range 800–1000 and at 1004 cm⁻¹) and the bands in the range 1250–1350 cm⁻¹, with positive/negative peaks in this spectral region (amide III). PC4 has spectral features of small importance, mainly from lipids and/or proteins.

Figure 3 presents the mean *S* value of the first four principal component vectors. By applying ANOVA to pairs of *S* values (N×BCC, N×MEL, BCC×MEL), we found that S2 presented statistically significant differences between N and MEL and between BCC and MEL ($p < 0.05$), and that S3

FIG. 3. Plot of the mean and standard deviation of the first four principal components scores (S1–S4) for each tissue type to be used in the classification model. *Statistically significant differences among selected groups (ANOVA, $p < 0.01$).

FIG. 4. Scatter plot of the principal components scores S_2 versus S_3 . Separation of normal skin (N), basal cell carcinoma (BCC), and melanoma (MEL) was done by the mean Euclidean distance among groups (straight lines).

presented statistically significant differences between N and BCC and between N and MEL ($p < 0.05$), indicating that these two scores have the ability to discriminate tissues. Figure 4 shows the scatter plot of the $S_2 \times S_3$ values and the resulting discrimination model, after calculating and plotting the mean Euclidean distance from the data points of a group to the center of the other groups. Table 1 shows the sensitivity, specificity, and accuracy results for the discrimination model using these scores.

To verify the agreement of the PCA discrimination model with a representative tissue spectrum, Fig. 5 presents a plot of a N sample spectrum, the reconstructed spectrum (obtained through $\sum PC_n \cdot S_n$, where n is the number of principal components considered in the model), and the residual (by subtracting tissue spectrum and the reconstructed one). The excellent agreement observed indicates that these four components carry the spectral information relevant for tissue diagnosis.

Discussion

The biopsy of suspicious lesions followed by histopathological analysis is considered the gold standard for skin

FIG. 5. Plot of a sample Raman spectrum of normal tissue, the corresponding reconstructed modeled spectrum using the principal component analysis (PCA) variables and the residual spectrum.

cancer diagnosis when it is performed by well-trained specialists for differential diagnosis of neoplasias from common skin pigmented and unpigmented lesions. Up to 50% of early diagnosis of skin malignant lesions may escape detection during clinical routine examinations, whereas experts achieve 80–90% accuracy.¹⁹ A technique with the diagnostic capability to identify important biochemicals such as Raman spectroscopy would help to increase overall accuracy.

As mentioned, several previous reports have proposed the use of multivariate statistics such as PCA to classify biological tissues according to malignancy.^{22,27,31–34} In skin, our previous work revealed that PCA could successfully discriminate BCC from N with sensitivity and specificity of $\sim 96\%$ and 92% , respectively,²⁷ using Mahalanobis distance as a discriminator.

We found that the first four principal components explained $>95\%$ of all spectral variations, and the scores S_2 and S_3 have the capability to discriminate tissues with high accuracy, as demonstrated in Table 1. These first principal components presented spectral features related to tissue biochemistry,^{30,37} with peaks and valleys in the positions of the Raman bands of proteins, lipids, and melanin. This confirms our speculation that the principal components could reveal tissue biochemistry by revealing biochemical information observed in the spectrum of each tissue type.

The spectral differences among BCC and MEL compared with N revealed by PCA indicated differences in the amount of proteins, lipids, and melanin, which is in accordance with the recent literature.^{18,22–29,38} S_2 is capable of discriminating MEL from N and BCC, and S_3 is capable of discriminating N from BCC and MEL. Because PC_2 presented spectral information from melanin, it was expected that S_2 would help in estimating melanin content in melanomas and discriminating pigmented lesion from unpigmented one. Despite the presence of triolein bands in PC_2 , this biochemical was not relevant for the discrimination. PC_3 presented remarkable spectral features of proteins (mainly the amide III, the proline/hydroxyproline/tyrosine, and the phenylalanine ring bands), with which it is possible to distinguish the contribution from actin, which has a higher intensity at the 1004, 1318, and 1342 cm^{-1} bands and from collagen, which has higher intensity at the 1248 and 1271 cm^{-1} bands and the bands in the region of $800\text{--}1000\text{ cm}^{-1}$. S_3 showed N tissue

TABLE 1. RESULTS OF THE DISCRIMINATION MODEL IN TERMS OF ABSOLUTE VALUES AND SENSITIVITY, SPECIFICITY, AND ACCURACY

Histopathology	Discrimination based on PCA score and Euclidean distance		
	N	BCC	MEL
N (30)	28	0	2
BCC (96)	1	93	2
MEL (19)	0	6	13
Sensitivity	99.1% ^a ; 68.4% ^b		
Specificity	93.3% ^a ; 97.9% ^b		
Overall Accuracy	92.4% ^c		

^aConsidering malignancy both neoplasias: BCC+MEL compared to N.

^bConsidering the differential diagnosis between BCC and MEL.

^cFor all discrimination groups.

PCA, principal component analysis; N, normal skin; BCC, basal cell carcinoma; MEL, melanoma.

with positive scores, which could indicate higher collagen contents because collagen bands are positive in PC3, and S3 showed BCC tissues with negative scores, which would suggest greater actin content, because PC3 has negative features in the 1004, 1318, and 1342 cm^{-1} bands (same as actin) and S3 is negative for BCC. Biochemical studies have indicated that actin expression plays a role in the organization and growth of carcinoma cells³⁹ and has been proposed as a marker of invasiveness in BCC,⁴⁰ increasing the contribution of actin bands to the BCC spectra. In terms of collagen, certain growing tumor cells release collagenase that destroys native collagen fibers,³⁵ and reduces the extracellular matrix,³ reducing the contribution of collagen bands to the BCC spectra. This indicates a change in the molecular composition of tissue proteins, as suggested by Gniadecka et al.²⁸

This result is comparable to other studies in which the spectra of basal biochemicals were used to correlate the differences in the spectra presented by neoplasias. In a recent study, we developed a biochemical model to discriminate skin tissues and found changes in the amount of proteins in BCC and MEL compared with N tissues: collagen decreased and elastin and actin increased for BCC.²⁹ Stone et al.³ found an increase in the amount of actin and a decrease in the collagen in urological carcinoma lesions.

The main advantage of a discrimination model based on multivariate statistics such as PCA is that no information about sample constitution is needed to develop the model and subsequently discriminate the samples among the selected groups. The main disadvantage is that, because of its statistical nature, one needs a representative data set (sometimes enormous) and the correct information about which group each sample belongs to, and if a new sample needs to be added to the model, the principal components must be recalculated. Also, the use of a retrospective data analysis is limited by the fact that the model is constructed and validated using the same data set, and any information arising from an instrument- or environment-dependent variable that is not present in the prospective data set could corrupt the classification model.

Raman spectroscopy has the potential to become a technique for biochemical analysis of skin cancer biopsies, or even in real time, *in vivo* and nondestructively with fiberoptic Raman probes, differentially diagnosing BCC from MEL using PCA and Euclidean distance. Studies are under way in order to automate data collection and evaluate the performance of the model at suspicious skin tissues *in vivo*, as it provides a possible local, clinical diagnosis with rapid and reliable results during examinations or even margin detection during surgery.

Conclusions

PCA and Euclidean distance were successfully employed to discriminate Raman spectra of skin fragments of BCC and MEL from N *in vitro*, with a sensitivity, specificity, and accuracy of 99.1%, 93.3%, and 92.4%, respectively, for diagnosis of malignant tissues compared with normal tissues. Principal components vectors revealed spectral information useful to unveil differences in tissue biochemistry related to pathological conditions.

Acknowledgments

Landulfo Silveira Jr., Renato Amaro Zângaro, and Marcos Tadeu T. Pacheco acknowledge CNPq for the Productivity Fellowship. This work was supported by São Paulo Research Foundation - FAPESP (Grant 2009/01788-5).

Author Disclosure Statement

No competing financial interests exist.

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