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Label-free Infrared spectroscopic imaging for characterization of necrotic tissue areas on cutaneous squamous cell carcinoma

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Abstract

In this study, FTIR spectroscopy was used to evaluate the overall biochemical status of necrotic tissue areas of cutaneous squamous cell carcinoma chemically-induced on mice. FTIR hyperspectral image collected from specimen showed high correlation with the photomicrograph obtained by light microscopy, in which we were able to identify clusters associated to keratin, necrosis and regions with no tissue. Alterations in the protein content were documented in the necrotic tissue areas, indicating changes on protein conformation.

Keywords: FTIR microspectroscopy, cutaneous squamous cell carcinoma, death mechanisms, necrosis, KMCA

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1. INTRODUCTION

Fourier Transform Infrared (FTIR) spectroscopy is a label-free analytical technique able to provide information about the common molecular units of tissue samples (carbohydrates, proteins, lipids and nucleic acids) based on its chemical vibrations that are infrared active [1]. In the last years, many proof-of-concept studies have been performed using the spectral signatures measured by FTIR spectroscopy for the diagnosis of cancerous and non-cancerous diseases as well as to monitor the effects of drug treatments on disease progression/regression [2-6]. However, few studies have focused on evaluate the spectrochemical signatures of necrotic cell death. Necrosis is a nonspecific mode of cell death induced by different factors that lead to the irreversible cell injury such as mechanical injury, infections, hypoxia, and a variety of toxic chemicals. Cell death caused by necrosis is accompanied by ion and water efflux and dispersal of organelles, resulting in a rupture of cytoplasmic and nuclear membranes, the swelling of mitochondria, and the flocculation of chromatin [7]. The techniques routinely used to study the cellular and molecular mechanisms triggered by necrosis may include histological examination of tissue biopsies, immunohistochemistry (IHC), in-situ hybridization (ISH), polymerase chain reaction (PCR), etc. These methods are laborious, time-consuming and require multiple assays/staining. Thus, the present study aims to evaluate the spectrochemical signatures of necrotic tissue areas on cutaneous squamous cell carcinoma in order to develop FTIR microspectroscopy as a new optical diagnostic tool to support conventional lightscopical and molecular techniques to understand the mechanisms associated to necrotic cell death.

2. MATERIAL AND METHODS

2.1 Chemical carcinogenesis

After approval by the Committee on Animal Research and Ethics (164/15-CEUA-IPEN/SP), female Swiss mice aged up to 10 weeks and body mass about 25 g were submitted to a well-established two-stage chemical carcinogenesis protocol. In the first phase (induction), the shaved backs of the animals were submitted to a single dose of 7,12-dimethyl-benzanthracene (DMBA, CAS number 57-59-6, Merck KGaA, Darmstadt, Germany) (50 µg diluted in 200 µl of acetone). After one week, the second phase of the protocol was initiated (promotion), in which the animals underwent twice a week to applications of 12-O-tetradecanoyl-phorbol-13 acetate (TPA, CAS number 16561-29-8, Merck KGaA, Darmstadt, Germany) (5 µg diluted in 200 µL acetone) during 12 months [8]. In the first phase of the

experiment, animals were submitted to general anesthesia using ketamine (0.001 ml/g) and xylazine (0.0005 ml/g) intraperitoneally applied. The whole procedure for the application of the carcinogens were performed in a laminar flow to ensure the safety of the manipulator. Animals were euthanized at the end of experiment via overdose of anesthetics and the cutaneous tissues were extracted with a scalpel.

2.2 Sample preparation

Specimens extracted from animals were formalin-fixed for 24 hours, diaphanized in two baths of pure xylol for 30 minutes, dehydrated in ethanol baths with increasing concentrations (50, 70 and 100%) and paraffin-embedded (FFPE). Two adjacent histological slices (5 and 10 μm thickness each) were obtained using a microtome. The first slice (5 μm thickness) was prepared in a conventional glass slide, hematoxylin and eosin (H&E) stained according to the international standards and coverslipped. The second slice (10 μm thickness) was placed on calcium fluoride (CaF_2) substrates (Crystran Ltda., Poole, Dorset, UK). No chemical dewaxing or chemical staining was performed on the second slice and it was not coverslipped. Specimens H&E stained were examined by a pathologist.

2.3 FTIR spectroscopy

Data acquisition

FTIR hyperspectral images were acquired in transmission mode using a Spotlight 400N FTIR imaging system (Perkin Elmer, Waltham, MA, USA) equipped with an AutoImage microscope system operating with a $\times 40$ Cassegrain objective. Spectral measurements were acquired over the spectral range $750\text{--}4000\text{ cm}^{-1}$ with a pixel size of $6.25\text{ }\mu\text{m} \times 6.25\text{ }\mu\text{m}$ at a spectral resolution of 4 cm^{-1} . Background measurements were acquired on a region with no tissue with 120 scans per pixel whereas 8 scans per pixel were recorded from the sample.

Data pre-processing and Spectral analysis

FTIR spectra were vector normalised and smoothed with Savitzky-Golay filter using a polynomial of second order in an eleven-point window. RMie-EMSC algorithm was applied to the images aiming to correct dispersive effects induced by resonant Mie scattering. FTIR images were segmented/reconstructed via K-means clustering analysis (KMCA) using the spectral region over the range $1000\text{--}1800\text{ cm}^{-1}$. The reconstructed image were compared to photomicrographs acquired from H&E stained specimens in order to identify the clusters associated to necrotic areas. All pre-processing steps and spectral analysis were performed using Matlab® R2015 (MathWorks, Natick, MA, USA).

3. RESULTS

Figure 1 depicts a photomicrograph obtained by light microscopy from a histological section of a specimen collected from a mouse submitted to 12 months of chemical carcinogenesis. The lesion was classified as invasive squamous cell carcinoma based on the microscopic histological aspects observed. The specimen depicts intense epithelial hyperplasia with solid pattern of invasiveness (arrow), poor connective tissue with intense inflammation (arrow), extensive areas of necrotic tissue (*) and hyperkeratinization (pentagram).

FIGURE 1. Representative histopathology aspect presented by histological specimen stained by hematoxylin and eosin (100X original magnification). (*): Necrotic tissue. (pentagram): Hyperkeratinization. (Arrow): Intense inflammation and epithelial hyperplasia.

A FTIR hyperspectral image was collected from a specific region of the histological specimen exhibiting intense keratinization and keratin pearls surrounded by extensive area of necrosis (Figure 2.A) in order to evaluate the spectroscopic profile of necrotic tissue. Figure 2.B and C show the chemical map and the reconstructed image obtained using KMCA (4 clusters) as segmentation algorithm.

FIGURE 2. **A:** Representative histopathology aspect presented by histological specimen stained by hematoxylin and eosin (100X original magnification). **B:** Chemical map. **C:** Hyperspectral image reconstructed using K-means cluster analysis

At a first glance, the FTIR image and the photomicrograph obtained by light microscopy are highly correlated. The necrotic area is depicted by blue pixels (cluster 4), keratin regions are represented by green pixels (cluster 3), regions of no tissue are colored in black (cluster 2) and grey pixels represent regions of mixed keratin and necrosis (cluster 1). Figure 3 shows the averaged second derivative obtained for each cluster.

FIGURE 3. Fingerprint region (1000-1800 cm^{-1}) of averaged second derivatives of each cluster obtained by KMCA.

Changes on vibrational modes over 1000-1400 cm^{-1} (carbohydrates, fibrous structure of proteins and nucleic acids) were not observed. Spectral region over 1500-1800 cm^{-1} comprises vibrations associated to protein content (Amide II: 1550 cm^{-1} and the sub-bands of Amide I: α -helix, 1660-64 cm^{-1} ; β -sheet, 1633-39 and random coils/turns: 1681-83 cm^{-1} [9]. Significant alterations were identified in the protein content region. Spectral signatures associated to secondary structure of proteins as well as to Amide II presented increased absorption intensities in the necrotic tissue areas as well as shifts on band peak position, indicating changes on protein conformation.

4. CONCLUSIONS

In this study, FTIR spectroscopy was used to evaluate the overall biochemical status of necrotic tissue areas of cutaneous squamous cell carcinoma chemically-induced on mice. FTIR hyperspectral image collected from specimen showed high correlation with the photomicrograph obtained by light microscopy, in which we were able to identify clusters associated to keratin, necrosis and regions with no tissue. Alterations in the protein content were documented in the necrotic tissue areas, indicating changes on protein conformation.

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