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Raman micro-spectroscopy for rapid screening of oral squamous cell carcinoma

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ABSTRACT

Raman spectroscopy can provide a molecular-level fingerprint of the biochemical composition and structure of cells with excellent spatial resolution and could be useful to monitor changes in composition for dysplasia and early, non-invasive cancer diagnosis (carcinoma in situ), both ex-vivo and in vivo. In this study, we demonstrate this potential by collecting Raman spectra of the nucleoli, nuclei and cytoplasm from oral epithelial cancer (SCC-4) and dysplastic (pre-cancerous, DOK) cell lines and from normal oral epithelial primary cell cultures, in vitro, which were then analysed by principal component analysis (PCA) as a multivariate statistical method to discriminate the spectra. Results show significant discrimination between cancer and normal cell lines. Furthermore, the dysplastic and cancer cell lines could be discriminated based on the spectral profiles of the cytoplasmic regions. The principal component loading plot, which elucidates the biochemical features responsible for the discrimination, showed significant contributions of nucleic acid and proteins for nucleolar and nuclear sites and variation in features of lipids for the cytoplasmic area. This technique may provide a rapid screening method and have potential use in the diagnosis of dysplasia and early, non-invasive oral cancer, the treatment of which involves much less extensive and complex surgery and a reduction in associated co-morbidity for the patient.

1. Introduction

Oral cancer is one of the more common cancers worldwide, and in non-developed countries is next in prevalence to skin and breast cancers. Tumours are primarily located around the tongue, floor of the mouth, gingiva and buccal mucosa (Cabral et al., 2010; Kelly et al., 2014). Although clinical diagnosis of late stage, invasive oral cancer is relatively straightforward, the clinical challenge remains to detect early cancerous lesions, which are significantly harder to identify. Surgical treatment of early oral squamous cell carcinoma involves much less extensive and complex surgery and leads to a significant reduction in co-morbidity. Currently, the gold standard for diagnosis is histological analysis of tissue biopsies. However, numerous studies are being conducted in order to improve histopathological techniques with the ultimate goal to obtain a non-invasive, high throughput detection method for screening suspect cancer cells (Peer, 2014).

In this context, optically-based spectroscopic techniques are prominent among potential candidate non-invasive and rapid diagnostic methods. These methods are widely used as a tool for analysis of many biological tissues and the technique has been referred to as an "optical biopsy" (Carvalho et al., 2010; Evers et al., 2012; Kallaway et al., 2013; Nawaz et al., 2011) or "spectral cytopathology" (Schubert et al., 2010) because of its capacity to show features of underlying pathological tissues when compared with normal samples. Raman spectroscopy is one of the most popular techniques with demonstrated potential in studies examining different types of cancer, including lung cancer (Wang et al., 2014), neural cancer (Tanahashi et al., 2014), breast cancer (Damayanti et al., 2013; Marro et al., 2014) cervical cancer (Lyng et al., 2007), gastric cancer (Luo et al., 2013), skin cancer (Mittal et al., 2013) and head and neck cancer (Singh et al., 2012).

The power of the method is primarily related to the molecular and biochemical signature it provides of the biological sample when the fingerprint region is analysed. The Raman effect is a result of inelastic scattering of monochromatic electromagnetic radiation due to interaction with molecular vibrations, such that the frequency of the scattered radiation is different from the incident radiation (Raman and Krishnan,

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1928). The Raman spectrum of a given molecule consists of a series of peaks or bands, each corresponding to a characteristic vibrational mode of that molecule. Each molecule exhibits a characteristic spectrum, and thus the Raman spectrum can provide a "fingerprint" of a substance from which the molecular composition can be determined (Lyng et al., 2007; Nawaz et al., 2011). Changes in molecular composition due to the presence of disease are often subtle, requiring the use of multivariate statistical analysis (Byrne et al., 2014). In this way, Raman spectroscopy has been demonstrated to elucidate biochemical signatures not only from cancer studies but from other different pathological conditions in tissues and cells. Carvalho et al. (2010), using Raman spectroscopy to discriminate buccal inflammatory processes and normal tissues, revealed that the discrimination is possible using Soft Independent Modelling of Class Analogy (SIMCA) methods of analysis.

Although there are increased numbers of studies involving Raman spectroscopy as an optical biopsy method, as the technique is based on optical microscopy, it can also be extended to the analysis of cytological samples at a cellular and subcellular level (Bonnier et al., 2010b). Thus, interactions of, for example, anticancer agents and nanoparticles can be explored at a subcellular level (Dorney et al., 2012; Nawaz et al., 2011). Of clinical relevance, Raman spectroscopy has been explored for routine cytological screening for cervical cancer (Bonnier et al., 2014; Vargis et al., 2012). However, only a few studies have been published on oral squamous cell carcinoma and Raman microspectroscopy (Guze et al., 2011; Lasalvia et al., 2015; Su et al., 2012). The present study demonstrates the potential of Raman spectroscopy to provide and differentiate the biochemical signature from subcellular regions of oral cell lines, such as the cytoplasm, nucleus and nucleolus, in a label free manner. Also, we illustrate the potential of the technique to discriminate between cancer, pre-cancerous dysplastic cells and normal oral cell lines.

2. Materials and methods

2.1. Oral cell lines

To determine the efficacy of Raman spectroscopy in distinguishing between the cellular states observed during carcinogenic transformation, three different types of oral cell lines namely SCC-4 (malignant cell line), DOK (dysplastic cell line) and primary cells (normal oral epithelial cell line) were utilised. It is important to note that both pathological cell lines were originally from tongue, one of the sites of highest incidence of oral squamous cell carcinoma.

2.1.1. SCC-4 and DOK cell cultures

The SCC-4 and DOK (HPA cultures, UK) cell lines were cultured in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FBS, penicillin/streptomycin (100 U/100 μg) and L-glutamine (2 mM). For the DOK cell line, hydrocortisone (5 μg/ml) was also added. The medium was pre-warmed at 37 °C before incubation of the cells. Cells were cultured until 90% confluency in a humidified environment at 5% CO_2, before being passaged. When confluent, the cells were washed in pre-warmed phosphate buffered saline (PBS) (0.01 M phosphate buffer, 0.154 M sodium chloride) and incubated with trypsin–EDTA (0.5% trypsin, 0.02% EDTA) for 5 min at 37 °C. Fresh, pre-warmed medium was added to deactivate trypsin, and the suspended cells were centrifuged at 250 *g* for 5 min. The supernatant was discarded and the cell pellet was resuspended in a sufficient volume of fresh pre-warmed medium, counted and passaged at a ratio of 1:3.

2.1.2. Primary cell culture

Human oral mucosa was recovered at the Dublin Dental University Hospital from patients undergoing routine third molar extraction in the Department of Oral and Maxillofacial Surgery. The sample was immediately placed into pre-warmed collection medium [DMEM, penicillin/streptomycin (100 U/100 μg), amphotericin B (2.5 μg/ml)] for 10 min before washing the tissue three times with pre-warmed 1× PBS and placing it in 0.17% trypsin overnight at 4 °C. The following day, the sample was washed with 1× PBS and the connective tissue was removed using a scalpel. The tissue was cut into small pieces (1 mm × 2 mm) and the small sections were placed in pre-treated T25 flasks (CELL+, Sarstedt), each with a small coating of keratinocyte growth medium (KGM). These were left to adhere to the flask for 1–2 h and the flask was subsequently flooded with KGM. Once sufficient growth of cells from tissue was achieved (2–3 weeks), KGM was replaced with Epilife medium (Invitrogen) to select for epithelial cell growth. Cells were passaged using 0.05% trypsin and spun at 250 *g* for 10 min at 4 °C and cultured to 90% confluency.

2.1.3. Sample preparation

To facilitate Raman spectroscopy measurements, cells were detached from the flasks using 0.025% trypsin–EDTA at 37 °C and pelleted at 250 *g* for 5 min at room temperature. The supernatant was removed and cells were counted and seeded at a density of 5×10^4 cells/calcium fluoride (CaF_2) disc in a multiwell plate and maintained, as previously described, until a monolayer of cells was stably growing on the disc. The cells were then fixed with 10% neutral buffered formaldehyde for 5 min, washed with 1× PBS and stored in 0.9% physiological saline solution prior to capture of the Raman spectrum.

2.2. Raman spectroscopy measurements

The study was conducted with a Horiba Jobin-Yvon LabRam HR800 instrument using a 532 nm laser as the source in a backscattering geometry, and a 300 lines/mm grating, providing a dispersion of ~1.5 cm^{-1} per pixel. The laser power was approximately 35 mW at the sample. Spectra were taken in the range from 600 cm^{-1} to 1800 cm^{-1} with a confocal hole diameter of 100 μm. A 100× water immersion objective (LUMPlanF1, Olympus, N.A.: 1.0) was used to focus the laser on the sample, immersed in distilled water, providing a spatial resolution of ~1 μm (Bonnier et al., 2010a, 2010b; Bonnier et al., 2012; Fullwood et al., 2014). Water immersion has been demonstrated to reduce any photothermal damage to the cells during measurement, and the signals were observed to be stable and reproducible (Bonnier et al., 2012). For each cell line, 20 cells were analysed. For each cell, three different subcellular regions were analysed, resulting in one spectrum for each subcellular region of each cell. Thus, for each cell line, 20 nucleolar, 20 nuclear and 20 cytoplasmic spectra were recorded, each for a period of 2 × 20 s.

2.3. Data preprocessing

Data preprocessing was performed using Matlab (Mathworks, USA). Before statistical analysis, a Savitsky–Golay filter (5th order, 7 points) was applied to smooth the spectra. As it has been demonstrated that, in water immersion, the background to the Raman spectrum is simply the spectrum of the overlying water (Bonnier et al., 2010a), and no background was subtracted. All spectra were (vector) normalized to remove point to point intensity variations and facilitate comparison of all spectra.

2.4. Data analysis

Principal component analysis (PCA) is a method of multivariate analysis broadly used with datasets of multiple dimensions (Varmuza, 2009; Laurikkala and Juhola, 2007; German et al., 2006). It allows the reduction of the number of variables in a multidimensional dataset, although it retains most of the variation within the dataset. The order of the principal components (PCs) denotes their importance to the dataset. PC1 describes the highest amount of variation, PC2 the second highest, and so on (Kelly et al., 2011; Martin et al., 2007). A PCA scatter plot groups similar datasets (spectra) according to the loadings of the PCs