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Raman Spectral Mapping in the Assessment of Axillary Lymph Nodes in Breast Cancer

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This paper is the first reported description of Raman Spectroscopy in the assessment of axillary lymph nodes in breast cancer patients. Raman Spectroscopy is an inelastic scattering spectroscopic technique appropriate for the assessment of unprocessed complex biological tissues. Spectra represent biochemical signatures of the tissue under scrutiny. The described method of Raman spectral mapping produces false-color spectral images of lymph node sections. These can be compared with standard histopathology slides and white light images of nodal tissue. This method has the potential to allow the detailed biochemical assessment of heterogeneous lymph node features, and to contribute towards the development of an optical diagnostic tool for use in a clinical setting.

Key words: Raman spectroscopy, Breast cancer, Axillary lymph nodes.

Introduction

Breast cancer kills 13,000 women every year in the United Kingdom and 44,000 in the United States of America. Many more survive months of investigation and treatment for the disease. Accurate staging of a newly diagnosed breast cancer is essential in assessing a patient's prognosis and in the planning of further treatment. In breast cancer, disease staging includes the assessment of the lymph nodes in the ipsilateral axilla. Lymph node metastasis is a predictor of local disease recurrence and lower survival rates. Diagnosis of lymph node involvement initiates management options including extensive dissection of axillary lymph nodes, chemotherapy and occasionally radiotherapy.

At present, few routine diagnostic techniques are effective in the diagnosis of lymph node metastases. Preoperative imaging modalities such as mammography, ultrasound imaging and lymphoscintigraphy do not reliably diagnose axillary lymph node metastases. Assessment of the axilla is usually substantiated during the operative management of the breast lesion with lymph node sampling or clearance, and more recently sentinel lymph node biopsy. Assessment of node pathology at this point is a focus of much debate and research.

Gold standard histopathology techniques include formalin fixation, wax embedding and microscopic examination of a varying number of sections from each node. This process takes several days to complete. However, intraoperative diagnosis has become increasingly important with the introduction of sentinel lymph node biopsy. Alternative histopathology methods can provide information within the time frame of an operative procedure, including frozen section assessment and imprint cytology. Frozen sections can pose diagnostic difficulties for the histopathologist as tissue section quality is variable. Frozen section

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processing results in a delay of approximately 30-60 minutes and requires availability of a histolopathologist. Imprint cytology is employed in some centers for intraoperative assessment of lymph node status. Two recent series assessed the value of imprint cytology, and reported sensitivity for positive node detection as low as 72% (1) and as high as 92%, with delay to diagnosis of 30 to 50 minutes (2).

There are several concerns with current routine histopathology process in axillary lymph node assessment. Most histopathology laboratories do not have the human resources to examine every section from every node. It is accepted that many micrometastases to nodes may be missed. Research has shown that more exhaustive histology assessment of revisited axillary nodal tissue results in 7 % to 33% of negative nodes reclassified as positive for breast carcinoma metastases (3).

Immunohistochemistry is available in many pathology laboratories as an additional tissue technique in axillary lymph node assessment. This method commonly employs antibodies to proteins unique to epithelial origin tissues, such as antikeratin and antimucin antibodies. Carcinoma cells are thus stained as distinct from the hematopoietic cells of lymph nodes. Immunohistochemistry can improve the detection of cancer with between 14%and 30% of negative nodes reclassified as positive (3). This technique cannot provide a diagnosis within the timeframe of a breast cancer operation.

Reverse transcriptase-polymerase chain reaction (RT-PCR) is an alternative technique explored to differentiate carcinoma from normal lymph node. However, this method is limited by the specificity of the tumor marker used. RT-PCR also requires blending of tissue for complete results, and so can only report on overall nodal status, not on macro- and micrometastases. Following RT-PCR, tissue is not available for validation with routine histology assessment.

The limitations of the techniques described may have consequences for both clinician and patient. If the axillary dissection is limited to the sampling of 4 nodes at primary surgery, and the nodes are found to be positive, the usual management would be a second operation to complete lymph node clearance. The lack of an intraoperative diagnosis of lymph node status may result in unnecessary removal of nodes. Extensive dissection of the axilla increases the likelihood of arm lymphedema, a potentially debilitating complication. The incidence and prognostic significance of axillary lymph node micrometastases is uncertain at present, but 2 large studies suggest a higher rate of recurrence and a lower 5 to 7 year survival rate in patients with 1 or more axillary node micrometastases when compared to matched patients with negative nodes (4, 5).

Various laser and spectroscopic techniques have been used to assess lymph node characteristics. Laser microscopy in the form of multi-photon fluorescence microscopy has been used to assess lymphocyte motility within lymph nodes (6). Nuclear Magnetic Resonance (NMR) spectroscopy has been applied to axillary lymph nodes in rat models and in humans with breast cancer. Differences in concentrations of choline-containing compounds, cholesterol and glycogen have formed the basis of the NMR spectroscopy diagnostic models (7, 8). Elastic scattering spectroscopy is currently being investigated as a portable device for intraoperative use. Researchers at University College London have reported some success in the identification of axillary lymph nodes invaded with metastatic breast cancer. However, this group reports limitations in the assessment of nodes containing a heterogeneous pattern of metastatic infiltration (9, 10). Raman spectroscopy has been employed in the identification of silicone inclusions in lymph nodes of patients with ruptured silicone breast prostheses (11). Silicone has a specific and strong Raman signal, and is used in a laboratory setting as a calibration tool. However, Raman Spectroscopy has not yet been

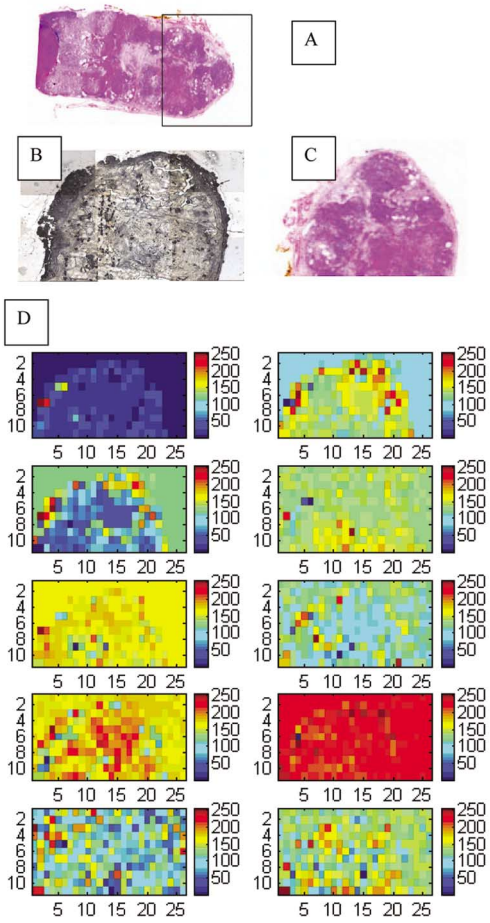

Figure 1: Axillary lymph nodes containing metastatic breast cancer. **A)** H and E slide showing lymph node section with heterogeneous infiltration with metastatic ductal carcinoma. **B)** White light image of tissue section for Raman processing. **C)** Parallel end of node section stained for histology (H and E). **D)** Raman spectral maps of the node section by principal component.

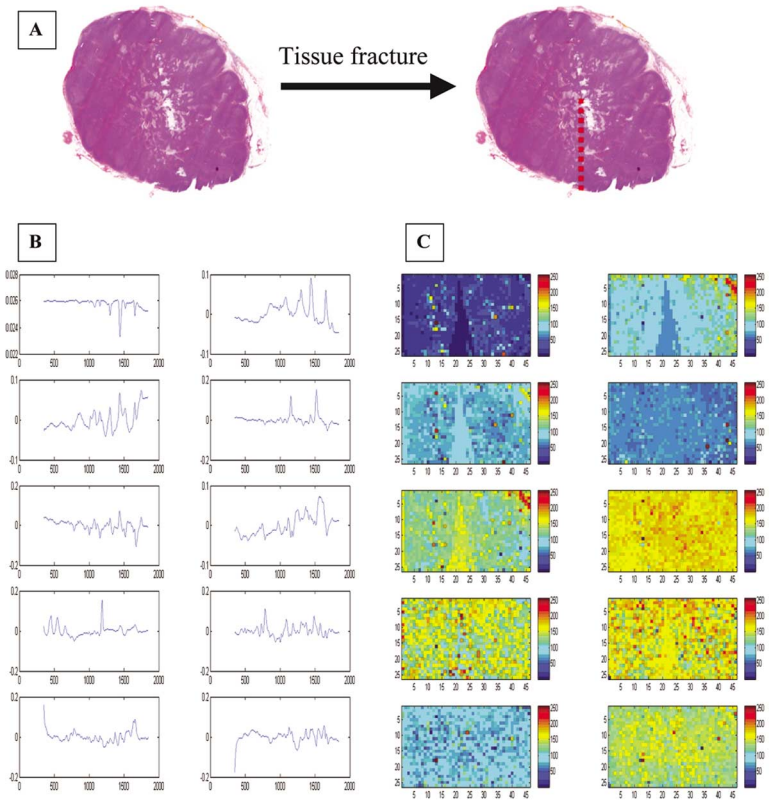

Figure 2: Axillary lymph node without metastases from a breast cancer patient. **A)** Histopathology slide section (H and E stained) showing position of tissue fracture evident on Raman spectral maps. **B)** Principal component spectra after Raman mapping of immediately parallel section of node. **C)** Raman spectral maps created by application of false color to principal component in each spot (pixel).

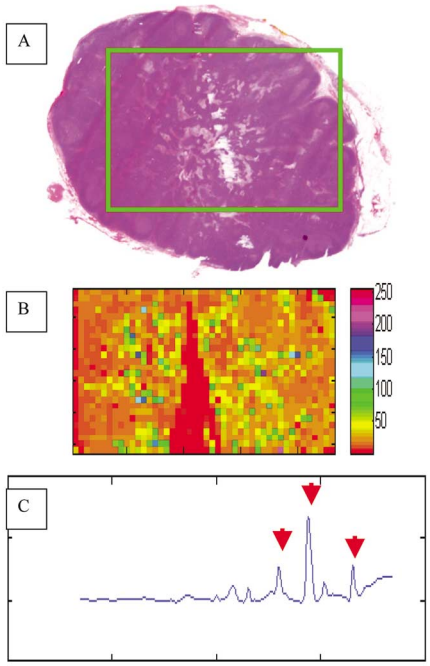

Figure 3: Axillary lymph node without metastases. **A)** H and E section; green box indicates approximate area Raman mapped. **B)** Raman spectral map of principal component 1: Histiocyte channels visible in central portion of node in A are suggested by yellow/green pixels of false color weighting. The red triangle is a fracture in the tissue section. **C)** Principal component 1 spectrum (flipped): Peaks indicated with red arrows are lipid peaks at 1300 cm^{-1}, 1440 cm^{-1} and 1654 cm^{-1}. False color for these "flipped troughs" indicates lipids in higher intensities compared to pericapsular regions.

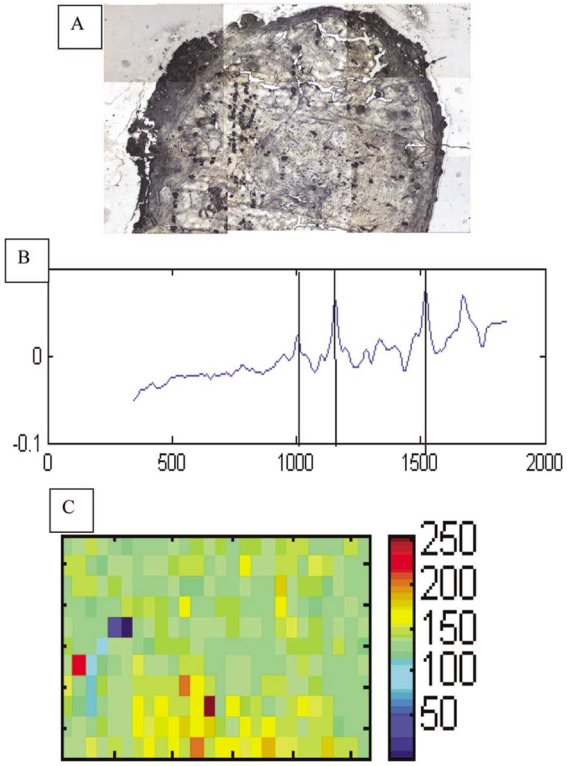

Figure 4: Lymph node with metastases. **A)** White light image. **B)** Spectrum of principal component 4. Carotenoid peaks are shown 1005, 1156, 1519 cm^{-1} **C)** Raman spectral map of principal component 4 showing increased intensity from carotenoids in the central area of section.

reported in the assessment of malignant spread of breast cancer to lymph nodes.

Background to Raman Spectroscopy

Raman spectroscopy is an optical technique employing the inelastic scattering of light to interrogate the biochemical states of molecular bonds. The technique has a proven application in the optical assessment of complex biological tissues. A measured incident photon beam is directed at a tissue sample. Dependent on vibrational and rotational modes of biochemical bonds and atomic nuclei in tissue molecules, incident photons interact with the tissue in highly specific ways. The intensity and wavelength of scattered light is detected. Incident and emitted photons are compared and the difference describes the Raman shift. Raman spectra are therefore specific biomolecular signatures of the scrutinized tissue samples. The intensity of spectral peaks changes in linear proportion to varying concentrations of molecules.

In contrast, histopathological tissue assessment is based on microscopic features of stained tissue architecture and cell components. In the development of cancer, specific histological stages may be identified, ranging from cellular atypias to carcinoma *in situ* to invasive cancer. Histological features of malignant progression are a result of biomolecular changes in cell nuclei, cytoplasm and membrane. Raman spectroscopy has the capability to distinguish between tissues classified as benign and malignant by the current gold standard of histopathology. This diagnostic potential has been demonstrated in many organs including the esophagus, prostate, bladder, cervix and breast (12, 13, 14). The potential of the technique to identify biochemical changes in tissue may allow detection of malignant change before histological features are present.

The practical characteristics of Raman spectroscopy make it a very suitable tool for rapid diagnosis in the clinical setting. Tissue does not require preparation or staining. Unlike some other optical techniques, water does not have significant representation in Raman spectra. Therefore, the high water content of tissue does not obscure results, and sample desiccation is unnecessary. The technique does not result in significant tissue change suggesting that *in vivo* use is safe. Rapid spectroscopic acquisition time coupled with appropriate diagnostic software could allow immediate assessment of lymph nodes in the clinic or operating room.

This study is the first description of Near-infrared Raman Spectroscopy in the assessment of axillary lymph nodes from breast cancer patients. The emphasis of this article is to describe a specific Raman spectral imaging technique. This forms part of ongoing research into the development of Raman Spectroscopy as a real-time tool in the diagnosis and management of breast cancer.

Materials and Methods

With approval from the Gloucestershire Research Ethics Committee, and with fully informed consent from patients, tissue was collected after surgical resection for breast cancer (axillary node clearance and axillary node sampling). A portion of one axillary lymph node from each appropriate case was collected. Tissue was snap frozen on acetate paper and placed in cryovials immediately after collection to maintain biochemical condition. Frozen tissue was cut on a cold cryotome providing a 7μm section on a calcium fluoride slide for Raman Spectroscopy. The remaining sample was formalin-fixed and wax embedded, then an immediately adjacent section was cut and hematoxylin and eosin (H and E) stained for comparative histology. Slides were reported by a Consultant Breast Histopathologist with the operative main specimen to avoid late alterations to patient prognostic score.

Nodal sections on calcium fluoride were defrosted then processed using a commercially available Renishaw Raman System 1000® spectrometer with an electronic stage: 830 ηm laser light was directed onto samples from a diode laser source. Spectra were collected in an automated spatial scanning mode in raster pattern with a step size of 100 μm in both X and Y directions. At each spot, 3 accumulations of 10 seconds were taken. The slit width was set at 50 μm for all spectra. The spot size was approximately 3 μm × 15 μm. An automated Raman focus function was applied to each spot to maintain focus on the surface of the tissue section. This function was set to adjust the focus to maximize the Raman peak in the 1400-1500 cm^{-1} range. For most runs, automatic focus was performed in 2 μm steps with a range of 20 μm per focus and an absolute focal range of 40 μm to 100 μm. Dependent on successful completion and sample size, running time for each section ranged from 12 to 120 hours.

Analysis

All resulting spectra were subjected to principal component statistical analysis. Spectral images were created by applying a false color weighting to each principal component in each spectrum. Therefore, each pixel of an image reflects the intensity of a principal component in that spot on the node surface. Thus, Raman spectral maps of axillary lymph node sections were constructed.

Results and Discussion

Raman lymph node maps were compared with white light images of the same section (Figure 1) and H and E histology slides of parallel sections (Figure 1 and 2). The intensity of principal component spectra can be examined for specific lymph node features described by histology opinion (Figure 3). Similarly, the relative presence of molecules identified in

principal component analysis, such as lipids and carotenoids, can be assessed in various areas of nodal section (Figures 4).

The long running time for each sample emphasizes that this mapping technique is not proposed for real-time tissue diagnosis. However, it is ideal for the detailed mapping of heterogeneous features common to lymph nodes, and for the construction of diagnostic models. Map spectra can be variably grouped according to histology features, such as histiocytosis, germinal centers, capsule, fatty infiltrate and metastatic breast carcinoma cells. The weights of the principle components for each pathology group can be entered into a linear discriminant analysis (LDA) model, which maximizes the variance between groups and minimizes the variance within groups. These analytical models can then be used to predict the pathology of new samples. The construction of a diagnostic model with a representative range of pathology groups is essential in the development of Raman Spectroscopy as a diagnostic tool.

When an adequate library of spectra corresponding to tissue features has been collected, a biochemical comparison of features may be possible. Peak heights and widths from principal component spectra can be compared. Peaks for carotenoids, nucleic acids, protein and lipids have been identified in lymph node maps, but maps from more samples are required. In addition, this mapping technique should allow comparison between the Raman spectra from axillary lymph node metastases, and from the breast lesion of origin. The potential exists for a biochemical comparison between the *in situ*, invasive and metastatic components of the same lesion.

Conclusion

This first description of Raman spectral mapping applied to axillary lymph nodes in breast cancer patients demonstrates the exciting potential of the technique as a tool to advance the understanding of the biochemistry of breast carcinoma, and to further the development of a novel optical diagnostic tool.

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