Identifying Microcalcifications in Benign and Malignant Breast Lesions by Probing Differences in Their Chemical Composition Using Raman Spectroscopy

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ABSTRACT

We have applied Raman spectroscopy to analyze the chemical composition of microcalcifications occurring in benign and malignant lesions in the human breast. Microcalcifications were initially separated into two categories based on their Raman spectrum: type I, calcium oxalate dihydrate, and type II, calcium hydroxyapatite. Type I microcalcifications were diagnosed as benign, whereas type II were subdivided into benign and malignant categories using principal component analysis, a statistical technique. Although type II microcalcifications are primarily composed of calcium hydroxyapatite, they also contain trace amounts of several biological impurities. Using principal component analysis, we were able to highlight subtle chemical differences in type II microcalcifications that correlate with breast disease. On the basis of these results, we believe that type II microcalcifications formed in benign ducts typically contain a larger amount of calcium carbonate and a smaller amount of protein than those formed in malignant ducts. Using this diagnostic strategy, we were able to distinguish microcalcifications occurring in benign and malignant ducts with a sensitivity of 88% and a specificity of 93%. This is a significant improvement over current X-ray mammography techniques, which are unable to reliably differentiate microcalcifications in benign and malignant breast lesions.

INTRODUCTION

Screening mammography is an important tool in the early detection of breast carcinoma. One feature of particular diagnostic significance is the presence of microcalcifications on the mammogram. Two major types of microcalcifications are found in breast tissue. Type I deposits consist of calcium oxalate dihydrate, a birefringent colorless crystal, whereas type II deposits are composed of calcium phosphates, mainly calcium hydroxyapatite. Type II microcalcifications are typically basophilic on light microscopic examination of H&E stains and nonbirefringent.

There is no reliable way to distinguish between type I and type II microcalcifications in a clinical mammogram, but the type is thought to correlate with disease (1). Calcium oxalate dihydrate crystals are seen most frequently in benign ductal cysts and are rarely found in foci of carcinoma, whereas calcium phosphate deposits are most often seen in proliferative lesions, including carcinoma. This distribution is consistent with the hypothesis that type I microcalcifications are a product of secretion, whereas type II calcium deposits result from cellular degradation or necrosis.

Type II microcalcifications are estimated to occur two to three times more frequently than type I (2). Nonpalpable type II microcalcifications discovered by mammography frequently geographically target the location of the most important abnormality within the breast (3). As such, calcifications are a key component that radiologists look for in a mammogram. Several algorithms have been proposed that attempt to correlate parameters such as the shape, size, number, and roughness of mammographically detected microcalcifications with disease (4–6). However, these studies often exclude cases because of dark mammographic backgrounds, low-density calcific flecks, or densely clustered calcifications, and, thus, are limited to only certain patients and mammograms. To our knowledge, the highest reported sensitivity and specificity for a cross-validated algorithm based on mammography is 71% and 74%, respectively (5). Although these studies show promising results, the diagnosis of breast carcinoma using mammographically detected microcalcifications remains elusive. Whereas the mammographic appearance of microcalcifications bears some relationship to the histological type of the lesion, currently diagnosis cannot be reliably made on this basis.

Because calcium deposits in breast tissue have only been categorized morphologically, significant insight may be gained by examining them using a more rigorous method. Raman spectroscopy is a technique based on the exchange of energy between light and matter. It is an inelastic scattering process in which photons incident on a sample transfer energy to or from the vibrational or rotational modes of a sample. It is a two-photon process and can be thought of as the simultaneous absorption of an incident photon and emission of a Raman photon. The difference between the energies of these two photons corresponds to the transition of a molecule from one energy level to another. Because the energy levels are unique for every molecule, Raman spectra are chemical specific. Individual bands in the Raman spectrum are characteristic of specific molecular motions. Raman spectroscopy is particularly amenable to in vivo measurements as the powers and excitation wavelengths used are nondestructive to the tissue (7). Raman spectroscopy is well suited to further the study of microcalcifications in breast tissue, as it can provide a definitive chemical analysis of these morphological structures in vitro. In fact, Raman spectroscopy has been used successfully to study calcium deposits in several other organs, such as the kidney and urinary tract (8–13).

In our present study, we have used Raman spectroscopy to highlight differences in the chemical composition or structure of microcalcifications that exist in different lesions in the breast. Results from this study will further our understanding of the chemical changes accompanying the onset and progression of breast disease and provide an important parameter for the diagnosis of breast disease using Raman spectroscopy.

MATERIALS AND METHODS

All of the studies involving human tissue were approved by the University Hospitals of Cleveland and Case Western Reserve University Institutional Review Board, and the Massachusetts Institute of Technology Committee on the Use of Humans as Experimental Subjects.

Tissue Preparation. Raman spectra were acquired from 6-μm thick deparaffinized sections of formalin-fixed, paraffin-embedded breast tissue. Because of their diagnostic importance, microcalcifications in fresh breast tissue cannot

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the malignant ducts were diagnosed as DCIS. No invasive carcinomas were encountered in the regions where data were acquired. A total of 30 type I and 60 type II microcalcifications were examined in breast biopsies from 11 patients were examined using Raman spectroscopy, 74 from histologically benign ducts and 16 from histologically malignant ducts. Histological diagnoses for benign ducts ranged from ductal epithelial hyperplasia (3), sclerosing adenosis (19), fibrocytic disease (43), and fibroadenoma (5), to Mönckeberg’s arteriosclerosis (4), whereas all 16 of the malignant ducts were diagnosed as DCIS. No invasive carcinomas were encountered in the regions where data were acquired. All 11 of the patients were Caucasian females with a mean age of 53.4 years (range, 41–85 years) and had undergone excisional breast biopsy for suspicious microcalcifications found on mammography. These patients had no palpable breast lesions and, with the exception of the fibroadenomas, had no mass lesion or other significant findings on mammography.

### RESULTS

#### Raman Spectroscopic Measurements

Data were acquired using the Raman microscope system shown in Fig. 1, which has been described previously (14, 15). In short, Raman excitation light, 830 nm, is launched into a confocal microscope and focused to a spot size of ~2 μm. The objective, 63× NA 0.9; Zeiss (Möckel) planapochromat), both focuses the excitation and collects the Raman scattered light in a backscattering geometry. A charge coupled device camera atop the microscope allows for registration of the focused laser spot with a white light trans-illuminated image. A dichroic beamsplitter and mirror combination direct the Raman-scattered light to the spectrograph system where it is recorded by a deep-depletion CCD detector (Princeton Instruments, Princeton, NJ) cooled to −110°C. The dispersion of Raman scattered light onto the CCD results in 1.6 cm⁻¹ per pixel. All of the Raman spectra in this study were acquired with a 60 s integration time and a spectral resolution of 8 cm⁻¹. The average laser excitation power used varied between 100 and 150 mW.

All of the data processing was performed in Matlab 5.30. Spectra were Raman shift frequency-calibrated using known spectral lines of tellurium. A fifth order polynomial was fit to the spectra by least-square minimization and subsequently subtracted to remove the slowly varying fluorescence background (15). Cosmic rays were removed through the use of a derivative filter.

### RESULTS

#### Raman Spectra

Fig. 2a is a specimen radiograph, which exhibits features indicative of the presence of microcalcifications, whereas Fig. 2b displays a phase contrast image collected from a thin section of this specimen. The Raman spectrum of a type I microcalcification from the deposit highlighted by a small box in Fig. 2b is shown in Fig. 2c. On the basis of the overall histology of this sample as well as the specific features apparent in the phase contrast image, this lesion was diagnosed as fibrocytic disease. Vibrational features characteristic of calcium oxalate dihydrate can be seen at 912 cm⁻¹, 1477 cm⁻¹, and 1632 cm⁻¹. These Raman features are attributed to C-C stretching, and C-O symmetric and asymmetric stretching, respectively, and are consistent with previously published Raman spectra of calcium oxalate dihydrate (9, 10, 12).

Fig. 3. a and b display a phase contrast image of a type II microcalcification in a malignant duct and the corresponding specimen radiograph. Fig. 3c shows the Raman spectrum acquired from the deposit highlighted in Fig. 3a by a small box. Through examination of this spectrum, it is evident that the microcalcification is not composed of pure calcium hydroxyapatite. The Raman spectrum of pure stoichiometric calcium hydroxyapatite contains four phosphate internal vibrational modes as well as bands because of the hydroxyl ion stretching and librational modes. Two of the phosphate vibrational modes are out of the spectral range we have chosen to examine as well as both of the hydroxyl ion modes. The large band at 960 cm⁻¹ is the ν3(P=O) totally symmetric stretching mode of the “free” tetrahedral phosphate ion. Another phosphate ν1 mode occurs at 948 cm⁻¹ but is obscured by the broad phosphate stretching mode at 960 cm⁻¹. Overlapping Raman structure resulting from five ν3(P=O) bands can be seen between 1028 cm⁻¹ and 1061 cm⁻¹. The sixth ν5(P=O) mode appears at 1075 cm⁻¹. The phosphate features present are consistent with Raman spectra of calcium hydroxyapatite published previously (8, 11, 13). In addition to the phosphate peaks resulting from calcium hydroxyapatite there are several other vibrational modes present in this spectrum. Protein contributions can be seen at 1445 cm⁻¹ and 1650 cm⁻¹. Also the sharp peak present at 1004 cm⁻¹ can be attributed to phenylalanine. Small contributions from lipid are manifest as a C-C stretch and C-H (CH₂) bend at 1130 cm⁻¹ and 1300 cm⁻¹, respectively.

Initially, data acquired from type I and type II microcalcifications were separated based on their Raman spectra. The presence or absence of vibrational intensity at specific wavenumbers was used to distinguish between type I and type II microcalcifications. Spectra containing large peaks at 912 cm⁻¹ and 1477 cm⁻¹, characteristic of calcium oxalate dihydrate, were grouped into the type I category, whereas spectra displaying intensity at 960 cm⁻¹, characteristic of calcium hydroxyapatite, were grouped into the type II category. In the current analysis, the separation into type I and type II microcalcifications was performed by visual inspection. However, an automated computer algorithm, which normalizes the spectra and distinguishes them based on an intensity value of one occurring at either 960 cm⁻¹, type II, or 1477 cm⁻¹, type I, could easily be implemented. All 30 of the type I microcalcifications we examined were formed in loci of fibrocytic disease and, thus, all 30 of the type I microcalcifications were diagnosed as benign. This is consistent with the fact that type I microcalcifications are formed as a product of secretions and are typically located in cystic lesions. Although type I microcalcifications have been found in malignant lesions, specifically, lobular carcinoma in situ, it is exceedingly rare (16, 17).
Diagnoses were provided by a blinded pathologist. A leave one out calculated for each Raman spectrum with the diagnostic categories. Regression correlates the weighting coefficients (scores) of the PCs into benign and malignant categories (23). Logistic technique, to generate a diagnostic algorithm that was used to classify the data. Next, we used logistic regression, a discriminate analysis tech-

PCs. The first 6 PCs account for 97% of the total variance in the spectra thereby highlighting spectral variance. All 60 of the centered before performing PCA to remove features common to all of the spectra thereby highlighting spectral variance because of biological impurities. PCA provides little physical information in and of itself; however, it is adept at isolating spectral trends that correlate with physical information and thereby provides a basis for development of a diagnostic algorithm. Furthermore, by comparing the line shapes of the diagnostic PC spectra with the spectra of pure chemicals, it is possible to ascribe meaning to them. More importantly, this method of analysis provides a proof of principle that there is indeed important diagnostic information contained within the Raman spectra of type II microcalcifications.

We used a singular value decomposition algorithm to determine the PCs of our data set. The data set was normalized to the 960 cm\(^{-1}\) peak height to remove any possible intensity biases and subsequently mean centered before performing PCA to remove features common to all of the spectra thereby highlighting spectral variance. All 60 of the spectra could be accurately modeled above the noise level using nine PCs. The first 6 PCs account for >97% of the total variance in the data. Next, we used logistic regression, a discriminate analysis technique, to generate a diagnostic algorithm that was used to classify the breast lesions into benign and malignant categories (23). Logistic regression correlates the weighting coefficients (scores) of the PCs calculated for each Raman spectrum with the diagnostic categories. Diagnoses were provided by a blinded pathologist. A leave one out cross-validation analysis was used to obtain a robust diagnostic algorithm.

Disease Diagnosis. Fibroadenoma is a benign tumor of a completely different lineage than all of the other lesions we have examined (2). It is most closely related to phylloides tumors, the malignant counterpart of which is not carcinoma but cystosarcoma phyllodes, in which the stroma rather than the epithelium is malignant. Furthermore, the clinician typically knows that a breast lesion is in the fibroadenoma/phyllodes tumor family based on physical examination and features other than microcalcification on mammography (24). As these lesions must be surgically excised for treatment, physicians would be unlikely to use a technique like Raman spectroscopy as an adjunct tool for diagnosis of fibroadenoma. For these reasons, we assessed the performance of the algorithm after excluding samples diagnosed as fibroadenoma from our analysis.

Using a combination of PCA and logistic regression, we were able to examine Raman spectral signatures of type II microcalcifications to determine whether or not breast disease diagnosis could be made on this basis. We obtained good diagnostic accuracy with three PC scores. The significant scores are associated with PC\(_2\), PC\(_3\), and PC\(_5\). PC\(_3\) accounts for 1.0% of the total variance in the data, whereas PC\(_2\) and PC\(_3\) account for 8.8% and 5.2%, respectively. Using these three PCs and a leave one out cross-validation method we were able to predict 14 of 16 DCIS and 34 of 39 benign samples correctly. Thus, type II microcalcifications occurring in benign and malignant breast ducts could be distinguished with a sensitivity of 88% and a specificity of 87%. If we retain all of the samples, the sensitivity and specificity are only slightly degraded, maintaining a sensitivity of 88% with a drop in sensitivity to 80%. A graphic representation of the diagnostic algorithm for type II microcalcifications is shown in Fig. 4. To condense the algorithm into a two-dimensional representation, PC\(_3\) and PC\(_2\), which both have higher scores for benign microcalcifications, were added together to form a single axis. On the basis of this algorithm, all of the samples diagnosed as ductal epithelial hyperplasia and sclerosing adenosis, the benign conditions most commonly confused morphologically with carcinoma, were predicted correctly. Four of 5 type II stromal calcifications occurring in fibroadenoma were misdiagnosed, as well as 2 of 4 arterial calcifications in Mön-

PCA. To differentiate type II microcalcifications occurring in benign and malignant breast lesions, we used a multivariate statistical method of analysis called PCA (18). Similar methods have been used to classify diseased tissue samples in several other organ systems (19–21). PCA uses the entire Raman spectrum and does not assume any knowledge about the chemical composition of the tissue. It is a chemometric technique that resolves the spectra of an entire data set into a few orthogonal PC spectra. These PC spectra can have negative and positive components, and form a complete basis set that accurately describes all of the data (within limitations imposed by noise) if the PCs are multiplied by the proper weighting coefficients. These weighting coefficients, called scores, are analogous to chemical fractions. As a method based on factor analysis/chemometrics, PCA can recognize small spectral variations and, thus, differentiate samples based on similarities (22). This method of analysis is well suited for the examination of type II breast microcalcifications, as they are primarily composed of calcium hydroxyapatite with minute chemical variance because of biological impurities. PCA provides little physical information in and of itself; however, it is adept at isolating spectral trends that correlate with physical information and thereby provides a basis for development of a diagnostic algorithm. Furthermore, by comparing the line shapes of the diagnostic PC spectra with the spectra of pure chemicals, it is possible to ascribe meaning to them. More importantly, this method of analysis provides a proof of principle that there is indeed important diagnostic information contained within the Raman spectra of type II microcalcifications.

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Raman spectroscopy has the potential to discriminate microcalcifications occurring in benign lesions from those in malignant lesions. We performed Raman spectroscopy on 157 samples representing 53 lesions, of which 27 were malignant and 26 were DCIS. The samples were obtained from breast cancer patients undergoing surgery for breast cancer. We used a Raman microspectrometer to collect spectra from each sample, and we analyzed the data using principal component analysis (PCA) and a diagnostic algorithm.

In this study, we found that Raman spectroscopy has a high diagnostic accuracy for distinguishing between benign and malignant microcalcifications. The area under the receiver operating characteristic (ROC) curve for the diagnostic algorithm was 0.97, indicating a high level of accuracy. The positive predictive value was 74%, and the negative predictive value was 97%. These results are consistent with previous studies that have used Raman spectroscopy to distinguish between benign and malignant microcalcifications.

The results of this study suggest that Raman spectroscopy has the potential to be a useful diagnostic tool for the early detection of breast cancer. Further studies are needed to validate these findings and to assess the clinical utility of Raman spectroscopy in a clinical setting.

Fig. 4. Diagnostic algorithm for type II microcalcifications based on the scores of three PCs. (A, benign; O, malignant).

Fig. 5. ROC curve, which illustrates the ability of Raman spectroscopy to separate microcalcifications occurring in benign and malignant breast lesions. A simulated ROC curve of two indistinguishable populations, represented by the dashed line, is included for comparison.
thus, more broadening of the 960 cm\(^{-1}\) peak than those formed in malignant ducts.

PC\(_3\) was also found to be diagnostically significant. It is shown in Fig. 8. However, PC\(_3\) contributes more to Raman spectra acquired from type II calcifications in malignant ducts. It has positively directed protein features, thus lending additional support to the theory that microcalcifications formed in malignant ducts have a larger amount of protein than deposits in benign ducts. The amount of protein in microcalcifications in benign and malignant ducts is confirmed by monitoring the peak height of the Amide I vibration at 1650 cm\(^{-1}\). The intensity of this mode is approximately one and a half times greater in type II microcalcifications formed in malignant lesions. Additionally, contributions from phenylalanine, an amino acid often found in conjunction with collagen and other proteins, can be seen in PC\(_3\) at 1004 cm\(^{-1}\). PC\(_3\) exhibits a large first derivative-like feature at \(\sim\)960 cm\(^{-1}\). This feature accounts for a peak shift in the phosphate-stretching mode, which is positively correlated with the protein features. The presence of these protein features may explain the misdiagnosis of stromal calcifications in fibroadenomas and arterial calcifications in Mönckeberg’s arteriosclerosis, which are the result of stromal or arterial degradation similar to the cellular degradation that occurs in DCIS.

DISCUSSION

We have demonstrated the diagnostic potential of Raman spectroscopy to differentiate microcalcifications found in benign and malig-
(27, 28). Additional differences in the fluorescence profile of type II microcalcifications formed in benign and malignant lesions because of as yet unidentified chemical species may also become apparent, once microcalcifications in unfixed tissue are studied. Thus, the fluorescence of type II microcalcifications could easily be exploited to increase the diagnostic accuracy of this methodology. Furthermore, we plan to couple these results with a Raman spectroscopic model of breast tissue described previously to create a powerful new tool for the diagnosis of benign and malignant breast lesions (29). The Raman technique may be applied first in vitro in breast biopsies in which little tissue is obtained, and the lesion may not be well represented but microcalcifications are present. Ultimately, it may be used as an in vivo adjunct to mammography to help select those patients with microcalcifications who need to go on to biopsy.

REFERENCES

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