Spectroscopic Study for Detection and Grading of Breast Carcinoma In vitro

Safaa K. H. Khalil, Mostafa M. Khodeir, Rasha Abd El-Hakam, Rezq Abd El-Monem Rezq

Spectroscopy Department, Physics Division, NRC, Cairo, Egypt.  
Pathology Department, Faculty of Medicine, Cairo University, Cairo, Egypt.  
Physics Department, Faculty of Science, Helwan University, Cairo, Egypt.

Abstract: The breast cancer–related changes in the molecular organic and inorganic compositions were investigated by Fourier Transform Infrared (FTIR) and atomic absorption (AA) spectroscopy techniques. Surgically excised human breast cancer specimens from fifty female patients aged from 26–74 years old were analyzed (in parallel with normal tissues obtained from the specimens) and diagnosed 50 normal (N), 9 fibrocystic changes (F), 11 invasive lobular carcinoma (ILC) and 30 invasive duct carcinoma (IDC). The quantitative analysis of the deconvoluted FTIR spectra for (ILC) and (IDC) groups showed remarkable decrease in the lipid/protein and collagen/protein ratios. The rates of decrease in the former ratio were 16.76 % and 32.94 % and those of the latter ratio were 9.3 % and 19.76 % for ILC and IDC, respectively. On the other hand, the F group had pronounced increase in lipid/protein ratio (26.58%) and less increase rate in collagen/protein ratio (8.13%). The nucleic acid/collagen ratio showed rates of increase for F (7.92%), ILC (11.88%) and IDC (17.82%) groups. Irrespective to the carcinoma type (ILC or IDC), grade III of malignancy showed much more rates of change in lipid/protein (-39.30%), collagen/protein (-27.90%) and nucleic acid/collagen (+12.87%) than their corresponding values of grade II, -15.60%, -11.62% and +5.94%, respectively. FTIR inorganic results showed calcifications of type Ð (calcium phosphate) with three different structures namely are; carbonate-bearing apatite, carbonate-free apatite and pyrophosphates. The AA analysis showed higher calcium concentration in malignant samples in comparison with the normal ones. In conclusion, the spectroscopic results are in good concordance with the histopathological evaluation. Therefore, the IR analysis is likely to characterize the molecular abnormalities in breast tissues to the order of carcinoma grades and identifying the microcalcification type.

Key words: Breast cancer, detection, FTIR spectroscopy, grading

INTRODUCTION

The cancer of the breast is considered as one of the most leading female cancers in the world. The most widely used screening method, mammography, has serious drawbacks such as, the high rate of false results and the exposure of the patient to repeated dose of ionizing radiation. The gold standard of diagnosis, histopathology, is time consuming and often prone to subjective interpretations (Ng et al. 2008, Murali et al. 2008).

Recently, many developments have been performed to allow a highly sensitive and specific diagnosis to be obtained within seconds, allowing guided biopsies and therapies during a single procedure (Fabian et al. 2006, Chiriboga et al. 2000, Andrus et al. 1998). FTIR spectroscopy is a non invasive analytical technique which provides information about the molecular composition and structure of the examined sample (Ooi et al. 2008).

Tissue spectroscopy for various diseases has made rapid strides in methodology and technology, with promising results for the detection of cancers (Cohenford and Rigas 1998, Bohor Foush et al. 1996). There are many studies carried out on natural hard and soft tissues using FTIR spectroscopy to contribute in enhancing the early detection rate of cancer (Badar and Hassab Elnaby 2007, Li et al. 2006, Romeo and Diem 2005).
Fan et al. (2004) studied the deconvoluted FTIR spectra of normal, benign and cancerous breast tissues and found that they were different in constitution and content of protein, nucleic acid and sugar. Their results also indicated that the content of collagen and nucleic acid increased obviously in cancerous tissues. Fujoka et al. (2004) studied the IR absorption spectra of the gastric cancer and normal tissues and found 10 bands discriminating between normal and malignant tissues in the spectral range (1660–925 cm\(^{-1}\)). The absorption in these 10 bands was greater in the malignant tissues than in the normal tissues. Eikje et al. (2005) showed that IR and Raman spectroscopy have the potential in characterizing and discriminating tumor skin and dysplastic skin from normal skin tissue.

Liu et al. (2006) used synchrotron radiation based FTIR absorption spectroscopy in discrimination of normal, benign, malignant breast tissues. The whole IR absorption spectrum in the energy range of 900–3600 cm\(^{-1}\) was analyzed carefully. They found some specific absorption peaks which may help to diagnose whether the breast tissue is healthy or diseased, or in which stage of progression to cancers.

Zeng et al. (2007) used FTIR spectroscopy in the characterization of normal, benign and malignant thyroid tissues. They observed the presence of shift in amide I band to lower wavenumber while amide II band shifted to higher wavenumber and the ratios of peaks intensities of 1640/1460 and 1640/1550 were raised up in malignant than normal. The authors concluded that the amount of the secondary structure of the protein and also the amounts of nucleic acid had increased in the malignant tissues.

Calcifications occur in 30–50% of breast cancers and constitute one of the most important diagnostic markers of both benign and malignant lesions. These calcifications can be recorded by mammography without identification to either type I or type II calcification (Morgan et al. 2005). Type I calcification (calcium oxalate) may be due to the product of an active cellular process while, type II calcification (calcium phosphate) is due to cellular degradation (Surratt et al. 1991).

Lagier and Baud (2003) demonstrated the presence of magnesium orthophosphate, whitlockite, which occurs in physiological or pathological conditions at extra or intra tissular sites, mainly in tissues of non-epithelial origin by X-ray or electron diffraction patterns. Olin et al. (2001) used X-ray diffraction to examine one of the most rare benign tumor types existed in parotid gland and the study revealed that the crystals found in this region was calcium pyrophosphate dehydrate.

In order to develop a reliable tool for tumor diagnosis, more research and large multicenter trials are necessary to demonstrate the efficiency and usefulness of these new tools (Sahu and Mordechai 2005). In this study, FTIR spectroscopy was adopted to detect the breast carcinoma-induced changes in the molecular structure and composition, and to identify the different carcinoma grades and microcalcification types.

**MATERIALS AND METHODS**

Fixed normal and malignant breast tissue specimens were obtained after mastectomy for 50 female patients aged from 26 to 75 years old with different types and grades of malignancy. All grossly neoplastic samples were analyzed in parallel with normal tissues obtained from the specimens and located at least 1 cm away from the neoplastic tissue. Breast tissue was obtained from patients who were undergoing surgical breast biopsy, reduction mammoplasties, and modified radical mastectomy with complete dissection of axillary lymph nodes, lumpectomy or simple mastectomy without axillary dissection. All were selected from Pathology Department, Kasr El Aini Hospital, Cairo University. Specimens were marked with India ink to indicate the region sampled, fixed in formalin, routinely processed, paraffin-embedded, and sectioned through the marked locations at 5μm thickness and stained with hematoxylin and eosin. The histological slides were examined by an experienced pathologist who was blinded to the outcome of the FTIR Spectroscopy analysis. The histopathological analysis diagnosed 50 normal (N), 9 fibrocystic changes (F), 11 invasive lobular carcinoma (ILC) and 30 invasive duct carcinoma (IDC) as shown in Fig. (1). Formalin–free tissue samples were lyophilized and pulverized into homogenous fine powder and analyzed by a Jasco 430 spectrophotometer using KBr disc technique in the absorbance mode at resolution 4 cm\(^{-1}\).

In order to get rid of all organic constituents and obtain pure inorganic constituents, the tissue samples were burnt off at 600°C for three hours until constant weight of the ash was obtained. FTIR spectra were also recorded for the normal and malignant ashed samples. Calcium level in the ash of normal and malignant samples was measured by atomic absorption spectroscopy. All samples in all groups were analyzed and the subsequent measurements were done in three replicates.
RESULTS AND DISCUSSION

FTIR Qualitative Analysis:

The FTIR spectra recorded for N, F, ILC and IDC groups are illustrated in Fig. (2). The spectra reveal dramatic changes in the peaks' heights but with no shift in the frequency. Over the spectral range, 4000–2000cm⁻¹, there are two absorption peaks at 2925 and 2854cm⁻¹ and an ill-defined one at 2952cm⁻¹ due to asymmetric and symmetric stretching vibrations of CH₂ and a symmetric stretching vibration of CH₃, respectively (Gao and Yunxiang 1999). The stretching vibration stemming from ester linkage of the fatty acid tail and triglyceride polar head group of lipids appears at 1745 cm⁻¹ (Diem et al. 1999, Gao and Yunxiang 1999). The stretching vibration of C=O of amide I and N–H in-phase bending are represented by an overlap absorption band at 1657cm⁻¹, which is characterized by α-helix secondary structure of proteins (Yu et al. 2005, Liu et al. 2006, Eckel et al. 2001). β-sheet secondary structure of amide II of protein appears at 1545cm⁻¹ (Sahu and Mordechai 2005, Yu et al. 2005, Liu et al. 2006). The peak at 1240cm⁻¹ assigned to asymmetric phosphodiester stretching vibration of nucleic acids υ₉ (PO₃⁻) (Lagier and Baud 2003, Gao and Yunxiang 1999).

The conventional FTIR spectra are deconvoluted mathematically by a program provided with Jasco spectrophotometer and are shown in Fig. (3). The deconvolution process investigates the overlapping absorption peaks. New absorption peaks which revealed by deconvolution have a diagnostic marker. Anti-parallel β-sheet secondary structure of proteins is demonstrated by the absorption peak at 1695cm⁻¹ while, the random turn secondary structure of proteins is demonstrated by peak at 1680cm⁻¹. Parallel β-sheet secondary structure of amide I of proteins can be found near 1635 cm⁻¹. Peak near 1553cm⁻¹ determines α-helical secondary structure of amide II of protein (Yu G et al. 2005). There are two peaks at 1204 and 1278 cm⁻¹ can be attributed to amide III/CH₃ wagging vibrations of collagen (Yu et al. 2005, Gao and Yunxiang 1999). Peak near 1149cm⁻¹ demonstrates glycogen molecule (Yu et al. 2005). These results are in agreement with those reported by Liu et al. (2006) who found some specific absorption peaks in the range 900-3600cm⁻¹ that may help differentiating between healthy and diseased breast tissue and in which stage of cancer progression.
FTIR Quantitative Analysis of Different Tissue Types:

In order to discriminate between N, F, ILC and IDC groups on quantitative bases, three absorbances ratios were calculated using the peaks' heights measurement. Variation of these ratios with the different groups of tissue types are exhibited in Fig.(4).

1) The absorbance ratio $A_{1745}/A_{1657}$ measures the lipid content relative to the total protein. The mean values of this ratio are 1.73 (S.D ±0.05), 2.19 (S.D ±0.06), 1.44 (S.D ±0.02), and 1.16 (S.D ±0.05) for N, F, ILC and IDC groups, respectively. The percentage rates of change of F, ILC and IDC from N groups are +26.58%, -16.76%, -32.94%, respectively.
Fig. 3: The deconvoluted FTIR spectra for normal (N) fibrocystic changes (F) invasive lobular carcinoma (ILC) and invasive duct carcinoma (IDC).

2) The absorbances ratio $A_{1204}/A_{1657}$ is used to measure the ratio of collagen to the total proteins. The mean values of this ratio are 0.86 (S.D ±0.07), 0.93 (S.D ±0.01), 0.78 (S.D ±0.01), and 0.69 (S.D ±0.02) for N, F, ILC and IDC groups, respectively. The percentage rates of change of F, ILC and IDC from N groups are +8.13%, -9.30% and -19.76% respectively.

3) The absorbances ratio $A_{1240}/A_{1084}$ measures the ratio of the nucleic acid/collagen. The mean values of this absorbances ratio are 1.01 (S.D ±0.04), 1.09 (S.D ±0.01), 1.13 (S.D ±0.05) and 1.19 (S.D ±0.08) for N, F, ILC and IDC groups, respectively. The percentage rates of increase of F, ILC and IDC from N samples are 7.92%, 11.88% and 17.82%, respectively.
Carcinoma Grading by FTIR Spectroscopy:

For carcinoma grading purpose, variations of the above mentioned three absorbances ratios with the tumor grades (II and III), irrespective to the tumor type (ILC or IDC) were plotted in Fig. (5). The number of cases in grade II and grade III of carcinoma was 27 and 14, respectively. This Fig. reveals significant decrease in the lipid/protein and collagen/protein ratios with the carcinoma grade, recording % rate of decrease in the former ratio 15.60% and 39.30% and in the latter one 11.62% and 27.90% for grades II and III, respectively. In contrast, the nucleic acid/collagen ratio shows slight increase with the carcinoma grade, recording % rate of increase 5.94% and 12.87% for grades II and III, respectively.

Based on the above results, one can conclude that the breast cancer type (ILC, IDC) and carcinoma grade (II, III) are strongly correlated with low lipids and collagen contents relative to high contents of the total protein and nucleic acid in the malignant tissues. This is in accordance with the results reported by Zeng et al. (2007), Badr and Hassab Elhaby (2007), Yu et al. (2005), Gao and Yunxiang (1999) and contradict with those results indicated by Ooi et al. (2008) and Fan et al. (2004) who found obvious increase in the collagen level in the breast cancer tissue. On the other hand, the fibrocystic changes are correlated with increasing
amounts of lipid, collagen and nucleic acid relative to the total protein. This finding concords with that of Gao and Yunxiang (1999) who detected high collagen level in fibroadenoma tissue by FTIR spectroscopy.

**Investigation of Tissue Calcification by FTIR:**

The recorded FTIR spectra of all ashed samples under study were classified according to their spectral features into three groups and are represented in Fig.(6).

![FTIR spectra](image)

Fig. 6: FTIR spectra representing different of calcified breasts tissue samples.

1) **Carbonate–bearing apatite** $[\text{Ca}_{10} (\text{PO}_4, \text{CO}_3, \text{OH})_6 (\text{OH})_2]_n$ group contains $(19/38)$ samples. The histopathological analysis for these samples were; N $(9/19)$ samples, ILC $(1/19)$ and IDC $(9/19)$ with different carcinoma grades. This group is characterized by a major content of apatite and minor content of carbonate.
Symmetric stretching mode of phosphate group is characterized by very strong sharp peak at 1030 cm\(^{-1}\) (Bohic et al. 2000, Ohmacht 1976). There are a medium sharp peak at 575 cm\(^{-1}\), strong sharp peak at 600 cm\(^{-1}\) and very strong sharp peak at 1080 cm\(^{-1}\) which are characteristics of apatite structure (Mizuno et al. 1984). The carbonate groups are assigned by two medium sharp peak at 1420 and 1457 cm\(^{-1}\), weak band at 875 cm\(^{-1}\) and medium shoulder at 960 cm\(^{-1}\) (Bohic et al. 2000, Ohmacht et al. 1976, Bellamy 1975).

Careful investigation of the spectra of this group showed that the presence of the absorption bands at 1420, 1385 and 770 cm\(^{-1}\) are strongly related to the N tissue samples (Fig. 6-G1a). The malignant tissue samples exhibited these bands at 1425, 1365 and 770 cm\(^{-1}\) with decreasing intensity, and in some cases the absorption bands at 1365 and 770 cm\(^{-1}\) disappeared completely (Fig. 6-G1b).

To record the phenomenon of decreasing intensity, the carbonate content was estimated quantitatively relative to the phosphate content. To obtain accurate results of CO\(^3-\)/PO\(^4-\), two absorbances ratios Ra (A\(^{1420}/A^{1030}\)) and Rb (A\(^{1420}/A^{575}\)) were calculated. The calculated mean values of the ratios Ra and Rb and their standard deviations are listed in table (1). This table shows that there is a sharp decline in the CO\(^3-\)/PO\(^4-\) of the malignant samples, recording % rate of decrease 52.63% and 54.83% for Ra and Rb, respectively.

Table 1: The relationship between the CO\(^3-\)/PO\(^4-\) ratios with the different tissue types.

<table>
<thead>
<tr>
<th>Absorbances ratio</th>
<th>Normal (± S.D)</th>
<th>Malignant (± S.D)</th>
<th>% Rate of decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ra</td>
<td>0.19 ± 0.02</td>
<td>0.09 ± 0.02</td>
<td>52.63%</td>
</tr>
<tr>
<td>Rb</td>
<td>0.34 ± 0.06</td>
<td>0.14 ± 0.01</td>
<td>54.83%</td>
</tr>
</tbody>
</table>

2) Carbonate–free apatite [Ca\(^{10}\) (PO\(^{10}\)\(_3\) (OH)\(_2\)]\(_x\) group includes (10/38) samples, histopathologically classified as IDC of grade II. There are two strong sharp absorption bands at 575 and 600 cm\(^{-1}\) and a strong broad peak at 1039 cm\(^{-1}\) which attributed to phosphate apatite structure with no evidence for the presence of carbonate anions (Fig. 6-G2).

3) Pyrophosphate [P\(_2\)O\(_5\)] group has (3/38) samples, histopathologically evaluated as ILC and IDC of grade II. The vibrations of P–O–P of pyrophosphate anions, P\(_2\)O\(_5\), are characterized by the presence of very strong sharp absorption peak at 1145 cm\(^{-1}\), strong sharp peak at 914 cm\(^{-1}\) and weak band at 730 cm\(^{-1}\) (Mizuno et al. 1984). There are a medium broad peak at 1430 cm\(^{-1}\) (which can be assigned to carbonate anions), a very strong sharp peak at 1066 cm\(^{-1}\) and a weak band at 570 cm\(^{-1}\) which can be assigned to phosphate anions (Fig. 6-G3).

Atomic Absorption Analysis:
All ash samples under investigation were analyzed by AA spectroscopy to measure level of Calcium ions content. The Ca\(^{2+}\) ions level in the normal tissue samples was 44.42 ± 0.03 mg/l. The other tissue types (F, ILC, and IDC) showed a pronounced high level of the Ca\(^{2+}\) ions, 71.87 ± 0.04 mg/l.

The comprehensive qualitative study of the FTIR spectra of the ashed samples lead to the conclusion that the calcification of the breast cancer is of type II, calcium phosphate, which is due to cellular degradation. This result is supported by the quantitative assessment of CO\(^3-\)/PO\(^4-\) ratio which showed that the malignancy is strongly correlated with much decrease in the carbonate substitution relative to the phosphate ions contents and the high Ca\(^{2+}\) level measured by AA technique. These findings agree with those reported by Stone et al. (2007) who demonstrated calcification type II with various carbonate substitution of phosphate in calcium hydroxyapatite in benign and malignant breast tissues.

Conclusion:
Quantitative FTIR results showed remarkable differences between normal, fibrocystic changes, invasive lobular carcinoma and invasive duct carcinoma regarding to the lipid, collagen, protein and nucleic acid constitutions. This technique was also able to differentiate between different grades of breast carcinoma. From the point of calcification view, FTIR technique was able to discriminate between normal and malignant tissues as general but couldn't differentiate between the different grades of the malignancy.

REFERENCES


