Diagnostic Segregation of Human Breast Tumours Using Fourier-transform Infrared Spectroscopy Coupled with Multivariate Analysis: Classifying Cancer Subtypes

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Abstract: The present study aimed to investigate whether attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy coupled with Multivariate Analysis could be applied to discriminate and classify among breast tumour molecular subtypes based on the unique spectral "fingerprints" of their biochemical composition. The different breast cancer tissues and normal breast tissues were collected and identified by pathology and ATR-FTIR spectroscopy respectively. The study indicates that the levels of the lipid-to-protein, nucleic acid-to-lipid, phosphate-to-carbohydrate and their secondary structure ratio, including RNA-to-DNA, Amide I-to-Amide II, and RNA-to-lipid ratios were significantly altered among the molecular subtype of breast tumour compared with normal breast tissues, which helps explain the changes in the biochemical structure of different molecular phenotypes of breast cancer. Tentatively-assigned characteristic peak ratios of FTIR spectra reflect the changes of the macromolecule structure in different issues to a great extent and can be used as a potential biomarker to predict the molecular subtype of breast tumour. The present study acts as the first case study to show the successful application of IR spectroscopy in classifying subtypes of breast cancer with biochemical alterations. Therefore, the present study is likely to help to provide a new diagnostic approach for the accurate diagnosis of breast tumours and differential molecular subtypes and has the potential to be used for further intraoperative management.

Keywords: Breast tumour; FTIR spectroscopy; Multivariate analysis; Characteristic peaks ratio

INTRODUCTION

Breast cancer is the most common malignancy worldwide threatening women's health, with the first incidence rate and fifth leading cause of cancer deaths in China [1, 2]. Such a high mortality is a result of late presentation and diagnosis at clinical stage III or IV. There is growing evidence that the different molecular subtypes and treatment time of breast cancer are associated with distinct outcomes [3]. Therefore, early detection is still the cornerstone for improving the outcome and survival of patients with breast cancer. Currently, the results of pathological diagnosis and immunohistochemistry (IHC) are often used as a diagnostic gold standard to identify the four major intrinsic molecular subtypes of breast cancer. Oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) statuses are well-established biomarkers for distinguishing breast cancer subtypes [4], while Ki-67 is employed as a prognostic indicator in these patients [3, 5]. To achieve this, breast tissue sections have to be stained by IHC for antibody of ER, PR, HER2 and Ki-67**,** respectively named Luminal A, Luminal B, HER2-positive and triple negative breast cancer (TNBC) [6, 7]. Since the early diagnosis and treatment of breast cancer patients is so important, it is time to find a novel approach to quickly and accurately diagnose tumour molecular subtypes in the early stages.

Recent studies have demonstrated the applicability of infrared (IR) spectroscopy in rapid, non-destructive and in situ diagnosis of various diseases and biological systems, including the characterization of cell cycle events, toxic damage and cancer diagnosis [8-10]. It is well known that the initiation and development of cancer is manifested at a molecular level before morphological changes occur, while the molecular changes cannot be easily detected by traditional methods or even pathological examinations [11]. Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy is based on the concept of light absorption, which subsequently causes vibrations of the chemical bonds of biomolecules in the mid-infrared (IR) region (4000-400 cm^{-1}) [12, 13]. As an emerging biochemical analytical tool, with a good signal-to-noise ratio, as well as high-resolution throughput over the entire spectral region, ATR-FTIR spectroscopy can provide biochemical information for proteins, nucleic acids (DNA/RNA), lipids and carbohydrates in biological samples [14, 15]. In addition, alterations in protein secondary structure and protein phosphorylation can also be identified from the vibrations of the respective functional

groups [16, 17]. In view of the different chemical bond absorption of biochemical samples in the infrared region (including 1800 cm^{-1} to 900 cm^{-1}), FTIR spectroscopy helps to investigate the chemical structure of the different molecules [18]. The vibrational spectrum in the 1800-900 cm^{-1} region is commonly referred to as the "bio-fingerprint" region [19]; absorbance in 1750-1700 cm^{-1} is primarily associated with C=O stretching vibrations of lipids [20, 21]; regions at 1700-1580 cm^{-1} (C=O stretch of proteins and particularly sensitive to changes in β-sheet formation) and 1580-1480 cm^{-1} (N–H bend of proteins) can be respectively assigned to protein secondary structures of Amide I and Amide II; the 1280-1185 cm^{-1} region is known as the nucleic acid and phosphate region; 1180-900 cm^{-1} region denotes vibrations related to carbohydrates [8, 22, 23].

A number of previous studies used FTIR spectroscopy to explore the classification and staging of various tumours, including the identification of benign and malignant tumours [24, 25]. The main purpose of this study was to use ATR-FTIR spectroscopy combined with multivariate identify in order to classify different molecular subtypes of breast cancer tissues. Further exploration of the characteristic spectral changes associated with different molecular subtypes, was also carried out.

Materials and Methods

Study participants

With the approval of the Ethics Committee (Approval NO.: 2015yx11kt46), we obtained fresh breast mass samples (42 cases) from the Department of Breast Surgery in the Guilin Medical University Affiliated Hospital. Age, histological type and WHO grade for all samples are summed up in Table 1. These pathologies are all derived from Chinese women, aged 18 to 82 years old, the median age is 46(The median age of the normal group is 37, and the median age of the tumour group is 47). Tissue blocks consisted of breast cancer (n=28 patients), breast fibroadenoma (n=7 patients) and normal breast (n=7 patients).

H&E staining

The isolated breast tissue was fixed in 10% formalin for 24 h and then paraffin-embedded. Three consecutive tissue sections were obtained using a microtome and the first 4-μm-thick tissue sections were transferred onto a glass slide after de-waxing and stained with stained with haematoxylin and eosin (H&E) for histopathological examination by two pathologists.

Immunohistochemical staining

Another 4-μm-thick tissue section from almost the same position of the same sample was collected to carry out the immunohistological staining. The tissue sections were submitted to roasting overnight, conventional de-waxing and hydration treatment. The tissue was circulated with a gradient alcohol, followed by incubation with 3% H_2O_2 in methanol at room temperature for 30 min to inactivate endogenous peroxidation enzyme. The sections were subsequently immersed in a cup of 0.01 M citrate buffer (pH 6.0) and placed in a high-pressure cooker for 2 min for antigen repair. Subsequently, the sections were incubated in 3% H_2O_2 blocking medium for 5 minutes, washed with distilled water and incubated for 60 minutes at room temperature with mouse monoclonal antibody against the following antigen: ER (1D5, 1:50; pH 7.3; Dako, San Jose, USA), PR (PR 636, 1:50; pH 7.3; Dako, San Jose, USA), HER2/neu (CB11, 1:50; pH 7.3; Novocastra, Newcastle, UK) and the cell proliferation marker Ki-67 (sp6) rabbit monoclonal antibody (REF275R-18). Slides were rinsed in TBS and then incubated for 35 minutes in DAKO Duet (code: K0492) biotinylated goat anti-mouse/rabbit secondary antibody (1:100). Followed by rinsing in TBS, dropping DAB working solution (code: 896102, Kem-En-Tec, Copenhagen, Denmark). Immunoreaction was visualized under a light microscope through adding DAB for 3 min. Staining was carried out according to the manufacturer's instructions by staining the slides with DakoREAL haematoxylin (code: S2020) for 1 minute and covering the glass with the mounting media. Internal positive control was normal breast epithelium for ER and PR. ER, PR and HER2 were positive for ER, PR and HER2 positive breast cancer, respectively. Negative control was evaluated by replacing the primary antibody with PBS.

Tissue preparation for ATR-FTIR

Parallel 10-μm-thick tissue section was obtained from the paraffin-embedded blocks and de-waxed by immersion in three sequential baths of fresh xylene (5 min),

then washed and cleared in an acetone bath for a further 5 min, and then fixed on a Low-E IR reflective slide (Kelvey Technologies, Chesterland, OH, USA) for ATR-FTIR spectroscopy [26]. The tissue slices were air-dried and then stored in a desiccator until analysed.

ATR-FTIR spectral measurement

The prepared samples on Low-E IR reflective slides were investigated using a Bruker Vector 70 FTIR spectrometer (Bruker Optics) equipped with a Helios ATR attachment containing a diamond crystal (≈ 250 μm × 250 μm sampling area) (Bruker Optics Ltd., Coventry, UK) and a HYPERION microscope, which contained a liquid nitrogen-cooled detector. The instrumental settings were optimized: ATR sampling mode, 64 scans, and 8 cm^{-1} spectral resolution giving 4 cm^{-1} data to spacing [27]. Tissue sections were acquired in a random manner to avoid deviations. Thus, 20 spectra of each sample were randomly obtained from different locations of the dried frond samples. Prior to starting the next slide, the ATR crystal was cleaned with distilled water and dried with dry tissue paper before the acquisition of spectral background. A new background spectrum was recorded after every 10 spectral measurements.

Computational analysis

IR spectra obtained from interrogated samples were converted into absorbance by Bruker OPUS software. Then the raw data was converted to TXT files [28]. Data pre-processing and multivariate analysis were then performed within IRootLab toolbox (http://irootlab.googlecode.com/)[28] running on MATLAB R2015b (The Maths Works, Natick, MA, USA). From the multidimensional analysis and consideration of the IR spectral dataset, the most important factors can be obtained by minimizing the dimension of the multivariate analysis techniques, for instance, by employing principal component analysis (PCA) followed by linear discriminant analysis (LDA) [19, 29, 30]. PCA is an unsupervised, data processing technique that allows for the reduction of variables, which can produce major components (PCs) that can capture more than 95% of the difference in the original variance dataset in the spectral dataset [31]. LDA is a supervised technique that forms a linear variable combination depending on the different classes. It can regenerate new variables, which are linear combinations called as “factors”. Each weighting factor is represented by a vector called "load vector". The

function of the load vector seems to maximize the difference between the classes responsible for diagnosing the separation of the category over the variance [31]. LDA is often used after PCA to reduce computational complexity and improve the accuracy of identifying different categories [9]. Factors derived from PCA-LDA techniques, the most important contribution to the few factors can be used as Cartesian coordinates generates 1, 2 or 3 dimension scatter plots. While the scores plot allows visual classification, the derived cluster vector and loading plot determine the responsible wavenumber separation [26]. Each spectrum was cut at the "biochemical fingerprint" region, followed by rubber-band baseline correction, normalization to the Amide I peak (~1650 cm^{-1}) and mean-centring [19]. After the above-mentioned pre-processing steps, cross-calculated PCA-LDA cross-calculated was applied to identify biochemical alterations that segregate the different groups from each other [18]. PCA was applied to the spectral dataset to reduce the dimensions of the datasets [32]. In order to interpret such complex biochemical information and avoid the spectral over-fitting, LDA was applied to discriminate differences between the classes [18, 33]. The scores plots and cluster vector plots were used to show the multivariate analysis' results. In the scores plots, proximity in LD1 space between two groups means similarity of biochemical structure, while distance represents dissimilarity and separation. Cluster vector plots after PCA-LDA contributed to revealing biochemical alterations associated with each category in the spectra dataset. In order to simplify the identification of major biochemical changes in each group, cluster vector were used to represent the top six peaks in the cluster vector plots [18, 34]. The statistical significance of each linear contribution determines the discriminant (LD) and the subtype of category interval was achieved in the GraphPad Prism 7 (GraphPad Software, USA) through unpaired t-test and ANOVA analysis with Dunnett's T3 post hoc test. A probability (p) value of <0.05 was considered to indicate the statistical significance for all tests.

RESULTS

Characteristics of breast tissues in H&E stained samples

Small red arrows in Fig. 1A indicate the microscopic appearance of female normal breast tissue, which is characterized by a larger duct and acinus. Amount of adipose tissue can be seen admixed with these elements. Fig. 1B shows a fibroadenoma, which is characterized by the disappearance of normal breast lobular structure, tumor tissue by

the proliferation of duct and fibrous tissue composition, and visible proliferation of fibrous tissue compression gland. Fig. 1C-F illustrate pathological sections in different molecular subtypes of breast cancer. After the use of the microscope it can be observed that the arrangement of cancer cells is very irregular, large nuclei and deep staining, showing an increase of mitotic figures.

Immunohistochemical identification

The percentage of positively stained tumour cells was observed and evaluated under a microscope. ER/PR staining positive in the nucleus, while HER2 staining was positive in the cell membrane. Staining positive intensity evaluation criteria: light yellow for the weak positive (+), brown positive medium (2+), tan is strongly positive (3+). The results were blindly performed and revised by two pathologists. Being dependent on the results of immunohistochemical staining, each breast cancer sample was divided into Luminal A, Luminal B, HER2-positive and TNBC, see Table 1 in detail. Fig. 2 shows one of the immunohistochemical results identified as breast cancer tissue. Fig. 2A illustrated that the nuclei were stained tan, and the pathology reported the following: ER (70%, strongly positive), PR (80%, strongly positive), HER2 (0, negative) and Ki-67 was 5%. Fig. 2B showed that the nuclei and cell membranes were stained with brown or tan, and the pathology reported: ER (60%, strongly positive), PR (90%, strongly positive), HER2 (3+, strongly positive), Ki-67 was 30%. Fig. 2C showed that the membrane was stained with brown, and the pathology reported that ER (0, negative), PR (0, negative), HER2 (3+, strongly positive) and Ki-67 was 20%. Fig. 2D showed that the nuclei and cell membranes were not stained as brown, and the pathology reported ER (0, negative), PR (0, negative), HER2 (0, negative), Ki-67 was 30%. Obviously, the immunohistochemical results were judged to be Luminal A, Luminal B, HER2-positive, and TNBC from A to D.

Diagnostic segregation of breast cancer with Multivariate analysis

Fig. 3A shows the average spectra for normal breast tissue (n =7) and breast tumours (n=35) after pre-processing. Overall, the IR spectra of breast tumours appear to overlap with the normal breast spectra in the biochemical cell fingerprint region, making it difficult to distinguish any subtle differences, except the absorbance at ~1250-1000 cm^{-1} (protein and nucleic acid), which is slightly increased compared to normal and benign tissue. The spectral data were subsequently explored by multivariate

analysis due to the evident overlap and the delicate biochemical changes in the breast tissue. In this more discriminatory approach, each spectrum becomes a point for determining the optimal separation and correlation wavenumbers. By this discriminant analysis, clear clustering of normal tissue and breast cancers was observed, as shown in the 2-D PCA-LDA scores plots (Fig. 3B). Fig. 3B revealed that the less degree of overlap in LD1 between the spectral point from breast cancer and normal tissue while no obvious separation in LD2. In other words, the contribution of LD1 in the tumour is relatively large relative to normal breast, while LD2 was less. Fig. 3C shows the difference of the spectral points (mean±SD) in LD1 between normal and tumour, the result shows distance in LD1 space from the spectral points (mean±SD) has been found statistically significant (p <0.0001). To be exact, breast cancer and fibroadenoma can be separated in LD1, especially in breast cancer. Fig.3D shows the corresponding cluster vector plot (peak detection plot) with wavenumbers discriminating the benign and breast tumour cases from normal tissue. Clearly, the major changes in benign tissue occur in protein molecules (wavenumbers around 1650 cm^{-1}, 1550 cm^{-1} and 1450 cm^{-1}), whereas malignant tissue differs in protein and nucleic acid molecules (wavenumbers around 1650 cm^{-1}, 1450 cm^{-1} and 1080 cm^{-1}) [22]. The corresponding loadings plots according to pairs are displayed with the top six wavenumbers marked in LD1 spaces (Fig. 3E & F). The wavenumbers derived from the spectral dataset of fibroadenoma were the following, with an importance order: 1541 cm^{-1} (Amide II), 1493 cm^{-1} (amino acid; ν[COO^{-}]), 1171 cm^{-1} (νC-O of side chain group in proteins), 1716 cm^{-1} (C=O stretching vibrations of lipids), 1458 cm^{-1} ($\sigma_{as}CH_3$ of protein and lipids), 1066 cm^{-1} ($\nu_s PO_2^-$ of nucleic acids). Those derived from cancer in the LD1 space were similar to fibroadenoma , the top six wavenumbers are 1452 cm^{-1} ($\sigma_{as}CH_3$ of protein and lipids), 1001 cm^{-1} (ν_sPO4= of RNA), 1655 cm^{-1} (Amide I), 1061 cm^{-1} (glycogen), 1238 cm^{-1} ($\nu_{as}PO2^-$ of nucleic acids) and 1541 cm^{-1} (Amide II) [35].

Inter-individual vs. disease differences

As shown in Fig. S1A (see ESI), there is almost no evidence of inter-individual variability in the spectral averages of all data from breast cancer, while the 2-D cross-calculated LDA score plot shows the individual differences in IR spectra of the patient. As shown in Fig. S1B (see ESI), the results from cross-calculated LDA are excellent, because the classes are almost separated; just a small overlap is seen among all subtypes. The difference in the mean spectrum of FTIR spectra originates from different cancer

subtypes which has been shown in Fig. S1C (see ESI). The average absorbance of all the breast tumour samples is higher than normal between 1750 -1650 cm^{-1} (lipid) and 1250-1080 cm^{-1} (nucleic acid), peaking at 1700 cm^{-1} and 1230 cm^{-1}. In addition, malignant and benign tissue changes followed an opposite trend at 1580-1480 cm^{-1} (protein) [22].

Classifying subtypes of breast cancer with Multivariate analysis

Two-category discriminant analysis was performed using cross-calculated PCA-LDA in FTIR spectroscopy to identify wavenumber in each spectrum to be the best separation and the spectral variance between the response categories. Then, 3-D, 2-D, and 1-D PCA-LDA scores plots were generated for visualization. Fig. 4A shows the overlap of data and spectral variations among each cancer subtype. It is difficult to identify segregation with other categories, except for TNBC and the normal group. Fig. 4B indicates these subtypes of 2-D plots that have been partially divergent in LD1 while overlapping in LD2. A clearer interpretation of segregation among each category by using PCA-LDA was presented using 1-D plots. Fig. 4C reveals a separation of the molecular subtypes with overlapping levels compared to normal breast. It is obvious that the contribution of the LD1 is partially overlapped with those tumours within a certain range, but the inter-class variance between tumour subtypes and normal breast tissue is significant. The difference of the spectral points (mean ± SD) in LD1 between classes is statistically significant ($p < 0.001$). Obvious separation is observed between the normal tissue and diverse types of tumours following ATR-FTIR spectroscopy, with Luminal A and Luminal B being the most apparent. Fig.4D demonstrates the corresponding cluster vector plot with wavenumbers discriminating the fibroadenoma and breast tumour subtypes from normal tissue. The cluster vector plot represents the discriminant variables in the corresponding fractional plot that is dependent on the peak intensity. The top six differences in the wavenumbers were presented. Figs. S2 (see ESI) shows the corresponding loadings plots identifying wavenumbers responsible for separation in LD1. The loadings plot of the contribution of LD1 shows the majority of the difference between normal. The wavenumbers in the loadings plots show that each molecule subtype has a strong change at 1068 cm^{-1} or 1066 cm^{-1} (nucleic acid) except for Luminal B. Luminal B showed the main changes at 1680-1380 cm^{-1} (Amide I and Amide II), while HER2-positive at Amide I, Amide II, nucleic acid and glycogen. Table 2 has listed the principal six biomarkers of each category [35]. Luminal A mainly

showed RNA (1001 cm^{-1}), Amino acid (1450 cm^{-1},1493 cm^{-1}) and Amide II (1541 cm^{-1}); Luminal B mainly showed Amide I (1653 cm^{-1}, 1614 cm^{-1}), Amide II (1574 cm^{-1},1541 cm^{-1}) and amino acid (1452 cm^{-1}, 1392 cm^{-1}); HER2-positive mainly showed protein (1159 cm^{-1}), Nucleic acid (983 cm^{-1}), Amide I (1662 cm^{-1}) and Amino acid (1479 cm^{-1}); and TNBC predominantly showed C=O stretching of Lipid (1716 cm^{-1}), Amide I (1683 cm^{-1}, 1647 cm^{-1}) and nucleic acid (1066 cm^{-1}).

Characteristic peaks ratio in IR spectroscopy

Since the specific wavenumber in FTIR spectrum can be used as a marker of biochemical structure in cells, the absorbance intensity after simple pre-treatment, (cut at the fingerprint region, rubber-band baseline correction and normalization to the Amide I peak) can be used to assess the biochemical structure of the target cells. It is necessary to further excavate the ratio of the intensity of the characteristic peaks derived from different spectra as a spectral marker for determining the molecular subtype of tumour. Specifically, the absorbance intensity of breast cancer in lipid-to-protein ratio was higher compared to normal tissue. The ratios for the two groups of Luminal B and TNBC, specifically, were significantly higher than the other subgroups (Fig. 5A). The RNA-to-DNA ratio was increased significantly in cancer tissue, especially for Luminal B and TNBC (Fig. 5B). Fig. 5C shows that the phosphate-to-carbohydrate ratio is decreased in fibroadenoma, while it increased in tumour tissue and in particular for the TNBC group. Fig. 5D indicated the significance of difference in the Amide I-to-Amide II ratio of various tumour subtypes compared to normal breast tissue. The absorbance ratio was higher in various tumour subtypes, with TNBC being significantly higher than any other subgroup. Fig. 5E and F show the ratio of RNA-to-lipid and nucleic acid-to-lipid, respectively, showing a completely synchronized tendency. The ratio of other tumour subtypes was significantly higher than that of normal tissues except from HER2-positive.

Discussion

In order to introduce a screening opportunity for early breast cancer cases in clinical practice, we introduced FTIR spectroscopy as a tool to identify breast cancer and classify its molecular subtype. This exploratory study has demonstrated that ATR-FTIR spectroscopy, coupled with multivariate analysis, has the potential to distinguish between normal breast and tumour tissue (Fig. 3), as well as between various subtypes

thereof (Fig. 4). The separation of cancerous from normal tissue was also found to be consistent with previous studies in a number of different tissues [26, 36, 37]. Results from the 2-D LDA score plot have indicated spontaneous clustering of similar molecular subtypes, which indicates the common biochemical characteristics of specific tumour subtypes within the cluster. In other words, breast cancer with different molecular subtypes has different biochemical characteristics, and it can be clustered by FTIR spectroscopy. Multivariate analysis with PCA-LDA and spectral visualization were also performed, which showed a good discrimination among the different subtypes and identified tentative biomarkers [38]. One-D score plots and wavenumber assignment in LD1 allowed the identification of molecular markers that can be utilized to separate normal breast tissue from tumours (Fig. 4C & D). To some extent, the result reflects the main changes in macromolecule structure and content in different subtypes of breast cancer. Specifically, the average spectra from malignant tumours showed increased intensity of nucleic acid region (1250 cm^{-1} to 900 cm^{-1}), which could be due to tumour cell overgrowth and replication. It was further found that there were significant differences in the following biological fingerprint regions, including 1580-1480 cm^{-1} (Amide II), 1280-1185 cm^{-1} (nucleic acid and Amide III) and 1750-1700 cm^{-1} (lipids). The most prominent result of the two-category discriminant analysis was the contribution of peak intensity in Luminal A, Luminal B, HER2-positive, and TNBC. Compared with the normal tissue, the cluster vector plot and the corresponding loadings plots illustrate the intrinsic molecular differences between the molecular subtypes and the associated contribution wavenumber. The results indicated that biochemical structure changes of nucleic acid in molecular subtypes of breast cancer are basically very similar, whereas the wavenumber that were representative of proteins and lipids showed significant differences. Obviously, the alteration of protein structure was the most complex in HER2-positive. This means that different molecular subtypes have different structural changes in the process of tumour formation. It may be closely related to the phosphorylation of the relevant signalling pathway in the process of tumour formation [39, 40]. Fig. 4C and D indicated that the inter-class similarities and differences in molecular basis from each category may be attributed to the inherent heterogeneity of breast tumour tissue [41]. Different molecular subtypes of breast cancer may contain separate matrix elements, including some changes in nucleic acid content and protein structure. Although there is a partial overlap of the spectra between the different categories, the important molecular biomarkers are identified by

wavenumber allocation, leading to the separation of normal tissue from the different molecular subtypes (Fig. S2, see ESI).

Our experimental results are the proof that the changes in normal tissue to tumour tissue are associated with structure and quantitative changes in lipid, protein and nucleic acid [42]. So, the absorbance ratio of the characteristic spectrum was used to explore and distinguish between normal tissue and molecular subtype in tumour tissue. Early studies have shown that the ratio of lipids to proteins is associated with the progress of malignant tumours [26, 43]. The ratios of lipid-to-protein, RNA-to-DNA, RNA-to-lipid or nucleic acid-to-lipid have also been used as potential biomarkers to predict cell proliferation or malignancy degree in normal or malignant tissue [44-46]. The results increased significantly among those categories, especially in Luminal B and TNBC, which may be closely associated with the degree of differentiation and malignancy in breast cancer [47]. This is similar to Argov and Mordechai's main findings [48, 49]. However, the group of HER2-positive may have its own characteristics due to its biochemical structure. In these breast molecular subtypes, TNBC has a clear advantage, which is consistent with the clinical results that more high degree of malignancy [50-52]. The Amide I and Amide II vibrations of the protein are almost independent of the side chain vibrations, which depend on the secondary structure of the main chain and are the most commonly used amide vibrations for secondary structure analysis [53]. So Amide I-to-Amide II ratio is used as a potential biomarker to explore the changes of protein secondary structure in tumour tissue. The result indicated that the proportion of tumour tissue was increased, especially with respect to TNBC. However, there is no difference of Amide I-to-Amide II ratio in Cohenford's study of cervical cancer smears [54]. We suspect that this ratio may be related to the alterations of the secondary structure (C=O stretch and changes in β-sheet formation) of the protein in different subtypes, and it might be related to the progression of the tumour [55]. Even more exciting was the fact that the trend of Lipid-to-protein ratio, RNA-to-DNA ratio, phosphate-to-carbohydrate ratio and Amide I-to-Amide II ratio remained consistent: all of the ratios in each subtype were higher than that in normal breast tissue with a different degree of increase, while normal tissue and fibroadenoma shared similar ratios (Fig. 5C). These results may provide information about the metabolism and transformation in cancer cells [56]. It may also predict a similar biochemical change in different subtypes of breast cancer. Considering that the difference in age may affect

the results obtained, we have further analyzed and explained the influence of age on our results. We try to stratify all cases by the median age (46 years), and explored the mean ±SD of the spectral points respectively. Spectroscopy results indicate that there is no significant statistical difference between the different age groups in the tumour tissue (Fig. 6, $p>0.05$). It needs further explanation that the statistical significance of normal tissues in different age groups may be caused by the sample size. In other words, age alone has not significantly altered our results.

Previous studies have used spectroscopy to study breast cancer but almost none took into consideration the different molecular subtypes as their main focus was to use spectroscopy as a screening, diagnostic or monitoring tool for breast cancer. [57, 58]. Other similar studies have shown that this tool can also classify grades of pathological breast tissue, monitoring treatment effects and identify distinctive biomarkers in each category [59, 61]. This proves that FTIR spectroscopy can not only identify breast cancer, but also precisely recognize peak assignments associated with the "bio-fingerprint" region and assess their corresponding contribution. In addition, it was also found that different molecular subtypes can be distinguished and they have distinct peaks ratios via the sensitive IR spectroscopic method. These peak ratios may be used biomarkers of breast cancer between the different molecular subtypes.

It is well-known that the prognosis of patients with breast cancer is increasingly dependent on early diagnosis and pathologic subtype results [62]. So far, histopathological identification has been the gold standard for the diagnosis of breast cancer and the principal means to identify different subtypes. The proliferation marker Ki-67 has been shown to be an independent predictor and prognostic factor in early breast cancer [63]. Additionally, identification and typing of breast cancer are widely used in decision-making and follow-up treatment programs for a mammary surgeon or oncologist. In terms of the current pathological diagnosis of breast tumour types, it is necessary that at least two professional and technical personnel are present for observation and analysis. However, the pathological tissue collection requires an invasive procedure, a certain pre-treatment time and, to a certain extent, is dependent on the subjective judgment of pathologists. Fortunately, we have found that using FTIR spectroscopy as a tool for tumour diagnosis and typing can overcome these disadvantages, and even has the potential to determine the surgical margins allowing for more accurately excised tumour tissue. In the meantime, the intensity ratio of the

associated characteristic peaks can be used as a proliferation index and biomarker for the different tumour subtypes. Recent research using plasma to diagnose and distinguish different diseases is also a future research direction [64]. Currently, however, we are still unable to precisely assign the spectral peaks to specific molecules, due to the complexity of the structural changes in the process of tumourigenesis. Other limitations of the study include the small number of cases studied and unpredictable disease severity. It is necessary to establish a database to identify distinct tumour subtypes using FTIR spectral markers in the future. Further validation of these approaches exploiting other bio-spectroscopic or nanotechnology-based techniques using larger and architecturally more robust datasets is also required.

Conclusion

In this exploratory study, we demonstrate that ATR-FTIR spectroscopy can easily identify and classify breast tumour subtypes after the interrogation of breast tissue from breast mass. Multivariate analysis of the spectral information revealed specific changes associated with malignant tumour subtypes, especially the structural changes of proteins in different molecular subtypes of breast cancer. The responsible changes for this segregation were primarily alterations in the lipid-to-protein, RNA-to-DNA, phosphate-to-carbohydrate, Amide I-to-Amide II, RNA-to-lipid and nucleic acid-to-lipid ratios, with a marked increase associated with tumour progression and subtyping. The difference of the peak intensity ratio of FTIR spectra reflects the change of the macromolecule structure in the tissue to a great extent and can be used as a potential biomarker to predict the molecular subtype of discrete tumour. In conclusion, ATR-FTIR spectroscopy can easily identify and classify breast tumour subtypes after the interrogation of breast tissue from breast mass, which could become an additional tool for the pathological interpretation of breast cancer.

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Compliance with ethical standards

Conflict of interest FLM is a shareholder in Biocel UK Ltd, a company seeking to develop spectrochemical tools and algorithms for commercial gain. All remaining authors declare that they have no competing interests.

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