X-ray diffraction method to identify epithelial to mesenchymal transition in breast cancer tissue

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Abstract. Breast Stromal tissue is significantly involved in the spread of cancer because of molecular variations. The main component, collagen, has a spatial arrangement allowing the investigation of its structural patterns in tissues by X-ray diffraction (XRD). Since detection and classification of tumor type and stage from the pathological examinations is a laborious task that additionally does not allow cancer detection in the early stages, development of new tools to improve the diagnostic power of pathologists is still needed. In this study, the potential used of XRD techniques as one of the method for early detection of breast cancer was done. Four pathological cancerous breast tissue samples were taken from four different patients; where two samples are with epithelial-to-mesenchymal transition (P-EMT) and two samples are without epithelial to mesenchymal transition (N-EMT). The latter was incorporated at an angle interval of 10° to 80° to obtain interference diffraction pattern of XRD of human malignant tissues. This was done to determine molecular structure changes of collagen fiber within the tissue and their potential relation to the changes in cancerous tissue toward metastasis. The results showed that the momentum transfer values for the first region of N-EMT,P-EMT cancerous tissue are not significantly different for both cancerous breast tissue types, being at 1.61 ± 1.74 nm\(^{-1}\). The average peaks of the second region were determined for N-EMT at 3.4 ± 3.5 nm\(^{-1}\) and P-EMT at 3.6 ± 3.7 nm\(^{-1}\) which is attributed to water content of the tissues, due to the greater intensity of the P-EMT compared to that of N-EMT. Principal component analysis (PCA) used to confirm statistical appropriateness of the results, showed a normal distribution within 95% confidence level. P-EMT clusters have a larger number of scatter plots compared to N-EMT, which indicates a higher similarity between P-EMT samples than between N-EMT samples, thus confirming the difference between positive and negative-EMT clusters. The current analysis can differentiate XRD features and thus confirms its effectiveness in detecting the molecular correlation of abnormal collagen fiber structure within epithelial change, and is useful for early diagnosis even case of dense breasts.

1. Introduction
Breast cancer is the most frequent malignancy in females with over 1.6 million newly-diagnosed cases yearly [1]. The early stage of breast cancer starts with uncontrolled growth of aggressive cells. When breast epithelial cells undergo regular periods of proliferation and apoptosis, their mutations may cause cancer. These tumor cells are usually diagnosable in a physical checkup or X-ray graph. However, the pathology can determine the grade of malignancy, which is extremely important as a clinical guide to the treatment options [2]. It is widely accepted that early-stage detection of breast cancer before the metastasis stage gives the patient a higher chance of cure [3]. The regular procedure of breast cancer diagnosis after a physical check-up is an ultrasound, mammography and magnetic resonance imaging
(MRI). Apart from methods to detect breast cancer, breast biopsy techniques are also used to
differentiate between cancer types, but these are costly, and time-consuming with inherent limitations.
for example, ultrasound may fail to detect some tumors due to the similarity in the acoustic properties
of normal and abnormal tissues [4], while in the case of MRI, in addition to being relatively costly and
time-consuming, it is also not appropriate for patients with claustrophobia or metallic implants [5].
Typically, in mammography, contrast between abnormal and fibro glandular tissues is low, which makes
it difficult to detect small lesions, causing up to 50% of breast carcinomas not being diagnosed in the
first round of mammography tests. Mammography is known to be generally less efficient with patients
under the age of 40 years and in detecting tumor sizes of below millimeter dimension or less than 105
cells [5]. Statistically, most of the tumors detected by mammography have a size ranging from 1 to 2
cm, while most of the MRI detected lesions are smaller than 1 cm.

The use of XRD to analyze the molecular structure of crystalline materials has been the traditional
practice. It has been shown that coherent scattering can be used to determine the average inter-molecular
spacing in biological samples [6]. Many XRD measurements on biological materials have highlighted
the changes in unhealthy tissues which can also be seen as cancer fingerprint [7]. These changes can be
related to an improper structure of collagen, caused by a breast malignant lesion [8]. Many studies have
indicated the potential of XRD to discriminate tissue types due to the alteration of collagen fiber within
tissues [6][9][10]. The early stages of metastasis are typified by the tumor cells migrating and advancing
by way of the stroma into the circulation via epithelial-to-mesenchymal transition (EMT) [11]. The
EMT is a well-defined developmental progress frequently adopted by tumor cells during the metastatic
process. Cells acquire a more spindle-like fibroblastic morphology and reach the distant organs, losing
epithelial cell adhesion and cell-cell contacts increase their stroma interactions and invasive behavior
[11][12]. But, the biological-effects of EMT are assorted and complicated. Linked features of a tumor-
cell may be different depending on the tissue type [13]. In the specific case of breast cancer, many
studies have proposed that cancer cells can hijack EMT to invade, showing the EMT’s importance in
breast cancer’s invasion, growth, and spread. Elevation of cell adhesion and collagen, as well as high
motility of cells, are signs of the tumor invasion [14].

In this study, the molecular structure of the breast cancer was studied in terms of collagen fiber
alteration and its association with EMT, to provide improvement for early diagnosis of breast cancer.
To achieve this, two different breast cancerous tissue types were used to examine the ability of the XRD
technique to discriminate the histopathological behavior between malignant tissues based on their stage.

2. Materials and methods

2.1 Tissue samples
Four cancerous breast tissue samples were taken from histopathology examination of four patients; two
samples P-EMT and two samples N-EMT. These patients underwent a breast-conserving mastectomy
where samples were between 3 to 5 μm thickness and not more than 2 × 2.5 cm² dimensions. To allow
tissue fixation and preservation, samples were immersed into 10 % neutral buffered formalin and then
impregnated with paraffin.

2.2 X-ray diffraction
XRD is a non-destructive test to analyse crystalline material and can be used to provide detailed
information on molecular structure of the material. It works based on the elastic or coherent scattering
of x-ray photons by atoms in an intermittent lattice. When X-rays incidence on material, they may
interact with the atomic electrons coherently or incoherently (Compton scattering). Diffraction occurs
through coherent scattering from the space between atomic planes (d) resulting in constructive interfere
with the scattering angle (2θ). Equation 1,

$$nλ = 2d \sin \theta$$  \hspace{1cm} (1)
where \( n \) is the order of reflection and is an integer, while \( \lambda \) is the X-ray wavelength. XRD patterns data were converted into values of momentum transfer by Equation 2, as follows:

\[
X (\text{nm}^{-1}) = \left( \frac{E}{hc} \right) \sin \frac{\theta}{2}
\]

where: \( E, h, c, \) and \( \theta \) are incident photon’s energy, the Planck constant, the light’s speed, and scattering angle, respectively. The XRD scan was performed with a BRUKER-binary V3 (RAW) diffractometer at room temperature with copper as the anode target material and value of Ka1, Ka2 and Kβ being 1.540, 1.544 and 1.390 keV respectively. The accelerating potential of electrons to produce X rays was 40 kV while the current was 40 mA. The range of scattering angles measured varied from 10° to 80° of the momentum transfer between 1.40 nm\(^{-1}\) and 11.03 nm\(^{-1}\).

The XRD scan was performed with a BRUKER-binary V3 (RAW) diffractometer at room temperature in a setup shown in Figure 1 with copper as the anode target material. The accelerating potential of electrons to produce X rays was 40 kV while the current was 40 mA. The range scattering angles measured varied from 10° to 80° corresponding to the momentum transfer interval of between 1.40 nm\(^{-1}\) and 11.03 nm\(^{-1}\).

2.3 Statistical analysis
The XRD data were analysed statistically by IBM SPSS Statistics 22.0 software to distinguish between tissue types and to classify the XRD pattern. The graphs were plotted using Minitab.

3. Result and discussions

3.1 XRD results
The momentum transfer values were calculated from XRD scattering results of P-EMT and N-EMT cancerous samples of four different patients as shown in Figure 1. Figure 1(a) shows XRD spectrum for N-EMT samples and Figure 1(b) shows XRD spectrum for P-EMT samples. In order to compare the results, XRD patterns were divided into two regions of scattering angle between 5° to 9° (region 1) and between 9° to 17° (region 2) that corresponded with the momentum transfer values of 1.4 to 2.4 nm\(^{-1}\) and 2.4 to 5 nm\(^{-1}\). In region 1 of both P-EMT1, P-EMT2, and N-EMT1, N-EMT2 specimen types (a and b), sharp peaks were observed at 1.61 to 1.74 nm\(^{-1}\) at scattering angle around 6° which is attributed to the prevalence of fibrous tissue characterized by water components, and in agreement with previous studies [9, 15-20] in peak position for initial photon energy of 40 keV, malignant tissue and, abnormal fibre portion around 47.8%. The number of uncommon structures of collagen fibres for different pathological types of tissue has been reported around 52.2 % [21].
Figure 1. XRD spectra of all of the cancerous breast tissues a) (N-EMT) b) (P-EMT).

Momentum transfer values are characteristics of the material and can reveal information on the spacing between atomic planes in its crystalline or amorphous structure [15]. Due to the molecular space the first two neighbours in these cells being larger and interconnected on a large spatial range, it results in new order with a higher degree [22]. The region of momentum transfer below 2.5 nm$^{-1}$ represents specimen interference found in most of biological samples [23]. Oliveira, et al. (2008) reported the signature peak of the connective tissue and the tumor to be at 1.5 ± 0.7 nm$^{-1}$ [10].

Table 1. The values of the diffraction pattern parameters obtained from region 1.

| Tissue type | Peak position (Θ) | FWHM (nm$^{-1}$) | d-spacing |
|-------------|-------------------|------------------|-----------|
| N-EMT1      | 5.9               | 0.49             | 0.33      |
| N-EMT2      | 6.2               | 0.42             | 0.34      |
| P-EMT1      | 5.7               | 0.53             | 0.31      |
| P-EMT2      | 6.1               | 0.31             | 0.77      |

From Table 2, the differences in the peak height noticed in Region 1 between N-EMT1 and N-EMT2 were found to be 0.116 to 0.119 while P-EMT1 and P-EMT2 were higher at around 0.121 to 0.123 respectively. Poletti et al. [9] stated that the values of the scattering intensity of tumor parts are greater than the health tissue region. Due to the existence of the collagen fibre [24], there is ample evidence indicating that of the extracellular matrix of breast tissue carcinoma undergoes change on its structure and composition [25]. The supra-molecular structure of collagen fibrils' alteration has been linked as a major component of the extracellular matrix because of the tumor existence [25][26]. Weaver [27] found that the healthy or malignant epithelial cells start migrating when they come into direct contact with collagen. Where interference effects are big and the angular distribution peak is at a certain angle, this angle depends on the photon's energy, type, and structure of the tissue. This lends support to previous findings [26]. Also, the peak heights among tissues can be related to the interaction between water molecules, which are a major component of glandular tissue. For instance, it has been found that a full width half maximum (FWHM) is more suitable than peak location for differentiation of water content between tissues [28]. From Table 2, the value of FWHM for all cancerous tissues was found higher in P-EMT from 0.31 nm$^{-1}$ to 0.53 nm$^{-1}$ than N-EMT with 0.42 to 0.49 nm$^{-1}$. Average peaks shown in Table
2 refer to the second region, showing the effects of the molecular interference for the case of all specimens. These sharp peaks started at position $\theta = 9^\circ$ to $17^\circ$. On average, it is concluded that momentum transfer of between 2.5 and $4.4 \text{ nm}^{-1}$ refers to the water content, which is in agreement with previous reports [18][19]. Also, [10][9] reported breast tissue momentum transfers of between 0.5 and $4 \text{ nm}^{-1}$ in agreement with the present findings.

Table 2. The values of the diffraction pattern parameters averagely obtained from region 2.

| Tissue type | Peak position ($\Theta$) | FWHM (nm$^{-1}$) | d-spacing |
|-------------|--------------------------|-----------------|-----------|
| N-EMT1      | 3.5                      | 0.32            | 0.0119    |
| N-EMT2      | 3.4                      | 0.25            | 0.0105    |
| P-EMT1      | 3.6                      | 0.28            | 0.0111    |
| P-EMT2      | 3.7                      | 1.89            | 0.0101    |

3.2 Principal component analysis result

PCA score plots were performed for all samples and two PCA models were built. From Fig. 2, score plots of the first component (PC1) for N-EMT samples and those of the second component (PC2) for P-EMT samples were categorized. Randomly, 30% of the two subgroups were picked out for all 4 samples. The score plots of the same tissue type samples have similar perpendicular alignment [29]. From the result, the level of confidence is 95%; cluster 1 has less similarity than cluster 2, which confirms the statistical difference between two clusters. The smaller number of scatter plots in cluster 1 indicates a big difference between N-EMT1 and N-EMT2. On the other hand, cluster 2 has a large number of score plots that indicates the big similarity between P-EMT1 and P-EMT2 tissues. As a result, this analysis has the potential to differentiate between cancer stages.

Figure 2. Principal component analysis of tissue type

Thus, the molecular structure of collagen fibre from a sample tissue type can help in identification of composition change in tissue. Furthermore, usefulness of PCA to detect a correlation between breast tissue types was shown. Therefore, this result encourages and suggests the use of XRD for bulk tissues classification and early detection of breast cancer [30].

4. Conclusion

In this work, a comparison was made between the malignant breast tissue types by XRD method considerable change of scattering angle was found between tissue types, and the intensity of the
diffraction pattern was the only parameter changed by difference in the molecular structure of collagen. This was found useful to distinguish between positive and negative EMT pathological tissue types. The peaks correspond to water and fibrous content within the tissue, from the P-EMT was found to have greater intensity compared to that of N-EMT.

The results of statistical analysis demonstrated a significant difference between P-EMT and N-EMT samples. PCA data provided support in differentiating various types of tissue using XRD pattern. The current work suggests that XRD has the potential to be used in early screening tests for detection of breast cancer patients, and thus can assist to classify the cancer tissue histopathology.

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