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Emerging Patterns in the Distribution of Trace Elements in Ovarian, Invasive and In-Situ Breast Cancer

Emerging Patterns in the Distribution of Trace Elements in Ovarian, Invasive and In-Situ Breast Cancer

A Al-Ebraheem[1], E Dao[1], K Geraki[2], and M J Farquharson[1]
[1] *Department of Medical Physics and Applied Radiation Sciences, McMaster University, 1280Main St W Hamilton, Ontario, L8S 4K1, Canada*
[2] *Diamond Light Source, Harwell Science and Innovation Campus, Didcot, Oxon, OX11 0DE,UK*
Email: farquhm@mcmaster.ca

Abstract: Breast cancer is the most common cancer and ovarian cancer is the 8th most common cancer affecting women worldwide. This study highlights the changes of trace element levels accompanied by the progression from ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC) of the breast, using micro probe Synchrotron Radiation X-ray Fluorescence (μSRXRF). The average values for the increase in Ca, Fe and Zn in tumour regions with respect to surrounding regions for the DCIS samples were significantly higher compared to the increase in the IDC samples (P <0.01).This study was also carried out to find a connection between ovarian cancer and breast cancer with respect to the cellular distribution of Ca, Cu, Fe, and Zn. For IDC, DCIS and ovarian cases, the statistical analysis reveals a significant increase in the levels of Ca, Cu and Zn concentrations in cancer tissue when compared to the normal surrounding tissue. For Fe, the differences between tumour regions with respect to surrounding regions were found to be not significant in IDC and ovarian cases. In DCIS cases, the results reveal a significant increase in the levels of Fe in cancer tissue when compared to the surrounding normal breast tissue (P <0.01).

Introduction

The most common type of breast cancer for women is mammary ductal carcinoma and it presents in two forms: invasive ductal carcinoma (IDC), an infiltrating, malignant and abnormal proliferation of neoplastic cells in the breast tissue; or ductal carcinoma in situ (DCIS), a noninvasive, potentially malignant cancer, where abnormal cells are restricted to the ducts. About 50% of untreated DCIS lesions are believed to rapidly transit to IDC, with a high degree of variability in this progression [1]. Our work to date has investigated the cellular distribution of Ca, Cu, Fe and Zn in IDC of breast tissue using micro probe Synchrotron Radiation X-ray Fluorescence (μSRXRF) [2-5]. These studies reveal an average increase for Ca, Cu and Zn levels in areas of malignancy. The levels of Fe on average showed an increase but in some samples there was a decrease in the tumour regions. The first part of the study reported here will focus on the investigation to determine whether the progression from DCIS to IDC is accompanied by changes of trace element levels.

Ovarian cancer is the 8th most common cancer affecting women in the world [6]. Rosen DG *et al*. reported that ovarian cancer is responsible for 140,000 deaths around the world each year and has the lowest survival rate of all female cancers. Only 45% of patients are likely to survive after 5 years of diagnosis [7]. The most recent data regarding ovarian cancer reports that in 2008, there were 12.7 million new cancer cases; 225,000 of which were ovarian cancer [6]. Ovarian cancer is often diagnosed as a secondary cancer alongside an initial primary cancer. Cases have been reported where ovarian cancer is diagnosed in addition to breast cancer and it has been found that women inflicted with breast cancer or with family history of breast cancer are also at an increased risk of developing ovarian cancer. Consequently with ovarian cancer being so closely linked to breast cancer, the second part of the study reported here was carried out to find a connection between ovarian cancer and breast

cancer with respect to the cellular distribution of Ca, Cu, Fe, and Zn using μSRXRF. Finding a connection between the two diseases may provide insight with regards to many unknown factors of the two diseases as it has been found that breast and ovarian cancer tissues have similar properties, and thus the same biomarkers are able to be used to diagnose and monitor the disease progression in both cancers [8]. Utilising in vivo experiments and the use of chelators in mice, Merlot, *et al*. found that a slight decrease in Fe levels could be observed in the cancer cells [9]. It was also found that Cu levels typically tend to be elevated in both breast and ovarian cancer tissues. This trend is seen in different age groups and geographical locations of those inflicted with the diseases. The levels of Zn were also investigated and found to remain constant.

Samples:

The IDC, DCIS and ovarian samples were obtained from the Cancer Research UK Tumour Pathology Group, Department of Clinical Laboratory Sciences, John Radcliffe Hospital, University of Oxford. All samples were Formalin Fixed Paraffin Embedded (FFPE) tissues arranged as micro arrays of 1.0 mm diameter and 10μm thickness. For each tissue micro array (TMA), two slices were cut from the paraffin block, one being 10 μm thick, the other being 5 μm thick and cut adjacent to the 10 μm slice. The 5 μm thick slice was mounted on a standard glass slide and then stained using Hematoxylin and Eosin stain (H&E) staining. The H&E slide was then optically imaged to produce high resolution images which clearly identify tumour regions in the samples and can be used as a reference slide for the elemental distribution maps produced from the experimental slide. The 10 μm thick slide was mounted on 4 μm Ultralene XRF film that was stretched and held in a bespoke frame.

Method:

The IDC, DCIS and ovarian sample measurements were carried out using the Synchrotron Radiation X-ray Fluorescence (SRXRF) at beamline I18, DIAMOND, UK and FLUO beamline at ANKA, Karlsruhe, Germany**.** Figure 1 shows the sample micro-array slide and a schematic of the experimental set-up. Figure 1 also shows the 90º geometry between the incident beam and the detector, which is maintained to utilise the strong polarization of the synchrotron photon beam. This arrangement provides significant suppression of the scattered incident photons in the plane of the electron orbit, while the fluorescence signal which is required for the measurement remains unaffected. The sample position is set at 45º to the incident photon beam. The experimental set up took on a similar form at the two facilities with specific details at each Beamline as given in table 1. The data collected at each point in the scan is in the form of an elemental spectrum that is collected using a Si (Li) detector (Oxford Instruments) at the FLUO beamline and a 4-element Vortex SDD detector at beamline I18. These spectra were analysed using AXIL and PyMCA programs respectively both of which are designed to fit the XRF peaks, remove the background and separate the overlapping peaks. The final metal distribution for each metal is composed by the element's peak intensity in each sample position. Maps of the Zn, Fe, Cu and Ca distributions were produced using IDL v6.3 (ITT Virtual Information Software). This program enabled regions of interest to be set on the maps encompassing tumour cell regions and regions of surrounding non-tumour tissue which is useful for further statistical analysis. Statistics obtained from these regions including mean pixel value and the standard error of the means. The parameter compared is the mean of Ca, Fe, Cu and Ca levels in tumour (T) and normal (N) regions. When comparing the changes of trace element levels between IDC and DCIS cases, the parameter compared is the average value for the percentage difference in the metal levels in tumour regions with respect to surrounding normal regions for each case (i.e for each element ((Tumour content-Normal content)/Normal content)*100%. The Shapiro–Wilk test was used for each element in each category to test if the data set was consistent with a Gaussian distribution function. For IDC, DCIS and ovarian tumour and normal data, Wilcoxon Rank test was used for comparing the difference in Ca, Fe, Cu and Zn levels for non normally distributed related data, while paired t-test was used for normally distributed related data. For comparing the percentage difference in Ca, Fe, Cu and Zn levels

in IDC samples with respect to DCIS samples, *P* values from Mann Whitney U and Independent t test were obtained.

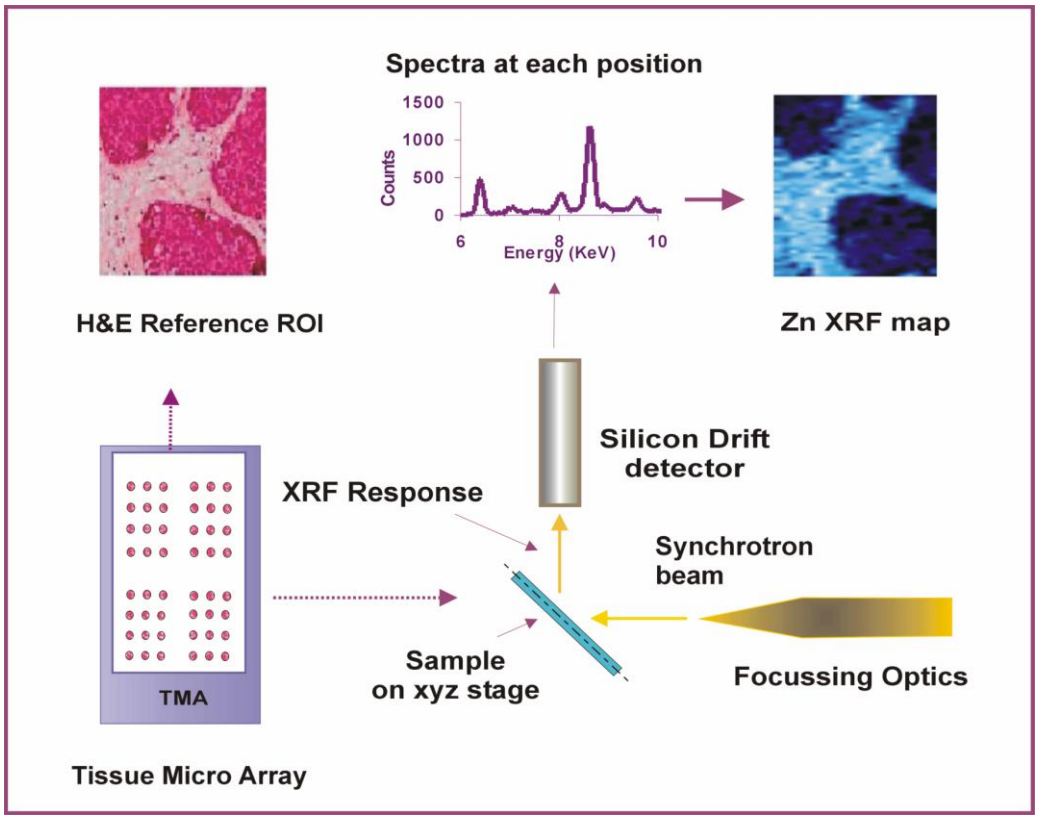

Figure 1: Schematic diagram showing samples arranged as a tissue micro array and mounted on a sample holder held on an xyz translator stage. The sample array is positioned at 45° to the synchrotron beam.

Beam line	FLUO at ANKA	I18 at DIAMOND
Incident Energy	10.1 keV	11 keV
Beam Size	5 μm x 15 μm	5 μm x 5 μm
Time/Pixel	Varied	4 seconds
Detector	Si(Li) detector (Oxford Instruments)	4-element Vortex detector
Fitting software	AXIL program	PyMCA program
Final parameter compared	Total metal Kα counts	Total metal Kα counts normalised to the incident scatter peak area

Table 1: μSRXRF beamlines experimental set up details.

Result and Discussion:

1- Elemental Mapping :

DCIS, IDC and ovarian carcinoma samples were scanned using a μSRXRF technique. Figure 2 shows an example of the elemental distributions maps of Zn, Fe, Cu, and Ca in two samples of DCIS tissue, two samples of IDC tissue and two ovarian tissue samples. The dark areas of the H&E stained reference image show the tumour areas, and the lighter regions are the normal tissue. Correlations were found between the stained reference images and the elemental distributions indicating an increase in element concentrations in most of the tumour regions of the sample (darker coloured areas correspond to higher metal content), but it can also be seen that the elemental distribution of Fe in some samples displays the reverse behaviour within the tumour areas with respect to the normal.

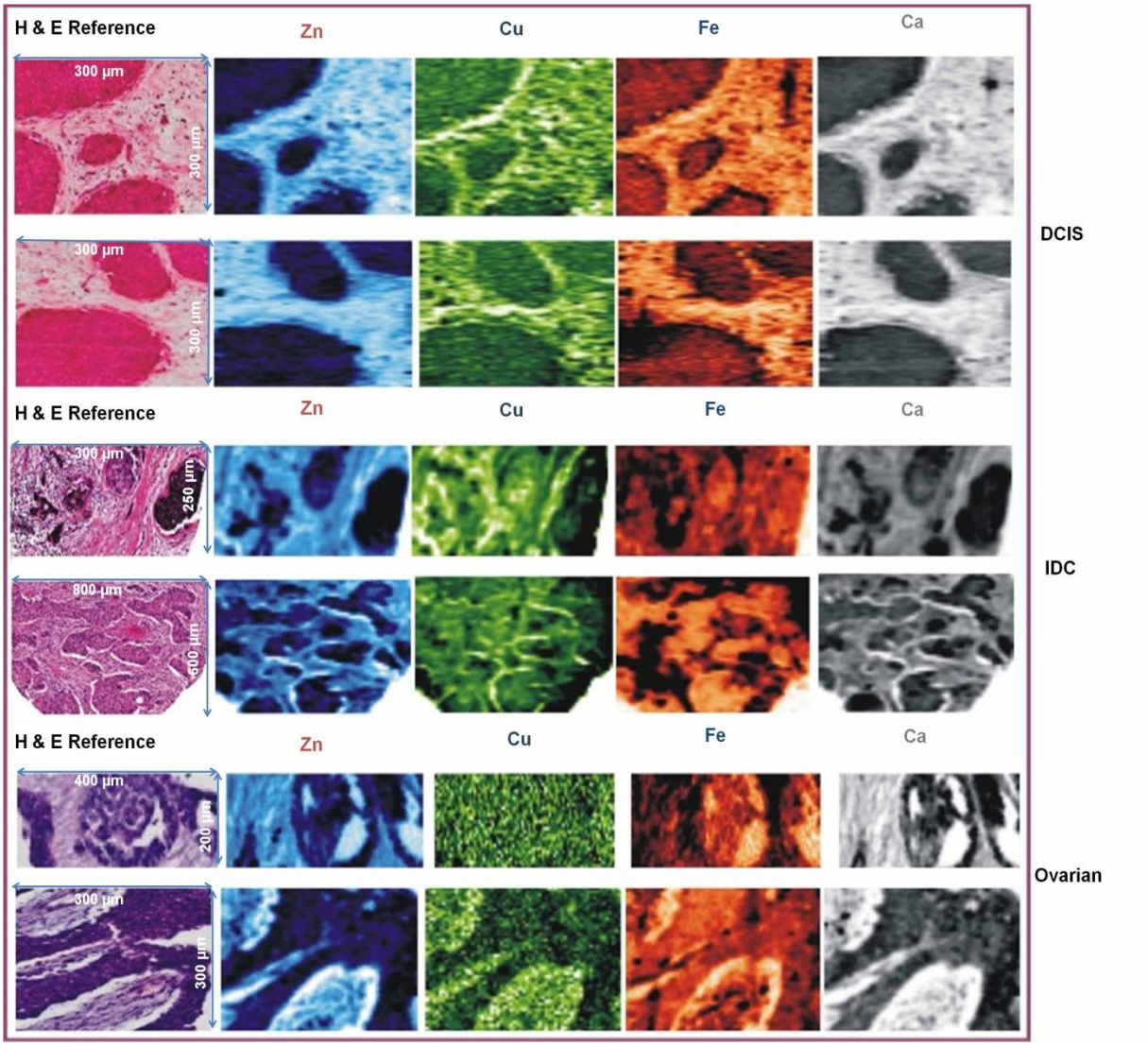

Figure 2: Examples of sample maps, showing the reference H&E stained reference image with the corresponding Ca, Zn, Cu and Fe distribution maps. Examples are shown of DCIS, IDC and ovarian carcinoma.

2- Elemental concentration in IDC and DCIS samples:

The relative levels of Zn, Cu, Fe and Ca were estimated in 20 IDC and 20 DCIS FFPE samples and normal surrounding breast tissue. Table 2 shows a summary of the descriptive statistical parameters including the mean, the standard error of the means and the *P* values for comparing the difference in Ca, Fe, Cu and Zn levels in tumour regions (T) with respect to surrounding normal regions (N) for DCIS and IDC samples.

Groups	Mean (Counts) (n=20)	Std. Error Mean (Counts)	*P* values
IDC-Ca-T	2144.73	420.78	<0.01*
IDC-Ca-N	1083.15	208.05	
IDC-Fe-T	140.61	33.45	0.84
IDC-Fe-N	129.60	26.14	
IDC-Cu-T	51.07	17.41	<0.01*
IDC-Cu-N	38.06	11.97	
IDC-Zn-T	282.84	75.62	<0.01*
IDC-Zn-N	109.83	22.79	
DCIS-Ca-T	3223.68	180.55	<0.01*
DCIS-Ca-N	1050.95	79.48	
DCIS-Fe-T	96.30	10.57	<0.01*
DCIS-Fe-N	38.12	4.98	
DCIS-Cu-T	80.70	7.95	<0.01*
DCIS-Cu-N	57.76	5.00	
DCIS-Zn-T	201.75	12.61	<0.01*
DCIS-Zn-N	41.39	2.81	

Table 2: Statistical summary of the mean and the standard error of the means of Ca, Fe, Cu and Ca levels in tumour (T) regions with respect to the normal (N) regions in DCIS and IDC sample. The corresponding *P* values from Wilcoxon Rank test (2-tailed).*Significantly different at $P < 0.01$.

The statistical analysis reveals an average increase in the levels of Ca, Cu and Zn in IDC tissue when compared to the normal surrounding breast tissue ($P < 0.01$). For Fe, the differences between tumour regions with respect to surrounding regions were found to be not significant in IDC cases ($P = 0.84$). In DCIS cases, the results reveal a significant increase in the levels of Ca, Fe, Cu and Zn levels in DCIS tissue when compared to the surrounding normal breast tissue($P < 0.01$). Table 3 shows a summary of the descriptive statistical parameters including the percentage difference of the mean levels in tumour regions with respect to surrounding normal regions for IDC and DCIS samples (i.e for each element ((Tumour content-Normal content)/Normal content)*100%, the standard error of the percentage difference of the means, and the *P* values for comparing the percentage difference in Ca, Fe, Cu and Zn levels in IDC samples with respect DCIS samples.

Groups	% Difference of the means (Counts) (n=20)	Std. Error Mean (Counts)	*P* values
IDC-Ca	91.42	9.99	<0.01*[2]
DCIS-Ca	229.80	24.91	
IDC-Fe	19.29	15.13	<0.01*[1]
DCIS-Fe	178.32	37.61	
IDC-Cu	33.84	5.38	0.397[2]
DCIS-Cu	40.96	6.31	
IDC-Zn	127.75	17.80	<0.01*[1]
DCIS-Zn	419.65	44.44	

Table 3: Statistical summary of the percentage difference of Ca, Fe, Cu and Ca levels in tumour regions with respect to the normal regions for IDC and DCIS samples, the standard error of the percentage difference of the means, and the *P* values for comparing the percentage difference in the mean metal levels in IDC samples with respect DCIS samples. The corresponding *P* values from Mann Whitney U[1] (2-tailed) Independent t test[2] (2-tailed). *Significantly different at $P < 0.01$.

The null hypothesis in this part of the study is that: the percentage differences in the metal levels in the two groups of cancer (IDC and DCIS) are equal. The null hypothesis is rejected when the p-value is less than 0.05. This test will enable us to assess if the progression from DCIS to IDC of the breast is accompanied by significant metal changes. The average values for the percentage increase in Ca, Fe and Zn in tumour regions with respect to surrounding regions for the DCIS samples were significantly higher compared to the percentage increase in the IDC samples ($P < 0.01$). The percentage difference between tumour and normal regions of Cu is not significantly higher in DCIS cases compared to the IDC cases. The results presented in our most recent publication [5] that combined data from 128 formalin fixed paraffin embedded IDC samples indicate the importance of correlating the changes in elemental levels to the clinical prognostic factors such as oestrogen receptor (ER) status. In order to take this into account the DCIS and IDC samples were classified on the basis of their ER status. Table 4 shows a summary of the results including only the ER +ve group DCIS and IDC sample. The clinical data was not available for all the cases, therefore a comparison of the ER negative (-ve) group has not been included.

Groups ER +ve	% Difference of the means (Counts) (n=6)	Std. Error Mean (Counts)	*P* values
IDC-Ca	114	18	0.14
DCIS-Ca	203	72	
IDC-Fe	-9	11	0.033*
DCIS-Fe	268	119	
IDC-Cu	30	8	0.36
DCIS-Cu	56	26	
IDC-Zn	166	43	0.04*
DCIS-Zn	337	77	

Table 4: Statistical summary of the percentage difference of Ca, Fe, Cu and Ca levels in tumour regions with respect to the normal regions for IDC and DCIS ER +ve samples, the standard error of the percentage difference of the means, and the *P* values for comparing the percentage difference in the mean metal levels in IDC samples with respect DCIS samples. The corresponding *P* values from Independent t test (2-tailed). *Significantly different at $P < 0.05$.

For the ER +ve DCIS and IDC samples, the statistical analysis reveals that the percentage difference in Fe and Zn in tumour regions with respect to surrounding regions for the DCIS samples were significantly higher compared to the percentage in the IDC samples.

3- Elemental concentration in ovarian cancer samples:

This part of the study examined the cellular distribution of the trace of the same four elements in 20 FFPE ovarian cancer samples and normal surrounding ovarian tissue. Table 5 shows a summary of the descriptive statistical parameters including the mean values and the *P* values for of the comparisons.

Groups Tumor (T) and Normal (N) ovarian tissue	Mean Normalised Counts (n=20)	Std. Error Mean Normalised Counts	*P* values from
Ca-T	0.026434	0.00226	<0.01[1]*
Ca-N	0.011694	0.00105	
Fe-T	0.004483	0.00024	0.14[1]
Fe-N	0.004053	0.00017	
Cu-T	0.000651	0.00002	<0.01[1]*
Cu-N	0.000614	0.00002	
Zn-T	0.006005	0.00038	<0.012*
Zn-N	0.003191	0.00021	

Table 5: Statistical summary of the mean and the standard error of the means of Ca, Fe, Cu and Ca levels in tumour (T) regions and the normal (N) regions of the ovarian samples. The corresponding *P* values from paired T test[1] (2-tailed) or Wilcoxon test[2] (2-tailed).*Significantly different at $P < 0.01$.

The null hypothesis in this part of the study is that: the trace element levels in tumour and normal ovarian tissues are equal. When the p-value is less than 0.05, the null hypothesis is rejected. The statistical analysis reveals a significant increase in the levels of Ca, Cu and Zn concentrations in ovarian cancer tissue when compared to the normal surrounding ovarian tissue. For Fe, the differences in between tumour regions with respect to surrounding regions were found to be not significant.

Conclusion:

In the first part of the study, the analysis reveals a significantly higher percentage difference in Ca, Fe and Zn in tumour regions with respect to surrounding regions for the DCIS samples compared to the percentage difference in the IDC samples. When the samples were reclassified in terms of their ER +ve status the statistically significant differences between the two types of breast tumour are again observed for Fe and Zn. The Fe result is in agreement with our previous study [5] which showed that imbalance in Fe concentration (deficiency) should be viewed as an important risk factor that is associated with aggressive features of the cancer. The second part of the study shows that the trend of the differences in the levels of Ca, Fe, Cu and Zn elements is similar between the IDC and ovarian cancers. For IDC and ovarian cases, the statistical analysis reveals a significant increase in the levels of Ca, Cu and Zn concentrations in cancer tissue when compared to the normal surrounding tissue. For Fe, the differences between tumour regions with respect to surrounding regions were found to be not significant in IDC and ovarian cases. This research will serve as a base for future studies aimed at exploring advanced cancer treatment options such as chelators that target metal concentrations.

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