The distribution of trace elements Ca, Fe, Cu and Zn, and the determination of copper oxidation state in breast tumour tissue using μ SRXRF and μ XANES.

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Micro distribution of trace metals and oxidation state of copper in breast carcinoma

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Abstract

A micro beam synchrotron x-ray fluorescence (μ SRXRF) technique has been used to determine the localisation of metals in primary invasive ductal carcinoma of breast. Nine samples were examined all of which were formalin fixed tissues arranged as micro arrays of 1.0mm diameter and 10 μ m thickness. Cu was the particular interest in this study although 2D maps of the elements Ca, Fe and Zn, which are also of physiological importance, are also presented. The distribution of these metals was obtained at approximately 18 μ m spatial resolution and compared with light transmission images of adjacent sections that were H and E stained to reveal the location of the cancer cell clusters. Correlations were found between these reference images and the elemental distributions indicating an increase in all element concentrations in the tumour regions of all samples, with the exception of Fe, which in some cases showed a reverse of this trend. On average over all samples the percentage difference

from the normal tissue elemental concentrations are $\text{Ca} \approx 67\%$, $\text{Cu} \approx 64\%$ and $\text{Zn} \approx 145\%$. Micro X-ray absorption near edge spectroscopy (μXANES) was used to estimate the oxidation state of Cu in 19 normal and 17 tumour regions spread over 5 samples. The shape and the position of both normal and tumour regions suggest that they contain mixtures of copper ions with a significant fraction of Cu^{2+} . However, the shape of the spectra does not exclude the presence of Cu^+ . Tumour regions were found to have a higher fraction of Cu^+ compared to the normal samples.

Introduction

The study of elemental concentrations in relation to a number of diseases, including breast cancer, has been an active area of research for several decades. Garg *et al* (1994) and Ng *et al* (1997) used Neutron Activation Analysis to study paired breast tissue specimens (healthy and tumours), while the same technique was employed by Kanas *et al* (1994) to compare samples of fibrocystic disease and fibroadenoma (benign breast tumour). X-Ray Fluorescence (XRF) has been used by Rizk and Sky-Peck (1984) to study paired samples, while Total Reflection XRF was utilised by Majewska *et al* (1997) to compare benign to malignant breast tumours. The physiological justifications for relating elemental concentrations and cancer are manifold, in accordance with the various functions of the elements. The trace element of particular interest in this work, copper, can be associated with functions that protect the body from disease but also with processes that facilitate its spread. For example, copper acts as a cofactor for antioxidant enzymes (superoxide dismutase, SOD) that have a role in the defence of the human organism against disease. Conversely, copper is also a cofactor for lysyl oxidase involved in invasion and extracellular matrix deposition. Copper ions can generate hydroxyl radicals more readily than iron can, and these may be related to tissue destruction. Previous work by this group has included the utilisation of an XRF system with a synchrotron radiation source to quantify levels of iron, copper, zinc and potassium in healthy and cancerous breast samples. The measurements were made on bulk samples of approximate mass 1g, and the results from these studies indicate elevations of the levels of all elements measured in the tumour samples. For details of this work see Geraki *et al* (2002, 2004). These previous measurements have clearly indicated the increased levels of copper in breast tumours when compared to healthy breast specimens, however, because these measurements were performed on bulk samples we have no means of localising where the metal is accumulating. Further evaluation of cellular localisation would be of interest for future therapy e.g. is the copper in the growing edge of the tumour, inflammatory cells or blood vessels in the stroma, or a response of normal tissues to cancer? This could be studied by analysing the localisation of the metal in the tumour and normal surrounding area.

Recent evidence has indicated that copper has a key role to play in angiogenesis and tumour growth (Martin *et al*. 2005). Copper-binding drugs have a prolonged stabilisation effect in

the anti-growth function of copper chelators is mainly due to their inducing the inhibition of angiogenesis, the creation of blood supply that sustains a growing tumour (Pan *et al*, 2002). However, the particular steps in the complete mechanism have not yet been fully unravelled. A possible function of copper is its role in inhibiting the function of superoxide dismutase 1 (SOD1) which is important for endothelial signalling and therefore tumour proliferation. Copper may also have a role in the metabolism of breast cancers, where the oestrogen receptor negative tumours that show the more aggressive phenotype actually expresses the RNA for this ceruplasmin gene. This is a major copper carrying protein and previously it was thought that this is only made in the liver and it is a key copper binding protein. Work carried out by the group in Oxford has also investigated superoxide dismutase 1 expression and lysyl oxidase expression and found that both these copper dependent enzymes are highly expressed in tumour cells compared to normal cells in breast cancer. However, this is not the same as being able to detect copper, which is also present in many other enzymes and may be bound to chaperones and it is of major interest to understand if some types of cancer have higher copper levels than others. However, there is no suitable stain sensitive enough to assess this in tissue sections. Our studies to date have shown it is possible to analyse the copper distribution within tissue micro array samples (Farquharson *et al* 2007), which have a relatively high throughput assessment compared to previous methods. Understanding which cell types express or contain copper at the highest level and differences between individual tumours based on other biology such as oestrogen receptor will be helpful in future development of copper chelation therapy.

Oxidative changes at cellular level are a main contributor to DNA damage and the subsequent carcinogenesis process (Borrello *et al*. 1993). Increased oxidative DNA base damage has been detected in the DNA of human breast tumour tissue (Malins and Haimanot 1991). It has also been shown that there is a clear association between excessive exposure to estrogens and the development of breast cancer (Harris *et al*. 1992). Cu^{2+} can mediate the oxidation of catechol estrogens, accompanied by the reduction of Cu^{2+} to Cu^{+} . This process involves generation of Reactive Oxygen Species (ROS) that cause oxidative DNA damage and can be blocked by adding a Cu^{+} specific chelator (Li *et al*. 1994). Moreover, Cu^{2+} plays a primary role in

promoting the Fenton reaction, in which ROS are also produced (Kremer 2006). Correlative increase of zinc and copper concentrations were found to be higher in breast cancerous tissue sections which might be explained by their association with the Cu/Zn (SOD) enzyme. SOD has different metal site stability and many studies have been concerned with the structural difference of copper sites between the reduced and oxidised Cu/Zn SOD enzyme. For each superoxide molecule, acyclic reduction of Cu^{2+} and reoxidation of Cu^+ is required in order to dissociate free radicals inside the cell (Bannister *et al.* 1987). Therefore, changes in the Cu or Zn binding ion may result in alteration in the regular function of SOD (Elliott 2001). X-ray absorption near edge structure (XANES) spectroscopy provides information about the valence state, local atomic structure and the binding configuration of the investigated element in the sample. Investigation in this experiment attempts to detect the valence state of Cu in healthy and cancerous breast tissue.

Recently, most XANES studies on biological tissue have focused on the Fe oxidation state (Chwiej *et al.* 2007; Dziedzic-Kocurek *et al.* 2006; Ide-Ektessabi and Rabionet 2005; Kwiatek *et al.* 2004). The reason for this, other than the role of Fe on the studied diseases, is the good agreement between the theoretical calculation and the experimental K-edge XANES spectra for Fe^{2+} (Chaboy *et al.* 2006). However, this is not the case for Cu^{2+} as the theoretical calculations failed to reproduce the experimental spectra as stated by (Briois *et al.* 1995) which may result in limiting the use of high resolution XANES analysis of Cu in living organisms (Ascone *et al.* 2005). Micro beam XRF and XANES spectroscopy were used to investigate Cu, Zn and Fe in biological samples that had neurodegenerative disorders and the quantification of Zn and Fe was possible in this study, while the low signal to noise ratio of Cu peaks limited this possibility (Ide-Ektessabi and Rabionet 2005). Other possible reasons for the limitation of using XANES in analysing the K edge of Cu in biological tissue is due to the photo reduction or radiation damage to the sample during data collection and heterogeneity of the sample. The photo reduction process depends on the metal site, the intensity of the beam and the exposure time. Despite these limitations, μXANES has been applied to investigate the Cu oxidation state of human Central Nervous System (CNS) tissue. The results of the study showed that most of the copper in the neurons for the sample population occur in the second oxidation state (Szczerbowska-Boruchowska *et al.* 2005). Moreover, XANES has become a

useful analytical technique for determining the oxidation states of metals in protein systems such as superoxide dismutase SOD, caeruplasmine and carbonic andyhydrase enzymes (Parsons *et al.* 2002).

Methodology

Measurements were made on individual samples (mounted on 4µm thick ultralene film) of a formalin fixed paraffin embedded tissue microarray of human primary invasive breast carcinomas. Measurements made on the ultralene film showed no signal from the elements of interest. The samples were obtained from the Cancer Research UK Tumour Pathology Group, University of Oxford, Nuffield, Dept of Clinical Laboratory Sciences, John Radcliffe Hospital, Oxford. Each section on the array was a section of 1.0mm diameter and 10µm thick.

The data was collected using the synchrotron X-Ray Fluorescence Microprobe (SY-XRF) at Hasylab, beamline L (HYMO) which is a powerful tool for simultaneous multi trace element analysis of microsamples. The white beam of a bending magnet source is monochromated by a double multilayer monochromator with a bandpass $\Delta E/E \sim 2\%$. The beam is focussed by a polycapillary half lens (X-Ray Optical Systems, Inc.) that provides a beam of 10-25 µm diameter, depending on energy. For the present study, the excitation energy was set to 12 keV, which is a compromise of maximum Cu signal due to high absorption cross section and minimum size of beam (18 µm FWHM@12 keV). By using this energy, data can be collected for Cl, Ca, K, P, S, Ti, Fe, Cu and Zn. Recent improvements in spectrometer sensitivity and detector count rate capability have improved the limits of detection for XRF. By reducing the measurement time per point, larger areas of sample can be scanned without loss of spatial resolution which is important as physiological relevant areas are often several square millimetres. At 20µm spatial resolution this results in many thousands of points per scan, and in order to keep such scans to an acceptable time (several hours), a continuous scanning mode for collecting data has been developed. In this mode the sample is moved continuously across the beam, not stepwise as in conventional mode. The multi channel analyser (MCA) opens for a pre defined time interval and subsequently the data is read out and written to memory. In addition the signals needed for normalisation e.g. ionisation chamber signals, detector deadtime, ring current, are saved. The sampling time for each data point is 5 seconds which

will allow samples to be scanned at the rate of approximately 6 hours each. The samples are supported on an XYZ table with a reproducible positioning of about 0.5 μm . The fluorescence signal is recorded using a Peltier cooled energy dispersive Si drift detector (Radiant VORTEX). The data is analysed using AXIL, a programme that fits the element $K\alpha$ and $K\beta$ peaks under consideration, taking account of line overlaps and subtracts the background. Automatic 2D scans are programmed and performed and trace element distribution maps are obtained with a matrix size of 63x58 pixels resulting in an area of 1.26x1.16 mm. Sections from the micro array were chosen for scanning based on light microscopy images of adjacent micro-array sections. These reference sections were haematoxylin and eosin (H & E) stained, which is a commonly used technique in routine pathology, in order to identify regions of normal and tumour tissue within the section. Using the 12 keV beam enabled the detection of the fluorescence from a germanium reference sample that could be incorporated into a fundamental parameters algorithm allowing quantitative results to be established.

The μXANES setup was identical with that used in μSRXRF with the exception that the multilayer monochromator was replaced by a Si(111) double crystal monochromator under remote control. The bandwidth of the incoming beam is $\Delta E/E \sim 10^{-4}$, therefore the typical count rate of Cu signal is reduced by a factor of 30. The regions of interest were obtained from the μSRXRF analysis of Cu distribution in the samples. The XANES data is measured in the fluorescence detection mode. The intensity of the beam (I_0) is measured before being incident on the sample. Subsequently part of the beam is absorbed by the sample and generates a fluorescence response. The intensity of fluorescent x-rays, I_f is measured using the energy dispersive Si drift detector, which was placed perpendicular to the beam, with a 50 mm^2 active area and 20 μm Be window thickness. The experimental absorption coefficient (μ), for a given element is proportional to I_f/I_0 . XANES spectra can be divided into three regions the first being the pre-edge part which has its origin in the 1s-3d electronic transition. The shape, position, and intensity of the pre-edge peak depend on the local atomic structure. The second region is the absorption edge with its crest and the shape and energy shift of this region gives information about the valence state of the absorbing atom. The intermediate multiple scattering is the third region, analysis of which gives information about the geometry of the scatter around the absorber. In this work, we attempt to find the relation between the valence

state of Cu in the breast samples and the shapes of XANES spectra by comparing the shape and position of the absorption edge with spectra obtained from references of Cu in different oxidation states. The standards include a Cu foil, CuI and Cu₂SO₄ compounds for Cu, Cu⁺ and Cu²⁺ oxidation state respectively. The Energy of the micro beam used for copper excitation was ranged from 8945eV to 9030ev, with 5, 0.5 and 1 eV energy step increments over the ranges 8945 to 8965, 8965 to 9005, and 9005 to 9030 respectively. The acquisition times over these regions are 10, 60 and 60s respectively for all tissue samples. Standard reference spectra were recorded under the same conditions as the samples except for a reduction in the acquisition time to 1 sec. Intensities were corrected to account for the dead time and the incident beam. The normalised data for each recorded spectrum was filtered using Fourier domain filtering (Table curve Program 2d v5.0). By using this procedure, the same lower and upper frequency was selected for all samples as well as for the standards in order to separate additional noise from the original data. XANES dactloscope software (Klementiev, 2006) was used for data analysis. For all spectra, a polynomial pre-edge background was subtracted to set a zero level. The spectra were normalised by the edge jump height, which represents the difference between the lowest and highest points in each spectrum. The absorption edge is located at the half height of the jump.

The main problem encountered in this experiment was the low signal to noise ratio which results from the low concentration of Cu in the tissue. The presence of holes, variation in the sample thickness, variation of elemental distribution, or any other sample imperfections may result in anomalies in measuring the value of μ for a sample. In order to handle these issues, pre-selected regions with relatively high Cu concentration, homogenous distribution, and were clear of any cracks or holes were chosen.

Results and Discussion

Elemental mapping

The trace element mapping using the XRF micro probe was carried out on 9 sections in the micro array, each section being entirely covered during the measurement. Figure 1 shows the results for one such section displaying the stained reference slice and distribution maps for the elements Cu, Zn, Ca and Fe. The scales on the right of each map show the concentrations of

the particular element in parts per million (PPM). The dark areas on the reference section are the clusters of tumour cells and the lighter areas are normal tissue. In this particular case the cancer is reasonably well defined and despite the registration error between the reference slice and the data slice, a good correlation is obtained with increased levels of all the elements in the tumour areas. The figure also displays the difficulties with mapping the Cu because of the very low concentrations, even in the elevated regions, typically a few parts per million (PPM).

Figure 1 : An example of the XRF mapping showing one reference section (1.0mm diameter) and the corresponding distribution maps for Cu, Zn, Ca and Fe. The scales are concentrations in parts per million (PPM)

The increase in the levels of elements in the tumour regions was consistent across all the sections measured, with the exception of Fe. In some of the sections the reverse was the case and the levels of Fe were decreased in the tumour regions as is clearly shown in figure 2.

Figure 2 : *In this section the Fe distribution shows a decrease in the levels of Fe in the tumour cluster.*

For each of the sections, regions of interest were set using pixel co ordinates over areas of tumour clusters and normal areas. The number of regions for a given slice depended on the distribution of cancer cells but at least two regions for cancer and normal areas were set for each sample. The background counts were also estimated by setting four regions of interest in each corner of the scanned area i.e. outside the diameter of the section. Figure 3 shows an example of these regions. The mean concentration in PPM was then calculated for each element in each region of interest and the mean background from the four regions subtracted.

Figure 3 : *Regions of interest were set to calculate the levels of elements (in this example, zinc) in the tumour, normal and background areas.*

Table 1 gives the statistics for this particular sample i.e. the mean concentrations, the standard deviation over the region of interest and the maximum pixel value. The background mean levels of each element are also given for the four corner regions.

	Tumour Region 1 (T1)			
	Mean (PPM)	SD (PPM)	Max (PPM)	
Ca	1087.64	182.261	1642.94	
Fe	15.6304	10.597	86.0982	
Cu	4.17361	0.83394	8.47255	
Zn	23.2951	3.63403	31.62	
	Tumour Region 2 (T2)			
	Mean (PPM)	SD (PPM)	Max (PPM)	
Ca	1715.53	551.163	2271.49	
Fe	14.1976	3.45711	19.8037	
Cu	4.54263	3.70077	21.2124	
Zn	27.689	9.44185	39.4964	
	Normal Region 1 (N1)			
	Mean (PPM)	SD (PPM)	Max (PPM)	
Ca	1145.53	170.743	1407.61	
Fe	14.8433	2.76695	23.5057	
Cu	3.55828	0.641774	5.17394	
Zn	18.9087	6.78953	40.8774	
	Normal Region 2 (N2)			
	Mean (PPM)	SD (PPM)	Max (PPM)	
Ca	954.396	206.181	1698.38	
Fe	12.6728	3.72245	24.1841	
Cu	2.5545	0.585949	3.86242	
Zn	11.8458	4.98397	47.4842	
	Background regions (mean value PPM)			
	1	2	3	4
Ca	6.56116	22.0052	32.7226	2.02316
Fe	0.359084	0.889828	5.54841	0.343807
Cu	0.912165	0.380091	0.498421	0.178994
Zn	0.354814	0.335083	0.43389	0.0714806
Table 1 : Mean, standard deviation and maximum concentrations of Ca, Fe, Cu and Zn in regions of interest shown for the sample in figure 3. All concentrations are given in parts per million (PPM)				

Table 2 shows a summary of the results for all 9 sample sections comprising the mean and standard deviation of the concentrations of Ca, Fe, Cu and Zn in the normal and tumour regions of interest. The values of the percentage difference between tumour and normal

regions are also given. The fact that some of the samples show a decrease in the Fe levels is indicated by the negative values. Taking the average over all samples the percentage difference from the normal tissue levels are $\text{Ca} \approx 67\%$, $\text{Cu} \approx 64\%$ and $\text{Zn} \approx 145\%$. It can be seen that there is an increase in the concentrations of all elements in all samples with the exception of iron. There are several possible reasons for this. Iron will be present in the blood vessels, contained within the red blood cell haemoglobin, and hence is going to be detected in the stroma where blood vessels are. Although tumours switch on pathways such as transferrin receptors to increase iron uptake from plasma iron transporters including transferrin, this is often insufficient to meet iron demand for cell growth and expansion. In figure 2, a ductal carcinoma in situ is shown. This is non invasive and the centre is necrotic, with dead cells and debris. Usually no new blood supply has developed at this stage, so delivery of iron from plasma iron transporters will be poor and growth is limited by poor oxygen supply, resulting in the central cell death. The striking lack of iron in the tumour is shown in this case. This would have implications for therapy, since iron chelators are being developed as anti cancer drugs, and it may be that tumours already so depleted would not be the obvious ones for such therapy.

XANES

Different features of the XANES spectra as well as the first derivative can be used to determine and characterise the oxidation state of Cu. Figure 4 shows the XANES features of the standard reference materials. The XANES spectrum of Cu foil is characterised by a shoulder at 8980 eV, which is due to 1s-4p electronic transition and a white line at 8992 eV (Lamberti *et al.* 2003; Prestipino *et al.* 2002). The Cu^+ spectrum is characterised by a single well defined peak at 8983–8984 eV, which is due to the 1s-4p electronic transitions and a lower intensity white line at 8990 eV. While the main characteristic features of Cu^{2+} are a weak absorption at about 8976–8979 eV, due to the 1s-3d electronic transition (Deutsch *et al.* 2004), a shoulder at 8987–8989 eV can be attributed to 1s-4p transitions, and an intense white line at 8994 eV.

Section No.	Ca					Fe					Cu					Zn				
	Tum. Conc PPM		Nor. Conc PPM		% of N	Tum. Conc PPM		Nor. Conc PPM		% of N	Tum. Conc PPM		Nor. Conc PPM		% of N	Tum. Conc PPM		Nor. Conc PPM		% of N
	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>		<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>		<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>		<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>	
1	1683	418	829	165	<i>102</i>	16.7	8.6	6.8	3	<i>145</i>	2.1	0.4	1.3	0.3	<i>55</i>	22	5.5	9.8	3.6	<i>129</i>
2	3449	820	811	195	<i>325</i>	178	184	3.6	1.9	<i>4814</i>	3.2	1.6	1.1	0.2	<i>183</i>	25	7.6	3.8	1.0	<i>562</i>
3	1472	370	964	288	<i>52</i>	32	10	56	39	<i>-42</i>	1.7	0.4	1.3	0.3	<i>30</i>	19	4.0	11	4.0	<i>75</i>
4	1385	366	1034	188	<i>34</i>	13	7.0	12	3.2	<i>9.6</i>	3.8	2.3	2.5	0.6	<i>50</i>	25	6.5	15	5.8	<i>67</i>
5	1131	279	651	90	<i>73</i>	28	12.	16	5.7	<i>75</i>	2.3	1.0	1.5	0.3	<i>52</i>	14	2.8	9.2	2.4	<i>53</i>
6	1463	619	1249	704	<i>17</i>	35	23	12	6.4	<i>190</i>	3.4	3.5	2.3	0.8	<i>67</i>	19	12	13	8.8	<i>48</i>
7	2784	493	1249	231	<i>122</i>	14	3.3	6.3	3.3	<i>132</i>	1.4	0.2	0.75	0.2	<i>82</i>	18	3.5	7.4	1.6	<i>150</i>
8	2999	554	1786	347	<i>67</i>	15	11	47	14	<i>-67</i>	1.9	0.3	1.6	0.2	<i>17</i>	17	5.3	9.3	3.5	<i>93</i>
9	1597	300	850	168	<i>87</i>	38	11	85	29	<i>-55</i>	1.8	0.4	1.2	0.2	<i>43</i>	25	4.6	11	2.4	<i>125</i>

Table 2 : Mean (*M*) and standard deviation (*SD*) of concentrations of Ca, Fe, Cu and Zn in tumour and normal regions of each samples. The figures in italics show the elemental concentrations as a percentage of the normal concentrations.

Figure 4 : Cu K-edge XANES spectra obtained for reference materials. The K edge of the most oxidised Cu compound is shifted to a higher energy.

Figure 5 shows the first derivative of the XANES spectra. The maximum derivative values for the Cu foil are at around 8977 and 8988 eV. For Cu^+ , the maximum derivatives are located at 8980 eV and 8988 eV. Cu^{2+} has its maximum values at 8986 eV, 8991 eV and a negative peak at around 8999 eV. These maximum values correspond to the inflection point of the 1s-4p electronic transition. The observed experimental values are comparable with the literature data that have reported absorption edge features for various copper compounds (Chaboy *et al.* 2006; Lamberti *et al.* 2003; Prestipino *et al.* 2002; Sarangi *et al.* 2005; Sipr *et al.* 2001).

Figure 5 : The first derivative of the Cu K-edge XANES spectra for reference materials.

Figure 6 shows averaged spectra of all normal and tumour tissue samples as well as the reference ionic materials. The XANES features of the normal and tumour tissue lie within the Cu^{2+} region. An intense white line is observed at 8995 eV. XANES features of the spectra suggest the possibility that the tissue samples contain mixtures of copper ions. When compared to Cu^+ , a strong decrease in 1s-4p peak intensity is observed, together with an energy shift (8983→8984), while the position of the edge and the white line is still toward Cu^{2+} spectra. The shift of the edge in the tumour spectrum towards the Cu^+ position may indicate that a fraction of the Cu^{2+} ions have been reduced to Cu^+ .

Figure 6 : Comparison of the Cu K-edge XANES spectra obtained for average normal, average tumour and for the reference materials.

Figure 7 shows the first derivative of the spectra shown in figure 6. The maximum value at around 8980eV in the samples is a prominent feature of the Cu^+ first derivative spectra and there is no such feature at this energy in Cu^{2+} spectra. The figure shows the maximum derivative at 8982.6eV and 8983.6eV for the average normal and tumour samples respectively. The relative intensity of the negative peaks in the first derivative of the average normal and tumour spectra are unequal. This may suggest lower levels of Cu^{2+} in the cancerous samples compared to the normal ones.

Figure7: Comparison of the first derivative Cu K-edge XANES spectra obtained for average normal, average tumour and for the reference materials

Using the information above it was found that in 24% of tumour samples, Cu was found in the Cu^{2+} state. A mixture of Cu ions was found in the remaining 76% of the tumour samples, with the relative amounts of Cu^+ varying. In Normal cases, Cu was found in the Cu^{2+} state in 36% of the samples. A mixture of Cu ions was found in remaining 63% of normal samples. In most of the tumour and normal samples, the mixture dominates and tumour samples were found to have higher fraction of Cu^+ as compared to the normal samples. The analysis of Cu K-edge spectra did not show significant differences between normal and cancerous breast tissue. Most of the copper was found as a mixture of Cu^{2+} and Cu^+ , however it would appear that Cu^+ is more abundant in tumour tissue than normal tissue. Zn/Cu SOD is a predominant Cu^{2+} binding protein while mitochondria and transport proteins are Cu^+ binding, and the Zn/Cu SOD enzyme has been suggested as a major target for chelation therapy. However, in order to estimate the best target for therapy more information is required about the relative abundance of all copper binding proteins, their oxidation state and their localisation at sub cellular level. .

Conclusion

The cellular distribution of Ca, Cu, Fe and Zn and the oxidation state of Cu were investigated using μSRXRF and μXANES respectively, with the aim of helping to obtain a better understanding of the role of trace elements in the carcinogenesis process. The combination of these techniques at the microscopic scale provides a methodology of characterizing the key

pathways through which trace elements are involved in invasive ductal carcinoma of breast tissue. The μ SRXRF study reveals an overall increase of Ca, Cu and Zn concentrations in areas of malignancy. The aim of micro mapping analysis of trace elements is to identify the relative differences of concentrations at cellular level between normal and cancerous tissue. This may clarify how the distribution of elements is related to the progress of the disease and it may also help in relating the distribution pattern of enzyme and protein (to which these elements are bound) to tumour carcinogenesis process. Additionally, the results obtained could be used in forming the basis for developing therapeutic strategies aimed at the inhibition of these elements in particular organs. The μ XANES study suggests that both normal and cancerous breast tissues have a significant fraction of Cu^{2+} , but does not exclude the presence of Cu^+ . However, the processes in which copper is involved and the site of possible targets for inhibition are not clear yet. Determination of specific sites and types of cells where copper accumulates may clarify the action pathway which is more involved in tumour progression and which can then be targeted and suppressed.

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