The Localisation and Micro-mapping of Copper and other trace elements in Breast Tumours using a Synchrotron Micro XRF System

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Abstract

Trace elements have critical roles in cancer biology. The quantity and the distribution of the elements Cl, Ca, K, P, S, Ti, Fe, Cu and Zn in samples of primary breast cancer has been assessed. The samples were formalin fixed tissue specimens formatted as microarrays of cores 1.0 mm diameter and 10µm thick each. The data were obtained using a synchrotron X-Ray Fluorescence Microprobe system. The spatial resolution of elemental maps was approximately 20µm. Maps were compared with light transmission images of the samples and images of the samples stained for cancer. The synchrotron system proved successful in producing data that could be mapped into high resolution images where clear structure could be identified. Correlation of these distributions with the concentrations of cancer cells was achieved in some samples.

Keywords

X-ray fluorescence (XRF). Synchrotron Radiation. Micro XRF. Breast Cancer. Trace elements

Introduction

An active area of research for over 30 years has been the study of concentrations of trace elements in relation to breast disease in order to help understand the disease process. Garg *et al* (1994) and Ng *et al* (1997) utilised Neutron Activation Analysis (NAA) 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. More recently studies by our group have shown statistically significant changes in levels of copper, iron, zinc and potassium in breast tissue, these changes being associated with cancer (Geraki *et al* 2002, Geraki *et al* 2004). The later studies were carried out using synchrotron radiation to excite an XRF response from elements of interest and utilising calibration samples for the quantification of the elemental concentrations.

There are several reasons for investigating elemental concentrations in cancers depending on the roles the elements play. Three trace elements of interest in this work were iron, copper and zinc. Copper and zinc are known to act as catalysts for antioxidant enzymes (superoxide dismutase 1, SOD). These enzymes have a role to play in the defense against disease. However, copper can also act as a catalyst for the production of hydroxyl radicals that are linked to tissue destruction and has an important role in angiogenesis. Iron is necessary for the growth of cancer and transporters for its uptake are often upregulated in cancer. Zinc is a co-factor for a group of enzymes that protects tumours from acidosis (carbonic anhydrases), which are also potential therapy targets. Our previous studies have shown that typical concentrations of these elements in breast tumours are approximately 1ppm, 7ppm and 15ppm for cu, zn and fe respectively. Potassium cannot be studied with the samples used because the fixing process removes this element.

This paper describes the study of the spatial distribution of a number of trace elements within breast cancer with a particular focus on the role of copper, and secondary on iron and zinc.

Recent evidence has indicated that copper has a key role to play in reducing angiogenesis and tumour growth (Lowndes and Harris 2004). Copper binding drugs can have a prolonged stabilisation effect in advanced cancer patients and are being studied in phase I and II trials. Although the exact mechanism is not clear, it appears that 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). Our group is also interested in the role of copper in the function of superoxide dismutase 1 (SOD). SOD is an enzyme involved in many diverse processes; the emphasis in relation to breast cancer is mainly due to its importance in endothelial signaling and therefore tumour proliferation. Both copper and zinc are essential for the physiological function of SOD which leads to the utilisation of copper chelators as a means of disrupting the function of the enzyme. Furthermore, caeruloplasmin, a key copper binding protein that was previously thought to be produced only in the liver, is another copper related pathway of interest. It has been shown that in certain groups of breast cancers, the oestrogen receptor negative tumours that show the more aggressive phenotype, the RNA for this gene is expressed. (Sotiriou *et al* 2003) An investigation of lysyl oxide along with SOD expression has found that both these copper dependent enzymes are highly expressed in tumour cells compared to normal cells in breast cancer.

Understanding which cell types 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. In particular it would be useful to differentiate between the metal content of the proliferating compartment of the tumour cells, cells near blood vessels and the vessels themselves. Also, inflammatory cells such as macrophages are known to be high in iron content and it would be of interest to see if they are also a source of copper that could then further activate the production of angiogenic factors.

In this paper we show a method of using a synchrotron based micro probe to determine the localisation of trace elements in breast tumours at a cellular level. Common techniques of histological staining can be either inapplicable or not sensitive enough for these cases, for

example immunohistochemistry could not be used to stain caeruloplasmin because, as it is a plasma protein, it is spread across the tissue section.

Methodology

Measurements were made on individual samples approximately 10 μ m thick (mounted on 4 μ m 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 10 μ m 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 monochromatised 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.) which provides a beam of 10-25 μ m 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 μ m FWHM@12 keV). By using this energy, data is 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 20 μ m 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.

Results and Discussion

A total of 10 samples were measured and elemental distribution maps obtained. Each map was compared to a transmission light image of the sample as well as a sample stained for cancer that came from a section close to the one data was collected from. Figure 1 shows the calcium and zinc distributions compared to the light transmission image. Note the scale on the right of each distribution map shows counts normalised to the ring current and can only be used for comparison purposes between the same element or elements that are close in atomic number. When comparison between different elements is attempted, an important factor that has to be taken into consideration is the variable efficiency of fluorescence production and detection based on the element's atomic number and fluorescence energy. For example, the expected levels of elements such as P and K are of the order of 10-1000 higher than those of Cu and Zn, a fact that is not mirrored on the intensity maps (see fig. 2 and 3). Since the fluorescence signals from the samples have not been calibrated against any standard the comparatively reduced production of x-rays from the lower Z elements (P and S) as well as the increased air attenuation of the same signals has not been accounted for. The dark areas on the right hand side of the zinc map are response from the microarray slide frame. In this case clear similarities in patterns can be seen between the elemental

distributions and the image. Although this offers some evidence of the technique being successful, it is of limited use in analysing where the concentrations of the elements are in relation to the distribution of the cancer cells. Indeed in general the light image of unstained samples has no structure that resembles the elemental distribution found in the maps (as in the cases shown in figures 2 and 3).

Figure 2 shows the elemental distribution of iron, zinc, phosphor and copper for a sample and is compared to the light image and also a further section obtained from the sample but has been stained for cancer. The distribution of elements is clearly seen. Note the levels of copper are the lowest of the elements shown, which was a consistent result across all samples and agrees with our previous measurements made on bulk samples. In this case the similarities with the structure shown in the light image are not obvious. Also when they are compared with the image of the stained section taken close to the measured section, there is still no obvious match. The darker areas in the stained section are cancer areas and in this section a concentration of cancer cells can be seen just off centre. This problem of matching the elemental distributions to stained sections taken from the same sample occurred across our sample range. This was because sections stained were a few sections away from those used for the elemental analysis.

One way to get around this problem was to stain the same samples the elemental distributions were obtained from after data collection was completed. The problem with this was that because samples are mounted on thin ultralene film (to maximise the efficiency of fluorescence detection) they often fail to remain intact during the staining process. However the technique was successful for two samples one of which is shown in figure 3. The figure shows the distribution of calcium, copper, phosphor, sulphur, iron and zinc. With the exception of iron, each of these elements show a clear and similar distribution pattern across the sample. The only real exception is the iron distribution. When compared to the light image there is no apparent correlation with structure. However, when the sample is stained

using haematoxylin, the cancer distribution (dark areas) matches the elemental structure very closely.

Conclusion

This study set out to test the use of a synchrotron based XRF micro probe as a suitable technique for mapping elemental distributions at a cellular level over small sections of breast cancers. The results presented show that the technique has promise and can produce elemental maps that show definite structure with regards to the distribution of elements. The main difficulty with the process is the comparison the measure data with the distribution of cancer cells across the sample. The best way to do this is to stain the actual section the data was obtained from post measurement but a more robust technique for this needs to be developed. An alternative would be to make sure the reference section was adjacent to the measured section such that the spatial variation was of the order of tens of microns.

The predominant element of interest for this study is copper however the maps from other elements are presented to demonstrate the capabilities of the technique. It can be appreciated that the maps obtained for copper are often less clear and that is due to the severely low concentrations of the element in breast tissue (of the order of a few ppm). This reflects in the counting statistics from the spectra obtained, mostly ranging in the region of 10 % to 30 % uncertainty (although occasionally it was as low as 4 %).

With up to 80 samples on a tissue microarray and improved scan times, it should be possible to relate the distribution of elements to other variables such as angiogenesis markers, SOD expressions, hormone receptor status and carbonic anhydrase expression and determine how the elemental pattern relates to tumour biochemistry and thence to selection of patients for therapy.

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Figure Captions

Figure 1 : The distribution of calcium and zinc compared to the transmitted light image. The scale on the right of the image is normalised counts and can be used for comparison purposes. Clear similarities in structure can be seen between all images.

Figure 2 : The distribution of iron, zinc, phosphor and copper compared to the transmitted light image and a section from the same sample stained for cancer. In this case it is difficult to see any clear matches in structure. Note also in terms of elemental level that copper is the lowest.

Figure 3 : The distribution of calcium, copper, phosphor, sulphur, iron and zinc compared to transmitted light image and same section stained for cancer. There is no clear match between the light image structure and the elemental distributions but if the section is stained then a clear correlation emerges.

Figure 1

Figure 2

Figure 3