

**Overcoming the Unknown:
New Approaches to the Diagnosis and Treatment of Carcinomas of Unknown Primary.
London 15th October 2009**

**The pathology work-up
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As you have heard already it is very important to remember that pathology is subjective. A comparison with some of the actual molecular tests you are going to hear later on, where you actually get, to some extent, a print out of the genes that are expressed. Pathology is dependent on interpretation at the microscopic level. It is crucial that that is done within an adequate clinical context. The clinical context also relates then to the timing of the tissue sampling, whether we do that early on in diagnosis, which would be according to the initially unknown CUP or according to the definitions we have heard earlier on, malignancy of unknown origin, compared to later on after most investigations nearer the true CUP – either provisional or confirmed CUP – which is approximately one third of the initially unknown.

Years ago it was probably closer to that but more and more we are doing these biopsies earlier on. That obviously impacts on what you are actually doing because it means that the differential diagnosis for the pathologists is wider. So I am not sure that one is definitely better but I think it generally depends on context. A lot of the time we are dealing with biopsies here.

What that clinical context does, is it means in the terms of the previous results in terms of the clinical history examination possibly blood tests, and especially radiology and endoscopy if they have been done, and that means is there is any suggestion where the tumour is and are there any clues with regard to the primary site.

So first of all for those of you who are not so familiar with actually how you get the sample the main issue a lot of the time with CUP biopsies is that they are often small. I am saying there that we are usually dealing with metastatic CUP, now that you may think is a given but actually a lot of the time what we are dealing with in terms of pathology is the issue between whether it could be a primary or is it a secondary and if so, is the secondary is the primary site for that known. So for example in Gauri's talk earlier on, when she was talking about the peritoneal carcinoma tosas there was an issue there as to whether that could be primary within the peritoneum or secondary from elsewhere and you need to be thinking about that all the time. So obviously the common metastatic sites are the solid organs like liver and lung, lymph nodes and the serious cavities. The sample is usually taken by needle, usually under radiological guidance, and that might be either a fine needle aspiration like cytology which yields a very small amount of cells and fluid but is increasingly amendable to the molecular testing. We relatively rarely get the larger biopsies like from the peritoneum so the samples are often very small and that will limit the amount of testing that is done, so again testing that enables us to work on small amounts of samples is really vital. I want to emphasise today as well is that the **pathologen** molecular tests that we are discussing at the moment are based on the tumour samples, not at the moment on systemic fluids or at least in my talk.

Processing the samples – this is just to let you know how we physically get it. The samples come like that. That is actually a fairly large one. It is then put in this plastic cassette that has been fixed in formalin usually over night. That is then processed through a machine through various solvents and embedded in wax. The wax block is taken from there and is then cut into sections which are put on a glass slide. That generally is not only the test bed, or substrate, for the pathology testing but it is also used for the molecular testing as well, so a lot of the molecular test you will hear about are derived from this type of material, often subsequently micro-dissected. For a standard pathology you then stain the section either with **8 anderythm ?? histochemistry** as you will hear. At the moment the pathologies standard tests, currently the molecular is usually additional and again further issues are that the standard tissue preservation at the moment is done in formalin. There are a number of other fixatives that are available but the most routine one, and the most common one in general practice of pathology, is formalin and it is not always optimal for molecular tests. But the alternative standard fixation, freezing, which is very good

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for molecular, doesn't give good morphology. As I say there are alternatives but it would require a more general frame shift at least in the UK.

What might be the optimal pathology work-up? However you do it from best patient management and also at the moment, as we are discussing for the comparison of it, the performance of standard pathology by comparing it with RNA or DNA methylation based molecular tests. You need optimal pathology.

What might that be? It might involve something like a stepwise assessment such as you have got here and I will go through that in a minute. It involves basically the morphology, which is the appearance by eye, and then tests for individual protein or other gene band markers standardly by immunohistochemistry. That really has to be in a panel, so again according to what you have heard already, it is important that you are not just doing one test, because those can occasionally vary and you might get odd results so you need to do it within a panel and it needs to look at things that you would expect in a given tumour type to be both positive and negative. That will give you the back up.

Again, the clinical context and the communications are absolutely vital. Quite often we will get biopsies just saying 'query mets in liver', or 'tumour in liver' or 'tumour lump, query ACC, query met'. Once we then make the diagnosis of an anodal carcinoma obviously that type of case is likely to be a CUP, so ordinarily people will go on for the full CUP work-up. But the full CUP work-up, as you have heard, takes a lot of time because you are doing it in a stepwise process as you don't want to get the faulty markers at once, quite often as you won't have enough tissue to do all of them. It is important to know whether you need to do that CUP work-up or not because, for example, sometimes we will get liver biopsies saying 'liver met, query collarette or primary' or 'collarette or primary' and that might be assumed but if you don't know that there isn't a definite collarette or primary you will just say 'well it looks like a collarette for met' and then not do anything else. So we really need to know up front that you are thinking of this as a CUP patient and we really need then to be linking in with the clinician to make sure that we are doing that diagnostic process in agreement. So maybe saying something like 'Full CUP work' or something like that would be useful.

Pathological diagnosis is very much based on morphology. This is very important. It is based on tissue architecture, so we need to know what is normal and for example, in this slide of squamous cancer, normally a squamous epithelium could be on the surface and not invading like this so cancer, basically when we are diagnosing it looks like cells where they shouldn't be. Also we use the appearance of individual cells or cytology. We look at the nuclei, which show the cell behaviour at the normal or malignant. So looking at this cytology sample, for example, that is a normal squamous cell with a small, fairly dark nucleus, whereas these are more malignant and they are showing basically nuclear irregularity and enlargement and the characteristics that are abnormal. The cytoplasm by contrast here is showing the cell type, like squamous or glandular and again that can help us when it comes to the diagnosis of CUP in terms of tumour subtyping and sometimes prediction of primary site. So again histology is core in excision biopsies that yield intact tissue, whereas in cytology, which is commonly got from, for example the neck nodes, you are only able to look at the cell appearance.

If you think about the stepwise diagnostic approach that we are taking to actually reach the diagnosis of CUP firstly you have to say 'Is there actually cancer present?' That may sound obvious but often there isn't. 'Is there a lesion present at all?' If there isn't, then you cut in, if there still isn't then you check with the radiologist how definite the lesion was. If there is a definite lesion then you re-biopsy if there is significant desire so to do. If there is a lesion then you need to know is it actually malignant, or in other words, is it cancer. If there isn't then you make the diagnosis of yes and then you move on. For example looking at the slide, that is normal liver and here is abnormal metastatic colon cancer within the liver. You can see that that colon looks quite different from the normal sample, so we know that that is abnormal and that it should not be in the liver.

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Then we need to identify the cancer type. You have all got the flyer called 'Carcinoma of unknown primary' so you would assume that we would go with this but actually to make sure that you know that it is carcinoma you need to be considering and excluding the other tumour types which are in particular a melanoma, lymphoma and sarcoma. So here you have got carcinoma epithelial cells aggregated together in tubules and islands and as ?????? background. You have melanoma, which is regarded as the great histological mimic, so it can look like a carcinoma, it could look like a sarcoma or it can, if you are lucky, have brown granules of melanin and thereby look like itself. You can have sarcoma, which tends to be more spindle cells and, thereby looks separate, or you can have lymphoma which is often sheets of relatively discohesive cells. If they are not clear in the histology then you can use various forms of IHC. For carcinoma you can use cytokeratins AE13 CK725 EMA ERA, melanoma - S100 **melana** and HNB45; lymphoma - there is a vast array depending on which lymphoma you are considering, but CNA would be your start and probably some plasma cell markers and sarcoma as well, again there is a vast array. Often people **menton** by menton but actually that stains many things so it is probably not the most specific but these will generally help.

There is a review in an issue of the Seminars in Oncology that Tony **Breckavere** edited, which has more IHC details. The thing in terms of the pathology is often not that you are not lacking the markers or that the markers have been stained for and misinterpreted, it is generally more that people haven't necessarily thought about a particular diagnosis as being possible, so you have not actually tested for it, so that is the main thing. Again from the clinical and the oncological perspective if you think that your pathologist, or if you want your pathologist to consider a particular diagnosis it is important to mention that, ideally early on.

One generic point - most of the biomarkers that we are actually using in CUP to test tumours are residual genes. Normal tissues corresponding to the previous tumours looks different from each other; they have different functions. Well differentiated tumours like **Wiess** look different from each other, so further testing are often unnecessary. So CK20 stains colon cancer and other GI things but actually it's there in the normal colon, it is a residuum from it like Weiss CDX2 on the slide. Most genes or proteins used for cancer typing, sub-typing and site predictions simply represent residual normal tissue differentiation and function remaining in those tumours. IHC or molecular tests into those residual genes can help type and sub-type the more poorly differentiated tumours if you can't do it on morphology. They all use variably sized panels of genes with particular patterns and so the panel again is important. You get a larger panel usually with the more **MR & A** based molecular testing but often it is similar genes. They are used because currently the primary site is the best predictive treatment response. Sometimes that is confusing because the best example of that is Ovarian Newsons Tumours. These are a slightly odd beast as the oncologists will know. Basically many of them are thought **motastacysts** from the GI tract anyway and tend to show morphology and gene expression profiles that can range from gastric to pancreatic to **colorectal** but even those that, as far as you can see, appear to be of ovarian origin if you give them chemo they tend not to respond to classical ovarian type chemo of pattern based **redulance** but they respond to GI based **dredulas** so they are responding more not according to their apparent primary site but according to their molecular profile and obviously in the future that may be supplemented or replaced by direct markers of tumour response.

The third thing to do in terms of the diagnostic approach is to identify a carcinoma's sub-type:

- Squamous adanocarcinoma
- Solid carcinoma like thyroid, liver, renal and adrenal, neuroendocrine and germs cell Mesothelioma mentioned there, not because it is a type of carcinoma, but because it enters the differential diagnosis as you heard earlier from Gauri. So those are the different appearances that you can see underneath on the slide.

What is the useful immunohistochemistry to do that? You can use a lot:

- squamous high molecular rate cytocheratins like these,

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- adenocarcinoma low molecular rate cytokeratins,
- Solid carcinomas, thyroid TTF1 and thyroglobulin, **hepatocellular** Hep Par 1 and CD10, renal LARC's and also CD10, but with a different pattern, and adrenal.
- You have heard me render **chromatin** markers and also germ cell tumour – you are going to hear more about germ cell tumour later on from Mark Erlander- that didn't actually show any of these markers so they can be false.
- Mesothelioma – positive with these and lacking some of the epithelial markers.

We have talked about that it is sometimes a lot of the testing that you are doing is actually to identify tumour type and sub-type not simply prediction of the primary site and indeed type and sub-type may be sufficient for optimal patient management including treatment. Like if you know that it is small cell or a particular form of germ cell or indeed if you predict that it is HCC.

How do you predict the primary site if possible? Which is really the last thing that you are wanting to do. For adenocarcinomas even a metastatic disease, albeit not necessarily CUP, morphology alone can predict primary site in up to 50% of cases and that was shown by Shennan and colleagues back in 1993. If a pathologist saw that he would tend to say that it was colon, or possibly upper GI tract occasionally. Serous papillary masses look like ovary, diffuse cancer which has a lot of signet ring cells is classically stomach, and again you can show those **meusem** signet ring cells with a meusem stain like that. If you cannot then there is useful **IC** out there and we performed a study which was trying to identify how best to use that. As you probably know the primary sites of adenocarcinoma, which is really a lot of the time what we are dealing with in terms of metastatic CUP the ones that most commonly present as mets are pancreas and lung and then colon, stomach, breast, ovary and prostate. So we did a study basically identifying candidate markers and then tested them in about 352 primary tumours from the seven common sites in the differential diagnosis in proportion to the presentations. We used amino and tissue micro arrays.

I just want to mention the importance of studying **anatomical** when you are looking at the success rate that is given with these types of studies. It's very important that you are aware that if, for example I use prostate in this study, or predominately prostate, we have good markers for that. Therefore I would be easily able to get a really high success rate. Likewise if you use for example leukaemia melanoma as, were done in a lot of the early studies, then those already can be told apart by histology normally so simply being able to add in a molecular test is not going to be of clinical benefit. So you need to have, within that, high success rate the tumours that you really trying to tell apart, that you can't currently already tell apart histological.

That study yielded a total of about ten markers and we put them together in a diagnostic table. These are the primary sites, markers, the number is the percentage positivity and the shading is intended to give an indication of how much, or how little, that positivity is. This is the kind of thing that is commonly used by pathologists. A lot of the markers here are familiar – you will see PSA and TTF1 but once you use them as a panel they are actually quite helpful, so for example prostate all positive for PSA, which is as you would expect, TTF1 high positive and lung but Gauri talked earlier about the issue of CK7 positive, CK20 negative cancers and a lot of that can be helped here. For example, most breast or ovary will be positive with ER but most ovary **????** will be positive also with mesothelin and C125 so that takes a lot of those tumours out. Likewise lung mostly TTF1 positive. So you are actually already getting most of those extra diagnosis identified and taken away.

The interesting thing has been to actually make a larger table which is including all of the differential diagnoses as well. What you are actually getting is the tumours from the similar place, like ovary series and endometrial have a rather similar profile. Likewise pancreas and **thyroidal**, stomach and oesophagus.

What we also did was to make a decision tree, basically because pathologists are familiar with diagnostic tables but the decision making process isn't obvious. We made a decision tree where

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most confidently from the top: PSA positive is prostate, if not then we are dying TTF1 is lung and likewise. In most confident here, less confident at the bottom with stomach and pancreas, which I guess is a bit galling since many of us are actually pancreatic and upper GI related. If you then use the diagnostic table decision tree 80% of the original first stage tumours were correctly classified, but that doesn't tell you much. You need to then test it independently and in a set of 130 primary impaired mets that rate was maintained.

The issues raised by that are:

- basically the marker profiles aren't confounded by normal tissue and that holds good as well for the molecular tests.
- The residual biomarkers were retained and mets that are truly useful.
- The errors are mostly primary and met pairs, some of those errors were actually likely to be errors in the original clinical diagnosis, not in the classification, but even the so called errors were when we were really trying to nail it down. They often enabled a site shortlist.
- We didn't at that point interpret the clinical information, histology and that is true for many molecular classifiers too. The inclusion should further improve the prediction.
- The errors were mainly between two sites stomach and pancreas, stomach and colon and pancreas and ovary, or ovary and breast or when no markers were positive.
- Pancreatic, capillary and gastro oesophageal are difficult to diagnose.
- Ovarian **neuroendocrine** tumours remain difficult to predict.
- We can probably identify further markers and ideally search for more specific markers.

The pathology work-up includes morphology and usually immunohistochemistry. It should be optimum in CUP but is difficult to standardise completely since pathology is interpretative. The IHC predictor has a good success rate which is similar to the molecular classifiers but there are particular gaps with some tumour and sites, particularly where markers are either absent or of no specificity, that remain difficult to predict. The cost for comparison is broadly somewhere about 150 – 200. We still aren't quite sure when is the best time to biopsy so that might be worth discussing later. Likewise when should the molecular test be used and this will be address over the course of the day. In a minority of CUP I would suggest where the optimal current assess yields no clear tumour type, sub-type or primary site or if there is significant doubt and there are treatment options that exist and that would be applied in that particular patient's case – that is up for discussion.