MOLECULAR GENETICS WILL MAKE HISTOPATHOLOGISTS REDUNDANT

INTRODUCTION

Arguably the most important scientific development in the new millennium has been the completion of the sequencing of the human genome. With it (and leading to it) have come new techniques that allow us to analyse the genetic sequence of individual cells. Histopathology on the other hand has existed for centuries, since Virchow in the 19th century but really since Hooke in the 17th. So is histopathology on the way out whereas genetics are on the way in?

The Oxford English Dictionary defines molecular genetics as "the scientific study of inherited variation in living organisms and the cellular and molecular processes responsible for this"[1], and as such it has existed since the 17th century when DNA was identified as the agent of inheritance. However, only in the last 50 years or so have molecular genetics become more prominent in clinical practice. The methods that are being used by pathologists today include electrophoresis to separate molecules of different sizes and charges, cloning of DNA sequences, polymerase chain reaction (PCR) to amplify DNA and RNA, DNA sequencing, microarrays to determine whether a large number of genes are expressed in a tissue sample,

imaging techniques for chromosomal and gene translocations such as fluorescent in situ hybridisation and immunohistochemistry, and many more.

Histopathology is the study of tissue changes associated with a disease or a disorder by a pathologist normally with a microscope after the tissue has been fixed and stained[2]. Different staining techniques can be used to visualise diverse cellular and tissue compartments. It is used daily by pathologists all over the world and most of our current staging and grading systems of tumours are based on it.

To assess whether molecular genetics will replace histopathology we first need to establish what the role of pathology is. For the purposes of this essay I will define it as

a) Diagnosis
b) Prediction of the prognosis
c) Guiding treatment
d) Understanding the pathogenesis
e) Audit pathological and clinical practice.

I will focus on tumours because they constitute a significant component of the workload of most pathologists. Molecular genetics have changed the diagnosis of breast tumours and lymphomas. A lot of research is currently being carried out in defining molecular markers for colon adenocarcinomas but as yet not yielding anything clinically useful.

COLON ADENOCARCINOMAS

In 1932 Culbert Dukes based his staging of rectal carcinomas on invasion through the muscularis propria (stage B) and the involvement of lymph nodes (Dukes' stage C). Tumours confined to the wall of the rectum are Dukes' stage A. This was a purely histopathologic definition and correlated roughly with survival (table 1). In order to standardise tumour staging the TNM classification, which assesses tumour size and local invasion (T), local lymph node involvement (N) and distant metastases (M), was introduced in the 1950s and has been updated since. As a rule of thumb, the higher the TNM staging is the worse is the prognosis. Many trials and follow-up studies have been carried out to establish the relationship of TNM stage to prognosis and response to treatment (table 1). The TNM staging is used in the UK and its requirements are reflected in the minimum datasets which are obligatory to be reported for every tumour. The colorectal dataset[3] includes e.g. maximum local invasion, the number of lymph nodes involved, the highest lymph node involved, extramural venous invasion and differentiation (or grading) of the tumour. These are all histological parameters. No molecular technique can provide this information. If molecular genetics were to replace histopathology here, either specific molecular markers would have to be found which closely relate to the TNM stage or new trials would need to be carried out to establish the relationship between genetic markers and prognosis and therapy.

A major drawback of the current (2006) TNM system is that there are wide variations in outcome in the intermediate stages (II and III), which are supposedly very similar. For example, 20-40 % of stage II patients will have recurrences, whereas up to 70 %

of stage III patients can have recurrences[4]. A study carried out by the US National Cancer institute[5] (table 1) also predicts better outcome for stage IIIA than for IIB, i.e. a better outcome for a more malignant tumour. This is one area where molecular genetics could help.

TNM staging	Dukes' staging	5 year survival rate
I	A	93 %
IIA	B	85 %
IIB	B	72 %
IIIA	C	83 %
IIIB	C	64 %
IIIC	C	44 %
IV		8 %

Table 1 - Correlation of TNM and Dukes' colon cancer staging with 5 year survival, taken from O'connell *et al.*[5]

Most colonic adenocarcinomas develop through 3 well identified sequences of neoplastic changes of the epithelia, progressing through adenomatous lesions, eventually developing into invasive carcinomas with metastatic spread[6]. Research has been undertaken to identify (common) defining molecular markers for each step. Genomic expression profiling such as microarray has already resulted in better prognostic markers for lymphomas, breast and lung tumours (see below). To perform microarray analysis the sample's nucleic acid is purified, amplified and labelled before it is hybridised with known complementary oligonucleutides (the "chip"). The labelling is quantified and correlates with gene expression. Eschrich *et al.* [7] used complementary DNA microarrays (for RNA rather than DNA) to establish expression of 43 genes in 78 human colon cancer specimens with intermediate staging (Dukes' stage B and C) and compared gene expression with Dukes' staging as a predictor of 36 month overall survival. Their results show that gene expression is significantly better at predicting survival than Dukes' staging. However, their study was small and the follow-up only three, opposed to the standard five, years. Yet as a preliminary

study it demonstrates that molecular genetics might play a role in colon carcinoma pathology in the future.

Colon cancers are a heterogeneous group in their development due to the three main, and many minor, pathways that may be involved. Therefore a genetic technique such as microarray analysis is necessary to assess all the (possible) abnormal genes simultaneously. The major drawback of microarrays is that they (presently) need to be carried out on fresh tissue, imposing strict rules on how the tissue has to be prepared and handled. Most centres in the UK would currently not be able to do this. Microarrays have also not been standardised yet, which results in differing results depending on which chips are used. Histopathology on the other hand uses formalin fixed paraffin-embedded tissues which are easier to handle. However, standardisation and automated processing are difficult to achieve in histopathology, but can be achieved comparatively easily with microarrays. Since thousands of genes are analysed simultaneously, robust statistics are needed to define e.g. the normalisation of data and the significance of the expression of one abnormal gene. The analysis of microarrays requires a specialist interpreter, probably someone other than the pathologist; this interpreter is not needed in histopathology.

BREAST CANCER

Breast carcinomas develop from epithelia responding to female sexual hormones. Hence many of them are oestrogen-receptor (ER) positive and require oestrogen stimulation to grow. Tamoxifen is an ER antagonist, inhibiting the stimulating actions of oestrogen[8]. The human epidermal growth factor receptor 2 (HER2) is involved in signal transduction resulting in growth and differentiation of the tumour cells. About 15-20 % of breast cancers demonstrate overexpression of HER2 which is associated with recurrence and worse prognosis. HER2 is also the target of the monoclonal antibody trastuzumab (marketed as herceptin) which is used as an adjuvant therapy to chemotherapy to improve disease free survival. Clearly patients (and the health budget) benefit from identifying which tumours will respond to treatment. Therefore ER status is included in the breast minimum dataset[9]. The NICE guidelines[10] regulate that ER status is to be determined on all tissues at diagnosis, before tamoxifen treatment is started. Trastuzumab[11] has some cardiac toxicity and is therefore not appropriate for all patients; if this is the case the tissue is not analysed for HER2 expression. If there is no clinical contra-indication for treatment, HER2 status is also routinely tested. Initially immunohistochemistry is used to define receptor status; FISH is then utilised in those cases where immunohistochemistry is equivocal.

The classification of breast cancers is currently undergoing remodelling, from a previously exclusively histological approach to one where molecular and histological results are integrated. This is especially important in morphological grade 2, or intermediately differentiated, tumours which comprise up to 60 % of all tumours and

have an intermediate risk of recurrence – not very helpful for clinical decision making. Their re-classification was begun by using microarrays. Perou *et al.*[12] for example defined four subgroups (basal-like, Erb-B2 +, normal-breast-like, and luminal epithelial/ER+) of breast carcinoma; these correlated with overall survival and were significantly better at predicting survival than grading into well or intermediately differentiated tumours. The subgroups are constantly being re-evaluated by statisticians: It is being investigated whether they also work for a greater number of tumours (Perou only used 65 specimens from 42 different patients) and tumours of different stages and metastatic disease. Admittedly microarray analysis provides the advantage of being able to determine precisely which genes are expressed. This is not particularly helpful, though, until we know what the expression of each of these genes means in terms of prognosis. Additionally there is the price, which is currently 3,500 US $ per specimen or more, compared with 100 to 500 US $ for immunohistochemistry or FISH and about 20 US $ per slide for a haematoxylin and eosin stain[13].

LYMPHOMAS

Molecular genetics have probably impacted most on haematological malignancies. The first translocation to be cloned and identified was the t(8;14)(q24;q32) translocation juxtaposing the MYC gene on chromosome 8 to the IGH gene on chromosome 14 in Burkitt's lymphoma in 1982[14]. FISH is used routinely today to demonstrate the translocation and hence diagnose Burkitt's lymphoma. It is not an absolute requirement, though, and a diagnosis can be made based on morphology

and the expression of other markers alone. We know that the movement of MYC to the proximity of the immunoglobulin heavy chain region results in overexpression of this proto-oncogene and hence understand the pathogenesis. Other molecular markers that are now routinely being investigated by FISH and aid diagnosis of other lymphomas include cyclin-D1 and Bcl-2 and Bcl-6 – more are sure to follow in the future.

However, histology is still important even in the world of lymphomas: for example the diagnosis of Hodgkin's lymphoma is highly dependent on the recognition of a Reed-Sternberg cell.

Diffuse large B cell lymphomas (DLBCL) constitute up to 30 % of non-Hodgkin lymphomas in the Western world. This group encompasses a wide range of different variants and attempts have been made to subclassify this group by morphological, molecular and immunophenotypical means - most of these have failed to show a definite relationship with prognosis[15]. Here analysis of a large number of genes may be helpful to establish a prognostic relationship. However, most DLBCLs have a comparatively good prognosis and respond to the routine CHOP-R chemotherapy regimen; therefore it should be the priority to differentiate more from less aggressive subtypes. B cell lymphomas intermediate between DLBCLs and Burkitt's lymphomas, for example, show a mixture of morphological and genetic features of both types. They are highly aggressive tumours that do not respond well to current treatment strategies. A combination of morphology, immunophenotype and genetics is required to identify these[16].

It is now common practice to assess the clonality of the T- and B-cell receptor in suspected haematological malignancies. The lymphocyte receptors are part of the immunoglobulin superfamily and contain up to three variable segments which can be combined to give a nearly infinite reservoir of receptor specificities. In a normal immune response all the lymphocytes will have different antigen specificities, i.e. different antigen receptors. Haematological malignancies are thought to arise from a single lymphocyte, hence their antigen receptors are all identical, i.e. clonal. Polymerase chain reaction (PCR) is the technique used routinely to assess clonality. In automated high resolution PCRs fluorescently labelled primers are used to amplify the genes (often the variable regions of the γ chain of the T cell receptor) – the fluorescent labelling allows the product to be quantified automatically. This technique is very sensitive, being able to pick up a difference of one base pair between probes[17], and highly specific with identification of monoclonality in 93 % cases[18].

However PCR does have drawbacks. Firstly not all tumours express clonality – e.g. lymphoblastic lymphoma arises from a very immature precursor which may not yet have undergone gene rearrangement; hence all cells are in germline configuration and not distinguishable from other immature lymphocytes in that tissue. Secondly, if the lymphocytes have undergone extensive remodelling through mutations, translocations and deletions, these prevent detection of clonality. Thirdly, the tumour cells may be below the threshold for detection of a clonal pattern by fluorescent PCR – i.e. there may be too few tumour cells within the tissue. Fourthly, the success of PCR depends on the selection of the right primers – ideally primers should cover all variable genes of the γ chain gene of the T cell receptor, in order to detect clonality. If this is not the case sensitivities of only 52 % or less may be achieved[19].

Nevertheless without clonality assessment (in certain cases) pathologists would not know whether an infiltrate was malignant or not since the histology is identical.

A very interesting scenario arises in the case of some benign inflammatory skin conditions such as pityriasis lichenoides et varioliformis acuta (PLEVA), which are admittedly very rare, but also show a dominant T cell clone. Dereure *et al*.[20] investigated 20 cases of clinically and histologically typical PLEVA with PCR (but not fluorescently-labelled PCR). They found that 13 out of the 20 cases had a dominant clone. The explanation for this is not fully established yet but it may be that the clonality reflects an immunological response to an unknown antigen or infectious agent. The differentiation between PLEVA and a T cell malignancy is very important, since most cases of PLEVA occur in young adults and normally disappear spontaneously after a few weeks. Primary cutaneous peripheral T cell lymphoma on the other hand only has a five year survival rate of 16 %[21]. Histology and genetics are crucial to make the correct diagnosis, to ensure that these benign conditions are not labelled as lymphomas.

CONCLUSION

When considering the main objectives of pathology, molecular genetics are not doing too badly: in Burkitt's lymphomas they assist the diagnosis and help us to understand the pathogenesis, in breast cancer they may lead to better prediction of prognosis and selection of the most appropriate treatment. However, because molecular genetics are still comparatively new they cannot provide enough

information for all malignancies yet and always need to be analysed in combination with histopathology. Their price and lack of standardisation mean that few techniques are currently routinely applied. Additionally let us not forget that the work of a pathologist does not end with tumours – e.g. in post-mortem tissue analysis molecular genetics play a very limited role today. Hence molecular genetics are not replacing histopathology yet but they have started to be used in areas where histopathology has its drawbacks. Whether they ever will replace histopathology is difficult to predict because as we see in the example of colon adenocarcinoma multiple tumourigenic pathways exist and we would need markers for all of them before we would rely on genetics alone. At the moment, molecular and histopathology co-exist and complement each other as “molecular pathology”.

Word count: 2,587

REFERENCES:

[1] Oxford English Dictionary

[2] Oxford English Dictionary and Wikipedia.org, visited 25th May 2009.

[3] Royal college of pathologists: *Standards and Datasets for Reporting Cancers.* Dataset for colorectal cancer (2nd edition). September 2007.

[4] Mutch MG: *Molecular profiling and risk stratification of adenocarcinoma of the colon*. J Surg Onc 2007;96:693–703.

[5] O'Connell JB, Maggard MA, Ko CY: *Colon cancer survival rates with the new American Joint Committee on Cancer sixth edition staging*. J Natl Cancer Inst. 2004 Oct 6;96(19):1420-5.

[6] Mutch MG: *Molecular profiling and risk stratification of adenocarcinoma of the colon*. J Surg Onc. 2007;96:693–703.

[7] Eschrich S, Yang I, Bloom G, Kwong KY, Boulware D, Cantor A, Coppola D, Kruhøffer M, Aaltonen L, Orntoft TF, Quackenbush J, Yeatman TJ: *Molecular staging for survival prediction of colorectal cancer patients*. J Clin Oncol. 2005 May: 20;23(15):3526-35.

[8] Baum M, Brinkley DM, Dossett JA, McPherson K, Patterson JS, Rubens RD, Smiddy FG, Stoll BA, Wilson A, Lea JC, Richards D, Ellis SH: *Improved survival among patients treated with adjuvant tamoxifen after mastectomy for early breast cancer*. Lancet 1983: 2 (8347): 450.

[9] Royal college of Pathologists. *Breast minimum dataset.*

[10] NICE Guideline: *Early and locally advanced breast cancer: diagnosis and treatment.* February 2009.

[11] NICE Guidelines: *Guidance on the use of trastuzumab for the treatment of advanced breast cancer.* March 2002.

NICE Guidelines: *Trastuzumab for the adjuvant treatment of early stage HER2-positive breast cancer*. June 2007.

[12] Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge Ø, Pergamenschikov A, Williams C, Zhu SX, Lønning PE, Børresen-Dale AL, Brown PO, Botstein D: *Molecular portraits of human breast tumours.* Nature 2000: 406(6797):747-752.

[13] Ross JS: *Commercialized multigene predictors of clinical outcome for breast cancer*. The oncologist 2008: 13 (5): 477-493.

[14] R Dalla-Favera, G Franchini, S Martinotti, F Wong-Staal, R C Gallo, and C M Croce: *Chromosomal assignment of the human homologues of feline sarcoma virus and avian myeloblastosis virus onc genes*. PNAS 1982: 79 (15) 4714-4717.

[15] Swedlow SH et al.: *WHO Classification of tumours of haematopoietic and lymphoid tissues*. IARC 2008.

[16] Swedlow SH et al.: *WHO Classification of tumours of haematopoietic and lymphoid tissues*. IARC 2008.

[17] Holm N, Flaig MJ, Yazdi AS, Sander CA: *The value of molecular analysis by PCR in the diagnosis of cutaneous lymphocytic infiltrate.* J Cut Path 2002: 29 (8): 447-452.

[18] Linke B, Bolz I, Fayyazi A, von Hofen M, Pott , Bertram J, Hiddemann W, Kneba M: *Automated high resolution PCR fragment analysis for identification of clonally rearranged immunoglobulin heavy chain genes.* Leukaemia 1997: 11 (7): 1055-1062.

[19] Ashton-Key M, Diss TC, Du MQ et al: *The value of the polymerase chain reaction in the diagnosis of cutaneous T-cell infiltrates*. Am J Surg Pathol: 1997; 21: 743

[20] Dereure O, Levi E, Kadin ME: *T-cell clonality in pityriasis lichenoids et varioliformis acute: a heteroduplex analysis of 20 cases*. Arch Dermatol 2000: 136; 1483-1486.

[21] http://emedicine.medscape.com/article/209091-overview. Visited on 31st May 2009.

Information about molecular techniques from:

Berg JM, Tymoczko JL, Stryer L: Biochemistry 5th edition. Available from http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=stryer.TOC&depth=2. Visited 25th May 2009.