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Impact of gene expression profiling in lymphoma diagnosis and prognosis

Christopher Osborne, Richard Byers

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Impact of gene expression profiling in lymphoma diagnosis and prognosis

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Review

Dear Dr Jack

Re: Re: Manuscript ID HISTOP-09-10-0484 entitled "Impact of gene expression profiling in lymphoma diagnosis and prognosis"

Please find attached revised manuscript in which each of the comments (below) have been addressed. The details of the response to each comment are detailed below.

Comment 1

Page 5, Note :

It would be helpful to describe what normalization is, why it is crucial to the overall process

Response

A brief description of the need for and methods for normalisation has been inserted on page 4, as copied below:

"Normalisation is important since array experimental conditions can strongly affect microarray hybridisation intensities, with resultant artefactual alteration of expression levels, especially for moderately expressed genes. Hence different areas of a given array may have different hybridisation efficiency, or different arrays in a given experiment may have different hybridisation efficiencies, with resultant differences in probe intensity. Normalisation is therefore a crucial preprocessing procedure prior to detailed analysis of the data. There is no gold standard method for doing so but methods commonly used include data scaling, normalisation against an array with median overall intensity or quantile normalisation, which adjusts the intensity values for each array so as to give an equal distribution of probe intensities for each array in a set of arrays.6"

Comment 2

Page 7, Note :

The concept of Leave one out validation needs to be explained for the general reader

Response

A brief description of the concept of Leave one out validation has been inserted on page 6, as copied below:

"in which the data from a single sample is used as the validation set and all other samples as the training set in a rotational manner such that each sample data set is used once as the validation data. This enables a data set to be used as both training and test groups without need to split the samples into two groups maximizing the power of smaller cohorts."

Comment 3

Page 12, Note :

Gene expression may not be currently used but may be practical technique with falling prices and the use of RNA derived from FFPE

Response

The feasibility of microarray GEP from FFPE is mentioned, and reference to Linton et al has been inserted, on page 12, as copied below:

"though with falling cost of arrays and improved methods for RNA extraction from formalin fixed paraffin embedded tissue it may be possible in the relatively near future.³³"

Comment 4

Page 18, :

This may be better discussed alongside DLBCL in terms of differential diagnosis, together with mediastinal B cell lymphoma

Response

This section has been moved to page 11 to follow directly after section entitled "Biology of DLBCL" and before section entitled "Immunohistochemical detection of GC-like and non-GC-like DLBCL". Figure numbering and reference numbers have been amended accordingly.

Comment 5

Page 25, Note (Orange):

There is also the question is to whether conventional gene expression profiling is already on the way to obsolescence and will be replaced in the next couple of years by deep sequencing

Response

The possibility the deep sequencing will render conventional gene expression profiling obsolete is mentioned on page 25, as copied below:

"and may in turn render conventional gene expression profiling obsolete in the medium term.⁶⁷"

Comment 6

Page 25, Note (Red):

The effective of RNA extraction from FFPE may be worth mentioning

Response

The feasibility of RNA extraction from FFPET for microarray GEP is noted on page 25, as copied below:

"Recent advances in methods for extraction of RNA from FFPET have enabled microarray gene expression profiling from archival FFPET samples, and this may open the way to either use of such arrays on clinical samples or identification of a new repertoire of biomarkers in FFPET that can be assayed by either real-time PCR or bead based platforms.³³"

Yours sincerely

Dr Richard Byers

Impact of gene expression profiling in lymphoma diagnosis and prognosis

Christopher Orsborne, Richard Byers¹

The Medical School, University of Manchester, ¹School of Cancer and Enabling Sciences,
University of Manchester, Manchester, UK

Running Title: Gene expression profiling in lymphoma

Address for correspondence:

Dr Richard Byers

Senior Lecturer in Pathology

Department of Histopathology

Manchester Royal Infirmary

Oxford Road

Manchester

M13 9WL

Email: richard.byers@cmft.nhs.uk

Tel: 0161 276 8816

Fax: 0161 276 6348

Abstract

Lymphoma classification has changed several times over time as understanding of normal and malignant lymphocyte biology has advanced. This has improved prognostication but there remain large diagnostic groups with diverse outcomes. In an attempt to refine diagnosis and prognostic power in these, global gene expression profiling (GEP) has been used to further improve understanding of lymphoma. This review will cover the impact of gene expression profiling on the diagnosis, prognosis, understanding of biology, and identification of novel treatments for the main types of lymphoma, as well its translation to clinical practice. Specifically, it will cover the use of GEP to identify prognostic subgroups within existing diagnostic categories in an attempt to improve prognostication in those subgroups with wide variation in outcome. Many of these studies have given additional novel insights into the biology of lymphoma, including the role of the immune system and the stromal environment. The improved understanding that these studies have given have suggested possible new treatments, linking diagnosis, prognosis, biological understanding and improved treatment.

Introduction

Lymphoma classification has changed several times as our understanding of lymphocyte biology and the origins of lymphoma have improved, though more recently the Revised European and American Lymphoma (REAL) and World Health Organisation (WHO) classifications have focused on clinical entities.^{1, 2} Despite this there remain large groups with diverse outcomes, especially diffuse large B-cell lymphoma (DLBCL) and follicular (FL) which between them account for nearly two thirds of all NHL (Non-Hodgkin Lymphoma).² In an attempt to refine diagnosis and prognostic power, several groups used global gene expression profiling (GEP), first in DLBCL but more in other subtypes of lymphoma. This has improved diagnostic categorisation, given insights into biology and identified candidates for novel treatments.

Global gene expression profiling was developed in the 1990s and first applied in the diagnostic arena by Golub *et al* who used oligonucleotide arrays to distinguish between acute myeloid leukaemia and acute lymphoblastic leukaemia.³ This was closely followed by Alizadeh *et al* who used a spotted array to discover subtypes of DLBCL.⁴ These two studies demonstrated the main different methodological and analytical approaches used in early studies as detailed below.

There are two main types of GEP (Figure 1a), printed or spotted arrays as detailed below and reviewed in Ramaswamy *et al*.⁵ Spotted arrays are produced by “spotting” oligonucleotide, cDNA or small PCR fragments directly onto glass slides, usually using an array of fine pins or needles controlled robotically. This technique was used early on in the field by researchers who were able to produce “in-house” printed microarrays from their own laboratories. They have the advantage of being able to be customised for each experiment or for the area of interest of one’s own laboratory. This was important in the early days of microarray use as the alternative of commercial printed oligonucleotide arrays had relatively few probe sets on the first generation microarrays, without full genome coverage as a consequence of which many genes one may have been interested in would have been absent, whilst now by contrast they can be used to assay a relatively low number of genes compared to commercial printed

arrays on which many genes not of interest may be present, with resultant higher cost and increased complexity of data analysis. They were also of relatively low cost once “spotting” hardware had been installed. Additionally, they enable direct comparison of gene expression between a test and control sample on a single microarray. However they have disadvantages of lack of standardisation, reducing cross comparison between studies from different groups and of quality control which, especially early in their use, required laborious inspection of the “spots” to ensure adequate and uniform deposition of “spotted” probe. At least in part due to this last point, together with the fall in cost of printed arrays they have now mostly been superseded by printed arrays. These are produced by photolithographic printing of sequential nucleotides onto silicon wafers, to generate short oligonucleotide sequences representative of a single gene chemically attached to the wafer. As a consequence although oligonucleotide probes are often used in “spotted” microarrays, the term “oligonucleotide array” most often refers to a arrays produced by this technique. The oligonucleotides printed vary from 25-mer probes on Affymetrix arrays to 60-mer probes on Agilent arrays. They have the advantage of high probe quality control compared to “spotted” arrays and of easy scanning using a proprietary linked commercial scanner as a consequence of which it is now the preferred microarray platform. There were several competing systems but Affymetix is now market leader. Another confounding problem is that of variable gene annotation and probe fidelity between platforms and releases, which may be partly responsible for reduced translation to clinical use as a result of unwillingness of diagnostic laboratories to invest given uncertainty as to which amongst competing systems would become the industry standard.

GEP analysis

Initial normalisation of data is performed followed by machine learning analysis. Normalisation is important since array experimental conditions can strongly affect microarray hybridisation intensities, with resultant artefactual alteration of expression levels, especially for moderately expressed genes. Hence different areas of a given array may have different hybridisation efficiency, or different arrays in a given experiment may have different hybridisation efficiencies, with resultant differences in probe intensity. Normalisation is therefore a crucial preprocessing procedure prior to detailed analysis of the data. There is no gold standard

method for doing so but methods commonly used include data scaling, normalisation against an array with median overall intensity or quantile normalisation, which adjusts the intensity values for each array so as to give an equal distribution of probe intensities for each array in a set of arrays.⁶ Machine learning analysis is of two principal types, supervised or unsupervised (Figure 1b). In supervised learning a class label is assigned in order to split a training data set into two or more, though usually just two, classes which are then used as the basis for cluster analysis. Typically for biological GEP studies, classes are assigned on the basis of a classification subtype or disease outcome. The resulting gene clusters are predictive of the starting classes and are then tested in a test data set for their ability to predict the same class structure. Those genes up or down regulated between classes are often displayed on a “heat map” in which the relative fold change of each gene in the predictive model is displayed as a colour gradient for each gene. Unsupervised learning uses cluster analysis to identify a given number of clusters, usually two or three, on the basis of similarity of gene expression between samples alone, without reference to predetermined class labels. It is therefore able to “discover” novel unexpected classes. The features of the resultant classes, either biological, behavioural, or both, can then be examined to determine the nature of the classes and their clinical utility, for example in predicting outcome.^{7, 8}

Initial GEP studies in lymphoma

The first use of GEP was in DLBCL, by Alizadeh *et al* and by Shipp *et al*.^{4, 9} Alizadeh *et al* used GEP to profile 96 samples. In addition a range of normal and activated B-cell subtypes were profiled followed by unsupervised clustering to group genes on the basis of similarity of their expression over all samples. Despite the complexity of the resultant “heat map” it was apparent that genes differentially expressed in germinal-centre B-cells were also differentially expressed amongst cases of DLBCL, from which it was hypothesized that this subgroup of DLBCL may resemble normal germinal centre B-cells. This was tested by clustering using only the genes defining the germinal centre B-cell signature from which two main molecularly distinct subtypes of DLBCL were identified. One expressed genes characteristic of germinal centre B cells, termed germinal centre B-cell like DLBCL (GC-like DLBCL) whilst the other expressed genes typically seen in activated peripheral blood B cells, termed activated B-cell

like DLBCL (ABC-like DLBCL) (Figure 2a). Additionally, these distinct genotypic subgroups, which appeared to map onto different biology, also had different outcomes, with GC-like DLBCL showing significantly better overall survival than ABC-like DLBCL.⁴ (Figure 2b)

In contrast Shipp *et al* carried out a supervised analysis of the expression of 6,817 genes in 58 cases of DLBCL and 19 cases of FL, a total of 77 samples.⁹ This demonstrated a clear difference between DLBCL and FL, with upregulation in DLBCL of lactate dehydrogenase, a previously known marker of DLBCL, together with genes associated with cellular proliferation (cyclin B1), and invasion and metastasis (cathepsins B and D). Genes upregulated in FL included regulators of apoptosis (HPD1 and WSL-Lr) and, interestingly, genes associated with follicular dendritic (clusterin) and T-cells (T-cell receptor- β , CD3- ϵ , CD3- δ , CD40 ligand, TXK tyrosine kinase,¹⁰ T-cell activation antigens, CD69¹¹ and V7¹² and the T-cell chemoattractant, SLC (also known as SCYA21)¹³). Furthermore the use of a 30 gene predictor correctly classified 71 out of 77 tumours as either DLBCL or FL using leave-one-out cross validation, in which the data from a single sample is used as the validation set and all other samples as the training set in a rotational manner such that each sample data set is used once as the validation data. This enables a data set to be used as both training and test groups without need to split the samples into two groups maximizing the power of smaller cohorts. They next used a supervised approach to identify a gene signature predictive of survival in DLBCL, from which a 13 gene model was derived for distinction of fatal/refractory DLBCL from cured disease, with upregulation of PKC- β (protein kinase C-beta) in the former (Figure 3). Immunohistochemistry (IHC) for PKC- β demonstrated its upregulation in fatal cases compared to those from cured DLBCL, pointing to its use as both a possible diagnostic marker and therapeutic target. This study also highlighted the problem of measuring gene signatures clinically by IHC in that of the 13 genes in the predictor, antibodies for IHC were only available for few.⁹ Since the predictive power of a gene signature is usually based on the expression of several, tens or even a hundred markers, reduction to just a few elements is likely to reduce its power. Other groups have looked at this by attempts to devise IHC algorithms for distinction of GC-like DLBCL from ABC-like DLBCL^{14, 15} (as detailed below) or smaller predictive lists of genes (17 or six gene models)^{16, 17}. Shipp *et al* attempted cross

validation of the ABC GC subtype by analysis of the original dataset of Alizadeh *et al* for 90 genes overlapping between the two microarray platforms, from which the resultant GC or ABC groups showed similar differential outcome to that reported by Alizadeh *et al*. However, there was no difference in outcome in the Shipp cohort when split into ABC or GC with the same 90 genes, from which they hypothesised that additional factors may be important in determining response of DLBCL to therapy.^{4,9}

In a follow-up study from the NIH group, Rosenwald *et al*, confirmed the existence of ABC-like DLBCL and GC-like DLBCL subtypes but additionally identified a third subtype the outcome of which matched that of ABC-like DLBCL.¹⁷ Specifically GEP was performed in 240 cases of DLBCL, all treated pre-Rituximab, and clustering performed in the gene space of 100 genes selected from a preliminary study predictive of ABC-like or GC-like DLBCL. This identified groups with gene expression profiles typical of either the ABC-like or GC-like subtypes together with a third group that did not express either set of genes at a high level; the level of heterogeneity in this group indicated that it may consist of additional subtypes of DLBCL (Figure 4). The GC-like subgroup was characterized by the t(14;18) translocation and amplification of c-rel which were exclusive to this subtype, suggesting that a limited number of features could accurately identify this subtype.¹⁷ The linkage of t(14;18) to GC-like DLBCL is of interest when considering the pathogenesis of FL transformation, nearly all cases of DLBCL arising from FL being of GC-like subtype¹⁸ (see below). The study also identified a novel 17 gene outcome predictor including GC-like genes, MHC class II genes, a "lymph node" signature and a proliferation signature, each of was differentially expressed in the three subtypes, specifically with the proliferation signature and BMP6 upregulated in the ABC-like subtype, and the GC-like signature upregulated in the GC subtype. This confirmed the finding of Shipp *et al* that a limited number of genes (in this case 17, in Shipp *et al* 13) could be used to predict subtype and outcome, additionally indicating that a single gene, c-rel, could identify one of the subtypes. It also confirmed the existence of the GC-like subtype, linking a specific maker to it, but also indicated that the non-germinal centre-like tumours may be more heterogeneous. Finally, this study, identifying genes involved in the immune system and

lymph node microenvironment, indicated a role for the host response in DLBCL, a possibility also alluded to for FL by Shipp *et al.*^{9,17}

Wright *et al.*, compared survival in patients classified as having either GC-like or ABC-like DLBCL by a Bayes rule statistical analysis, which calculates the probability of a case belonging to either the GC-like or ABC-like subtype.¹⁹ Subgroup prediction could be achieved using 27 genes, and was assigned to either GC-like or ABC-like subtype if it had a 90 or greater percentage probability of belonging to that group. Eighty seven percent of cases were correctly classified though only just over half of the type 3 subgroup were classified as either ABC-like or GC-like subtype, confirming heterogeneity of this subgroup (Figure 5). Interestingly, of the 27 genes used for prediction, only 14 mapped to the Affymetrix data from the study by Shipp *et al.*, but this relatively low number of genes still allowed classification in 82% of cases, with a similar proportion of cases being classified as of either GC-like or ABC-like subtype as those in the Lymphochip data set. This was despite the Lymphochip dataset initially being subclassified as GC-like or ABC-like, whilst the Affymetrix dataset had initially been clustered on the basis of outcome, rendering direct comparison difficult. However, *c-rel*, found exclusively in the GC-like cases in the Lymphochip dataset, was also highly expressed in the GC-like cases identified from the Affymetrix data set but not in the ABC-like cases, whilst *PKC-β*, which in the Affymetrix data set was associated with a poor outcome, was more highly expressed in the ABC-like cases from the Lymphochip dataset, indicating persistence of the ABC-like / GC-like subgroups of DLBCL across analytical platforms. Finally, using data from both the Lymphochip and Affymetrix platforms from the studies of Alizadeh *et al.* and Shipp *et al.* respectively, they found similar differential survival between the GC-like and ABC-like subtypes with either platform.^{4, 9, 19} This study also enabled the identification of a third group between GC-like and ABC-like DLBCL when the gene predictor was unable to assign a case to either with greater than 90% certainty, of interest given identification of a third group in other studies.¹⁹

Further studies in DLBCL

To overcome bias of clustering based on outcome Monti *et al* sought to investigate the underlying biology in DLBCL by identification of molecularly distinct subsets of DLBCL which were sufficiently robust to be captured by multiple methods.²⁰ Three different clustering algorithms (hierarchical clustering [HC], self-organizing maps [SOMs], and probabilistic clustering [PC]) were used together with consensus clustering to identify the top 5% of genes with the highest reproducibility across duplicate samples and largest variation across patient tumours. The most robust resulting substructure across all three clustering algorithms was one of three clusters, the biological nature of each of which was determined by GSEA. The first cluster (OxPhos cluster) was enriched in oxidative phosphorylation genes, the second (BCR/proliferation cluster) in cell-cycle regulatory genes, and the third (host response cluster) in genes associated with the host response (Figure 6). Immunohistochemistry confirmed increased infiltration of T-cells and S100+ve and GILT-positive dendritic cells in cases in the HR cluster.²⁰ Using the same strategy as Wright *et al* the three consensus clusters were analysed for cell of origin subtype but this failed to demonstrate any association of GC-like or ABC-like subtypes with the 3 consensus clusters, indicating that the 2 classification schemes are capturing largely different aspects of DLBCL biology.^{19, 20} This has implications for classification though the consensus method used enables identification of possible novel therapeutic targets. For example, OxPhos tumors have increased expression of proteosomal subunits and molecules regulating mitochondrial membrane potential and apoptosis. These tumours may be particularly sensitive to proteasome blockade or BCL2 family inhibition. In contrast, HR cluster tumors may be more sensitive to immunomodulatory approaches.²⁰ As an example, arising from this study Polo *et al* proposed BCL6 as a driver of the BCR cluster, from which they hypothesised use of BPI, a selective inhibitor of BCL6, as a novel treatment. In cell culture treatment of DLBCL cell lines with BPI resulted in cell death in lines with a BCR signature but had no effect in those with an OxPhos signature, supporting the hypothesis and suggesting use of BPI as a novel treatment for this subgroup of DLBCL.²¹

Robertson *et al* also tested the use of a possible novel treatment for DLBCL identified from GEP. Enzastaurin is a PKC- β inhibitor in phase 1 study as an oral agent in patients with advanced cancer, and, since PKC- β is upregulated in patients with fatal or refractory DLBCL,

it was tested in a phase II study in patients with relapsed / refractory DLBCL. Fifty-five patients were treated, and though only four underwent more than 20 cycles of treatment these four showed either complete remission or stable disease up to 50 months after study entry. A phase III study has been initiated but has not yet reported.²²

Biology of DLBCL

More recent studies have investigated both mRNA expression profiles and genomic abnormalities in parallel in DLBCL. These have indicated both a shared “stemness” and different oncogenic pathways for the different subtypes of DLBCL. Lenz *et al* carried out combined high resolution, genome-wide copy number analysis coupled with gene-expression profiling, identifying 272 recurrent chromosomal aberrations associated with parallel gene-expression alterations, of which 30 were differentially distributed amongst the three DLBCL subtypes, namely ABC-like and GC-like DLBCL and primary mediastinal B-cell lymphoma (Figure 7a & b). Specifically, FOXP1 and INK4a-ARF were upregulated in the ABC-like subtype, whilst PTEN was downregulated and the oncogenic Mir-17-92 microRNA cluster was upregulated in GC-like DLBCL.²³ Since INK4a-ARF and Mir-17-92 have been identified as possible oncogenes and PTEN as a possible tumour suppressor gene and that these alterations were present at the genomic level this study suggested that the different DLBCL subtypes use different oncogenic pathways.²⁴⁻²⁶ In contrast recent reports have shown that DNA methylation in cancer mostly occurs in genes repressed at the embryonic stem cell (ESC) stage, from which it has been hypothesised that aberrant DNA methylation occurs in cancer precursor cells, supporting the “cancer stem cell theory”. Detection of epigenetic changes in methylation may therefore represent a “memory of stemness”, indicating the existence of such a cancer stem cell.²⁷⁻³⁰ To test this hypothesis Martin-Subero *et al* carried out a large study combining microarray-based DNA methylation profiling with gene expression profiling in 83 mature aggressive B-cell lymphomas (maB-NHL), including Burkitts lymphoma and DLBCL (Figure 8).²⁷ Hierarchical cluster analysis was performed using the methylation status for 741 genes showing that DNA methylation profiling was not able to accurately differentiate between subtypes of maB-NHL. However, the genes methylated across the whole group of tumours was highly enriched for polycomb targets in embryonic stem cells,

regardless of the subtype of tumour, from which the authors suggested that maB-NHLs originate from cells with stem cell features or that stemness was acquired during lymphomagenesis by epigenetic remodeling.²⁷ This supports the concept that aberrant DNA methylation of genes already repressed in ESCs is a key initiating factor in the genesis of large B-cell lymphomas, and that maB-NHL originate not from differentiating cells but from precursor cells with stem-cell like features that have acquired aberrant methylation of PcG target genes.²⁸⁻³⁰ This is counter to the notion of a postulated normal counterpart cell for each of the subtypes of lymphoma referred to in the WHO classification, though the 2008 classification, whilst assigning a postulated normal counterpart cell to each subtype does not assert direct origin of the tumour from this cell, which may merely share phenotypic features with the tumour cell.^{2, 31} These two studies highlight contradictory homogeneity of DLBCL at the level of a possible stem cell and of difference in genomic makeup between subtypes, and further research combining GEP, genomic analysis and DMA methylation analysis is required to address this.^{23, 27}

Burkitt lymphoma

Burkitt lymphoma (BL) is strictly defined in the 2008 WHO classification, based upon the presence of an isolated c-Myc translocation.² Dave *et al* used GEP in 303 patients with aggressive B-cell NHL, demonstrating a signature for BL which correctly identified all 25 cases of classic BL in the cohort.³² This signature was characterised by high levels of c-Myc target genes, by increased expression of a subgroup of germinal centre genes and by low expression of MHC class I and nuclear factor kappa beta target genes. Interestingly this signature was also shared with cases of atypical BL and was present in eight cases with a diagnosis of DLBCL, each of which showed c-Myc translocation by FISH (Figure 9a & 9 b). Furthermore those patients within this group of discrepant DLBCL/BL who received intensive chemotherapy had better outcome than those receiving conventional DLBCL treatment.³² However, this was not merely a reflection of c-Myc status, since other cases of DLBCL with c-Myc translocation segregated from BL cases on the basis of the BL gene signature reflecting the fact that c-Myc translocation is not exclusive to BL but is not infrequently found in DLBCL in combination with other translocations.² This study highlighted the power of GEP to identify

biologically, and clinically, relevant subgroups of patients, raising the possibility of its use in difficult cases.³² (Figure 9)

Immunohistochemical detection of GC-like and non-GC-like DLBCL

GEP is presently impractical for clinical purposes though with falling cost of arrays and improved methods for RNA extraction from formalin fixed paraffin embedded tissue it may be possible in the relatively near future.³³ In order to overcome this present problem however, several groups have attempted to use IHC to distinguish between GC-like and ABC-like DLBCL. Hans *et al* used IHC for CD10, BCL6 and MUM-1 to subdivide DLBCL into GC-like or ABC-like / non-GC-like subtypes in a group of 152 samples that had been classified as GC-like or ABC-like on the basis of GEP, which was used as the gold standard. A diagnostic algorithm was developed identifying GC-like or non-GC-like subtypes, with resultant favourable outcome for the GC-like subtype (Figure 10a). Compared with the GEP gold-standard, the IHC algorithm had a positive predictive value of 87% for the GC-like subtype and 73% for the non-GC-like subtype.¹⁵ Other groups have investigated the use of IHC for outcome prediction in DLBCL, and Chang *et al* used a combination of GC (BCL6 and CD10) and ABC (MUM1/IRF4 and CD138) associated markers to define three patterns, pattern A (both GC markers and no ABC markers), pattern B (one GC marker and one ABC marker) or pattern C (one or both ABC markers and no GC markers). Pattern A had a favourable outcome compared to patterns B and C, both of which had similarly poor outcome.¹⁴ This is similar to the algorithm of Hans *et al* in that absence of any ABC associated marker (MUM1 alone in the Hans algorithm and MUM1 and CD138 in Chang *et al*) was associated with a favorable outcome. The use of an IHC algorithm has been widely adopted diagnostically, and has been incorporated into the British Committee on Standards in Haematology (BCSH) guidelines for lymphoma diagnosis and reporting (as shown in Figure 10b).³⁴ Taken together these studies underline the importance of the GC associated markers (CD10 and BCL6) in driving this distinction immunohistochemically, concordant with the more robust nature of this subtype as an entity, which is also highlighted by exclusive t(14;18) and c-rel amplification in this subtype.^{14, 15}

Role of the immune system

Rosenwald *et al* suggested a role for the host response, and in particular the immune system, in DLBCL, though this possible role was first highlighted most impressively by Dave *et al* in 2004, in FL.^{17, 35} FL is the second most common non-Hodgkin's lymphoma but has a variable clinical course, with an indolent course in some patients, whilst in others it rapidly progresses, with transformation to aggressive lymphoma and early death.² Dave *et al* performed GEP in 191 untreated patients with FL, from which an outcome based analysis identified two principal signatures, namely immune-response 1 (IR-1) and immune-response 2 (IR-2). Neither was present in the CD19 positive / malignant cell fraction and instead the IR-1 signature was highly expressed in T-cells, whilst the IR-2 signature was expressed in monocytes and macrophages, indicating that both prognostic signatures reflect non-malignant immune cells within the biopsy specimens (Figure 11).³⁵ To what degree this is reflective of a role for the immune response in tumour formation or response to treatment is unknown, though the study of Monti *et al* identified a host response signature in untreated DLBCL patients without reference to outcome suggesting the former.²⁰ Byers *et al* in a followup study used both IHC and real-time PCR to measure T-cell and macrophage associated genes in FL samples, demonstrating favourable outcome in cases enriched for T-cells and an unfavourable outcome in cases with high numbers of macrophages. Several other groups have also confirmed these findings.³⁶ Glas *et al* used IHC to measure numbers of T-cell and macrophages, finding association with high numbers of macrophages (greater than 15 per high power field) with a poor outcome.³⁷ Several groups have specifically identified high numbers of FOXP3 positive T-cells as indicative of a good outcome,³⁸⁻⁴⁰ whilst others have identified importance of CD57 or CD8 subpopulations^{41, 42}. FOXP3 positivity identifies regulatory T-cells which have a role in suppressing effector T-cells, depletion of which within carcinoma biopsies has been shown to correlate with poor survival.⁴³ However, in FL the reverse appears to be the case with association of higher numbers of FOXP3 positive T-regs with improved overall survival.³⁸ Farinha *et al* analysed the number and pattern of FOXP3 positive cells in cases of FL treated uniformly with multi-agent chemotherapy (BP-VACOP) followed by involved field irradiation to sites of original nodal involvement.³⁹ FOXP3 positive cells were distributed in predominantly either an intrafollicular / perifollicular pattern or a

diffuse pattern. An intrafollicular / perifollicular pattern was associated with a poor outcome and with an increased risk of transformation, whilst a diffuse pattern was associated with a good outcome. The authors of this study suggested reduced immunosuppression within the tumour microenvironment at the time of initial diagnosis in those with an intrafollicular or perifollicular pattern of FOXP3 cells. From this they suggested that the malignant cells may have the capacity to “recruit” Treg activity with consequent suppression of antitumoural T cells, allowing malignant cells to escape immune regulation. Additionally the majority of cases with high levels of macrophages had a follicular pattern of FOXP3 cells, suggesting cooperation of Treg and macrophages in tumour progression and transformation.³⁹ In contrast however, Wahlin *et al* found follicular infiltration of FOXP3 cells predictive of a good outcome, and in view of the number of contradictory studies in this area these markers are not recommended for use in clinical practice.⁴⁴

Monti *et al* highlighted the role of the immune system in the biology of DLBCL, interestingly without reference to outcome.²⁰ This group showed increased expression of multiple components of the T-cell receptor, CD2 and other molecules associated with T/NK-cell activation as well as increased levels of macrophage and dendritic cell transcripts. As noted above, this was confirmed by IHC, postulating a role of the immune system in pathogenesis. Host response (HR) cluster tumours had fewer genetic abnormalities than the other subtypes, occurred in younger patients and more commonly presented with splenomegaly and bone marrow involvement, further suggesting an alternative pathogenesis. The T-cell and dendritic infiltrates in these tumours resembled those of T-cell/histiocyte rich DLBCL, a subgroup of the WHO classification (2008) with similar clinical features to those for the HR tumours.^{2, 20} Interestingly patients in the HR cluster did not have better outcomes after CHOP chemotherapy, concordant with the outcome for T-cell/histiocyte rich DLBCL.²⁰ Lenz *et al* also identified stromal gene signatures associated with survival in large B-cell lymphomas by GEP of 414 DLBCL (181 treated with CHOP and 233 treated with R-CHOP).⁴⁵ Three gene-expression signatures predicting survival were identified, namely a favourable germinal centre B-cell signature reflecting GC origin, and two stromal signatures, a favourable stromal signature 1 enriched for extracellular matrix components and monocytic markers (such as

fibronectin, osteonectin, collagen and laminin isoforms and thrombospondin) and an unfavourable stromal signature 2 enriched for endothelial cell markers (such as VWF and CD31) and regulators of angiogenesis. Measurement of the signatures in CD19 selected malignant and non-malignant cells from three biopsies demonstrated higher expression of the GC-like gene signature in the malignant cells and of the stromal signatures in non-malignant cells, indicating importance of the host environment, both stromal and immune, in behaviour and outcome; interestingly this also applied to patients treated with Rituximab.⁴⁵

GEP in follicular lymphoma

Glas *et al* in a relatively early study in FL undertook GEP of 80 patients with FL identifying an 81 gene classifier which included genes controlling cell cycle, signal transduction and, interestingly, T-cell and macrophage activity. However, the authors noted the problems of translating an 81 gene classifier to clinical practice, suggesting either use of custom made mini-chips or multiplex PCR.³⁷ A major clinical problem in FL is that of transformation to DLBCL. Davies *et al* investigated the biology of this by GEP of 20 paired samples pre and post transformation.¹⁸ Classification of the transformed FL (t-FL) cases as either ABC-like or GC-like subtype showed almost all were of GC-like subtype, indicating a surprising degree of homogeneity in the underlying biology. They went on to identify seventy-six genes with a highly significant difference in expression between FL and t-FL, though no single gene demonstrated a uniform pattern of expression change across all samples upon transformation. Twenty nine genes were upregulated of which the majority were involved in cellular proliferation, together with genes involved in metabolism, cytoskeletal assembly and apoptosis. However, despite the predominance of a proliferation signature amongst the upregulated genes and the fact that nearly all cases were of GC-like subtype, only half of the case demonstrated upregulation of the proliferation upon transformation, indicating that transformation was due to other mechanisms in the remaining half of the cases, though the authors were unable to specifically identify these mechanisms. Those cases in which proliferation was upregulated also showed additional mutations, such as mutations in, or LOH (loss of heterozygosity), of TP53, or C-REL amplification, though these were absent in those without increased proliferation.¹⁸

Response of FL to radiotherapy

Very low-dose irradiation is a safe, highly effective treatment for localised FL, which is exquisitely sensitive to radiation. Knoops *et al* investigated the underlying biology of this focusing on the relationship between the immune response and outcome in FL.⁴⁶ GEP was performed in 20 patients before and immediately after a second 2 Gy treatment. Unsupervised clustering clustered pre-radiation and post-radiation samples from the same patient together for the majority of patients, indicating that global gene expression was not significantly altered by radiation, whilst supervised clustering identified 180 genes unregulated by radiation between the pre-radiation and post-radiation samples. These included many p53 targets suggesting activation of the p53 pathway, from which it found that low-dose radiation activated the intrinsic and extrinsic apoptotic pathways. Furthermore a large proportion of the genes induced by low-dose radiation were related to an immune reaction, reflecting activation of either macrophages and/or dendritic cell genes (confirmed by IHC for CD68 to identify macrophages), or of inflammatory and Th-1 related genes. These results point to the importance of the immune response to apoptosis in low-dose radiotherapy in FL, which together with the importance of the immune system in general in FL may explain the exquisite sensitivity of FL to radiotherapy, even at low doses. It is therefore perhaps not surprising that radiolabelled antibodies are so effective in FL.⁴⁶

Impact of Rituximab on GEP in lymphoma

A major problem in development of a prognostic model is the need for use of archival tissue from patients which by necessity will often have been treated with a different standard than currently available, with the potential for loss of predictive power when used in patients receiving modern treatment. In the case of DLBCL the introduction of Rituximab in the early 2000s has revolutionised treatment with improved outcomes. The early studies in DLBCL however, utilised samples from patients treated prior to introduction of Rituximab.⁹ Nyman *et al*, using IHC for BCL6, CD10 and MUM1 to classify cases of DLBCL as either GC-like or ABC-like subtype, measured survival in cases treated either with or without Rituximab.⁴⁷ There was differential survival between GC-like and ABC-like samples in those treated

without Rituximab but no survival difference between the two subgroups with Rituximab. This suggests that Rituximab overrides the causes of poor outcome in non-GC cases, though the mechanism underlying this was unclear. This finding cast into doubt the clinical relevance of much of the work in GEP of lymphoma prior to the introduction of Rituximab.⁴⁷ Rimzsa *et al* used a quantitative nuclease protection assay (Figure 12a) to measure gene expression of 36 genes (including MHC class II genes, germinal centre associated genes (including BCL6), stromal genes and proliferation genes) in formalin fixed paraffin embedded tissue (FFPET) from patients with DLBCL treated either with CHOP or with Rituximab-CHOP (R-CHOP).⁴⁸ In those treated with CHOP alone 15 of the 36 genes assayed were correlated with survival whilst for those treated with R-CHOP 11 of the 36 genes were correlated with survival including BCL6 and GCET1 (both GC associated genes), HLA-DQA1 & HLA-DRB (immune genes), ACTIN and COL3A1 (stromal genes) and myc (proliferation). In particular BCL6, HLA-DRB and MYC showed strikingly similar correlations with outcome in the two patient groups, and survival could be predicted on the basis of expression of myc and HLA-DRB alone (Figure 12b).⁴⁸

This study highlighted the continued survival advantage of germinal center associated genes (*BCL6*, *GCET1*, and *LM02*) in the R-CHOP era.⁴⁸ This was also supported by Natkunam *et al* in an IHC study which found that LM02 (a GC associated gene) was associated with a favorable outcome in patients treated with chemotherapy alone or in combination with Rituximab.⁴⁹ Furthermore Malumbres *et al* used RT-PCR in FFPET samples for prediction of outcome based on the expression of 6 genes (*LMO2*, *BCL6* and *FN1*, *CCND2*, *SCYA3*, and *BCL2*) in patients treated with R-CHOP. This six gene model, which had previously been used to predict outcome in patients treated with CHOP alone, was also able to predict outcome in the R-CHOP treated patients, indicating that the prognostic value of the 6-gene model remained significant in the R-CHOP era and could be applied to routine FFPET tissue from initial diagnostic biopsies.⁵⁰

Use of FFPET for prognostication is important clinically as the vast majority of diagnostic material is in this form and is likely to remain so for the foreseeable future. This is of

relevance to Rimsza's study in which a quantitative nuclease protection assay was used to facilitate RNA expression analysis from FFPET. The ArrayPlate method used by Rimsza *et al* enables gene expression measurement without the need for RNA isolation, reverse transcription and amplification, which is technically difficult in FFPET and can lead to confounding results. The ArrayPlate method avoids RNA processing with the use of short 50-mer probes that enable direct RNA hybridisation despite RNA fragmentation, usually to fragments approximately 200bp long, commonly present in FFPET.^{48, 51}

T-cell lymphoma

Compared to B-cell neoplasms relatively few studies have addressed GEP in T-NHL. Martinez-Delgado *et al* in an early study undertook GEP in a wide range of morphological subtypes of T-NHL, showing heterogeneity of the tumours at a genotypic level but also identifying deregulation of the NF κ B signalling pathway as a possible common feature.⁵² However, this study, which used a chip containing just 6386 genes, was unable to distinguish between different subtypes of T-NHL, with the exception of T lymphoblastic lymphoma vs other subtypes. A followup study by the same group investigated the expression of NF κ B associated genes using cDNA microarrays in peripheral T-cell lymphoma (PTCL) and reactive lymph nodes identifying two subgroups of PTCL, one, accounting for one third of cases, showing reduced expression of NF κ B associated genes and a second showing upregulation of these genes. Both groups contained multiple morphological types of T-NHL, including PTCL unspecified, angioimmunoblastic, cutaneous and natural killer/T lymphomas, with the exception that all cases of anaplastic large cell lymphoma (ALCL) had reduced expression of NF κ B associated genes. Furthermore reduced NF κ B gene expression was associated with reduced survival and was an independent prognostic factor in multivariate analysis.⁵²

Ballester *et al* also profiled a heterogeneous set of T-NHL cases but in contrast were able to differentiate morphological subtypes, specifically PTCL not otherwise specified (PTCL-NOS), angioimmunoblastic T-cell lymphoma (AITL) and ALCL, on the basis of GEP.⁵³ Within the

PTCL-NOS cases there was wide variation in GEP and this group of tumours could be further subdivided into three molecular subgroups, termed U1, U2 and U3. The U1 group included genes known to be associated with poor outcome in other tumours, such as CCND2, and the U2 subgroup showed overexpression of genes involved in T-cell activation and apoptosis, whilst the U3 subgroup was characterised by genes of the IFN/JAK/STAT pathway; no prognostic significance was reported for any of the groups.⁵³

Piccaluga *et al* concentrated on the PTCL-NOS subgroup and in a GEP of 28 cases showed that the molecular phenotype of this group matched more closely to activated than resting T-cells, though without any bias towards CD4 or CD8 positive cells.⁵⁴ Also included in the study were cases of AITL which clustered amongst PTCL-NOS in an unsupervised analysis, and cases of ALCL which clustered alone. Regarding possible molecular mechanisms underlying PTCL-NOS 155 genes were deregulated including genes associated with local invasion and metastasis (FN1, LAMB1, COL1A2, COL3A1, COL4A1, COL4A2, and COL12A1) and apoptosis (MOAP1, ING3, GADD45A and GADD45B). In addition PDGFRA, a tyrosine kinase receptor gene, was upregulated in PTCL-NOS. Furthermore, there was phosphorylation of PDGFRA in cultured PTCL cells which were sensitive to Imatinib suggesting a novel line of treatment for these tumours. More detailed analysis of the AITL cases demonstrated a molecular phenotype closer to CD4 than CD8 positive cells, and overexpression of the molecular signature of follicular T helper cells in AITL, concordant with previous IHC studies that had suggested origin of AITL from germinal centre regulatory T-cells and in particular T follicular helper cells on the basis of expression of CXCL13 which has been suggested as a novel diagnostic marker of AITL.⁵⁵ Several genes, including PDGFRA but also VEGF were upregulated in AITL, suggesting possible novel treatment regimes, and in particular, a possible role for Thalidomide and Bevacizumab as anti-angiogenic agents.⁵⁴

De Leval *et al* also used GEP to study both PTCL-NOS and AITL, an unsupervised clustering of which clustered most cases of AITL separately though some clustered with PTCL-NOS.⁵⁶ Interestingly all but one case with CD30 positivity clustered together, whilst the clusters observed also correlated with the percentage of tumour cells present in each case.

Furthermore, the molecular phenotypes of AITL and PTCL-NOS were distinct, with 442 genes overexpressed in AITL and 236 genes overexpressed in PTCL. The most significantly overexpressed genes in AITL were related to cell adhesion, matrix synthesis and remodeling, vascular biology (including vascular growth factors) and B-cell and plasma-cell related genes, whilst the genes upregulated in PTCL-NOS were reflective of nonspecific cell functions, including protein ubiquitination and regulation of transcription and metabolism. This suggested a role for the microenvironment in determining the molecular signature of AITL which was confirmed by more detailed study in 2 cases.⁵⁶ Furthermore the AITL transcriptional profile was enriched in genes characteristic of T follicular helper cells, such as BCL6, PDCD1, CD40L, NFATC1 and also CXCL13, confirming the findings of Piccaluga *et al.*^{54, 56}

Lamant *et al* concentrated on ALCL, demonstrating molecular distinction between ALK positive and ALK negative cases in an unsupervised analysis, confirming that they are different biological entities.⁵⁷ Specifically, BCL6, PTPN12, CEBPB, and SERPINA1, were overexpressed in ALK positive cases and CCR7, CNTFR, IL22 and IL21 in ALK negative cases.^{54, 56, 57} Interestingly Ballester *et al* also found distinction between ALK positive and negative cases at a molecular level. Regarding overexpression of the BCL6 oncogene in ALK positive cases, whilst BCL6 is typically expressed in germinal centre B cells it is also expressed in a subpopulation of germinal centre and perifollicular CD4 positive T-cells, which may correspond to follicular T helper cells, though CXCL13 was not found in ALK positive cases by Lamant *et al.*^{53, 57} SERPINA1 is also overexpressed in other tumour types, in which its expression is associated with worse survival, possibly as a result of its inhibition of natural killer (NK)-cell activity.⁵⁸ CEBPB overexpression has also been reported in ALCL cell lines in which its upregulation has been shown to be via the Akt/mTOR pathway activation, suggesting a possible role for Rapamycin in treatment of ALK.⁵⁹ Cuadros *et al* studied a relatively large cohort (n=35) of T-NHL cases comprising both PTCL-NOS (n=23) and AITL (n=12) using both custom cDNA and oligonucleotide microarray platforms.⁶⁰ Five clusters of genes were identified by unsupervised hierarchical clustering, each typified by genes involved in proliferation, inflammatory response, and T-cell or B-cell lineage; there was good agreement between the custom and oligonucleotide array signatures. There was an inverse

relationship between the proliferation and inflammatory response signatures, tumours with higher levels of proliferation genes had a lower proportion of infiltrating reactive cells; these tumours also had reduced survival.⁶⁰

Analysis of Complexity of GEP

GEP has enabled measurement of the entire transcriptome of a tumour, with resultant generation of highly complex data sets, as illustrated by the above studies. These have been analysed on the basis of outcome, as in Shipp *et al*, or biology, as in Monti *et al*, or on the basis of both biology and behaviour relevant groups, as in Alizadeh *et al*.^{4, 9, 20} However, each of these has conceptual problems associated with them. Outcome based analyses as demonstrated above can be rendered redundant with advent of new treatments, such as Rituximab, though such analyses are, from a clinical perspective, most immediately interesting. Biologically based analyses, such as Monti *et al*, are dependent upon accurate and detailed biological pathways, though these are likely to change with time as more detailed networks of gene interaction are derived.^{20, 61} Those studies in which there is an underlying biology and clinical phenotype are probably most compelling, though they are also prone to the same limitations of purely outcome based studies. Finally, in comparing the results of GEP to conventional diagnostic biomarkers, the former set out to define novel, more informative, diagnostic and prognostic categories, for which existing biomarkers may not be relevant, raising the problem of comparing a new predictor against a gold standard predictor that is neither as powerful as or relevant as the new marker. This may confound attempts to validate GEP identified biomarkers for clinical use (Figure 13).

Translation to practice

The above illustrates the recent rapid increase in GEP studies in lymphoma, many of which have pointed to diagnostic or prognostic signatures. However, few of these, with the notable exception of the activated B-cell vs germinal centre cell phenotype for DLBCL, have been translated to clinical practice and even for this there remains uncertainty as to its validity in the Rituximab era. There are two conceptual and practical obstacles to further translation of

these research findings to wider clinical use, namely choice of measurement platform and independent validation, which are to an extent inter-related.

Microarray signatures are usually predictive on the basis of a large number of genes, though attempts have been made to reduce these, notably by Lossos *et al* and Rosenwald *et al* who reported six and 17 gene models respectively; however, neither of these models have been used clinically and expression profiling of a large number of genes for signature measurement raises the practical problem of which platform to use for this.^{16, 17} Conventional microarrays require relatively large amounts of starting mRNA, which needs to be of high quality and preferably from fresh or frozen material, though recent advances in mRNA extraction and amplification have reduced the amount of starting material required and enabled microarray GEP using FFPE. However, conventional microarrays remain expensive, and contain many thousands of probe sets redundant for measurement of specific signatures. They also have problems of quantitation which are soluble by group normalisation in cohort studies but more significant when measuring gene expression levels for a single patient in isolation. Custom microarrays have been produced, specifically for measurement of P450 metabolism and prediction of outcome in breast cancer but so far no such microarray has been developed for lymphoma.^{62, 63}

Multiple genes can also be measured using real-time PCR which has the advantages that mRNA extraction and amplification issues have already been addressed, that this technology is already widely available in molecular diagnostic laboratories, and is quantitative and relatively inexpensive compared to microarrays. Byers *et al* and Sakhinia *et al* have used this approach for identification of diagnostic and prognostic genes in DLBCL and FL, demonstrating prognostic significance of mRNA expression level of YY1, low levels being associated with better outcome.⁶⁴ Additionally they performed global cDNA amplification of starting mRNA by polyA PCR generating a “molecular block” which could then be assayed by real-time gene specific PCR for any number of diagnostic or prognostic genes, which they termed “Indicator” genes. The advantages of this approach include the ability to analyse very small samples due to the exquisitely sensitive nature of polyA PCR, together with the ability to

indefinitely renew the cDNA “molecular block” generated, enabling its use for assay of large numbers of genes and for use in subsequent studies. However, measurement of large numbers of genes, typically more than 20, in most gene signatures would be cumbersome by real-time PCR, though reduction of predictive gene sets to 10 genes may render this approach feasible. Bead based high throughput flow cytometric systems may facilitate measurement of high numbers of genes but these have not been widely tested and have problems of quantitation, which is important for analysis of gene signature components.⁶⁵

Reduction to a very low number, under five, may facilitate measurement by immunohistochemistry, as reported by Hans *et al* and others for distinction of ABC-like and GC-like DLBCL, but such reduction of signatures to such a low number has not been achieved from the majority of gene signatures and, since the predictive power of such signatures is largely based on a multiplicity of genes there is an inherent problem in their reduction to a low number.^{15, 16} However, as indicated above, this has been achieved with a high degree of success for the ABC-like vs GC-like DLBCL phenotype, whilst other studies have identified single genes predictive of diagnosis or prognosis, such as PKC beta in DLBCL and CXCL13 in AITL, demonstrating that this approach is possible.^{9, 55} Furthermore, since such markers can usually be assayed by IHC in FFPET, they can more easily be adopted into routine clinical diagnostic practice than molecular techniques and this avenue is worthy of exploration. However reduced predictive power due to a low number of markers and reliance on availability of antibodies to these may force adoption of a more generic molecular solution if the problems associated with this noted above cannot be overcome.

As well as platform choice, there is a need for validation of predictive gene signatures between research groups and, ultimately, between diagnostic laboratories, though both of these require identification of the most appropriate measurement platform on which to validate the results. Additionally, for a test to be useful diagnostically it should be relatively inexpensive, robust, rapid and capable of widespread adoption, though complex molecular testing will, at least initially, naturally be undertaken in a relatively small number of

laboratories. Integrated haematological malignancy diagnostic services, as mandated by the NICE IOG for Haematological Malignancies, will facilitate this.⁶⁶

The above considerations however, only address issues of the most appropriate platforms for, and practicality of measurement of, individual gene signatures, though there are several other issues to be addressed prior to introduction of GEP in clinical practice. Gene signatures may require measurement using a combination of PCR and microarray methods dependent on which is most suitable after validation for each subtype of tumour. Consequently there may be signatures for which PCR measurement is suitable whilst others can be measured using microarrays alone. The same paradigm has been proven for breast cancer, for which both PCR and custom array methods of measurement exist. There will also be a need to develop algorithms for integration of the results of these tests with existing methods and though it is possible that some existing methods will be supplanted by new techniques, this will take time and in the interim there will be a requirement for their integration; integrated diagnostic services will facilitate this. It is likely that these new tests will be part of a constellation of analyses required, though their relative importance may vary from one subtype of lymphoma to another. Furthermore as new techniques are introduced it is expected that additional predictive signatures will be identified as a consequence of which new prognostic gene classifiers will have to be continuously validated, both against classical risk factors but also in the light of new treatments. Finally molecular analyses are relatively expensive and require more sophisticated infrastructure than current diagnostic methods and these additional costs will have to be considered in health economic calculations also taking into account the relatively high labour-intensity and requirement of scarce expertly trained personnel, both medical and scientific, of existing diagnostic methods, which molecular tests could reduce, offsetting their increased cost.

Future perspective

Microarray based GEP has revolutionised our understanding of the underlying biology of lymphoma, particularly DLBCL, and provided novel insights into the role of the immune and stromal response in tumour development and progression. These have suggested new

diagnostic and prognostic biomarkers, either as gene signatures or as single markers that can be measured routinely by IHC, and have also pointed to novel therapeutic interventions. There are many obstacles to be overcome in application of these to clinical practice and there is presently a relative paucity of biomarkers resulting from the huge amount of research that has been done in this area. The next steps are largely practical in determining best platforms for measurement, validation across large numbers of laboratories and integration with existing diagnostic methods, all of which may be best undertaken in integrated diagnostic rather than research laboratories. Recent advances in methods for extraction of RNA from FFPE have enabled microarray gene expression profiling from archival FFPE samples, and this may open the way to either use of such arrays on clinical samples or identification of a new repertoire of biomarkers in FFPE that can be assayed by either real-time PCR or bead based platforms.³³ Finally however, this is a field in continual development, largely driven by technological advances, including, most recently, next-generation high-throughput sequencing which promises ability to sequence the entire genome of a tumour for a relatively low price, and may in turn render conventional gene expression profiling obsolete in the medium term.⁶⁷ How the data from these new techniques will impact on this field remain to be seen but the resulting complexity of data, whilst enabling virtually completely comprehensive correlation with existing diagnostic and clinical parameters, will only compound the considerations noted above in discussion of the analysis of complexity of GEP. So, in conclusion, for those who fear a reduced role for histopathologists in this new era a thought from the Hitchhiker's Guide to the Galaxy in which two philosophers feared for their jobs from the computer Deep Thought designed to find the answer to life, the universe and everything. That programme ran for seven and a half million years and whilst the impact of GEP on lymphoma diagnosis and prognosis will take some time to establish, hopefully it will be less than that and be more useful and less enigmatic than 42. In the meantime however, figuring it out will give us plenty to do!

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For Peer Review

Figure legends

Figure 1a

Schematic of gene expression profiling using either oligonucleotide or spotted microarrays

mRNA is extracted from a test sample and labelled, either with Cy5 (for use on a “spotted” array), or with fluorescein (for use on an oligonucleotide array) and hybridised either alone to a oligonucleotide array or in competition with a reference mRNA sample labelled with Cy3 to a “spotted” array. Expression of mRNA for a given gene is measured in a oligonucleotide array by measurement of the absolute fluorescence level at each probe site whilst a “spotted” array provides a relative level of gene expression for each gene in the test relative to the level in the reference mRNA sample, usually normal non-malignant counterpart tissue in microarray expression studies in cancer.

Figure 1b

Schematic of supervised and unsupervised learning analysis of microarray gene expression data

Supervised learning uses a clustering algorithm to identify within a training dataset gene signatures associated with a pre-defined status, such as outcome or tumour subtype. The ability of the gene signatures to predict this status is then tested in a separate test dataset in order to validate the utility of the gene signature. The gene signature produced is therefore entirely dependent upon the status set for the training analysis.

Unsupervised learning uses an unconstrained clustering algorithm to identify a small number of independent clusters, independent of any status of particular samples within the training dataset. The characteristics of the resultant clusters and associated gene signatures are then investigated to determine whether they have any clinical or biologically meaningful or useful features, such as outcome prediction or distinct underlying biology. The gene signatures produced are therefore independent of any known status for the samples enabling novel biological entities to be identified.

Figure 2a

Discovery of DLBCL subtypes by gene expression profiling

Unsupervised hierarchical clustering of DLBCL cases identified two DLBCL subgroups, namely germinal centre B-like DLBCL (orange) and activated B-like DLBCL (blue) (reproduced with permission from Alizadeh *et al*, Nature 2000, 403, 503-511).

Figure 2b

Clinically distinct DLBCL subgroups defined by gene expression profiling

Kaplan-Meier survival analysis demonstrated survival difference between germinal centre B-like DLBCL (orange) and activated B-like DLBCL (blue) subgroups, defined by gene signatures, for all patients (a) and for those with low clinical risk, on basis of IPI score (c) (reproduced with permission from Alizadeh *et al*, Nature 2000, 403, 503-511).

Figure 3

Supervised analysis of DLBCL identifying 13-gene outcome predictor and protein kinase C-beta as a predictor of unfavorable outcome

a) Thirteen gene DLBCL outcome model

Gene expression level indicated in “heat map” with higher levels in red and lower levels in blue with intermediate levels represented on a red-blue colour scale. Genes more abundant in patients cured detailed at top of gene list and higher levels for these genes shown on the left side of the “heat map” and genes more abundant in patients dead of disease detailed at bottom of list and higher levels for these genes shown on the right side of the “heat map”; each column is a sample, each row a gene.

b) Kaplan-Meier analysis showing five-year overall survival for favorable (cured) and unfavorable (fatal) risk groups defined by the 13-gene model ($P = 0.00004$).

c) Immunohistochemical staining for protein kinase C-beta in patients cured or dead of disease with representative duplicate core samples from a cured DLBCL and a fatal DLBCL shown top and bottom respectively together with high power view for each (reproduced with permission from Shipp *et al*, Nat Medicine 2002, 8, 68-74).

Figure 4**Identification of subgroups of DLBCL by gene-expression profiling**

Upper panel shows hierarchical clustering of DLBCL identifying three subtypes, one with a gene expression profile characteristic of germinal-center B-cells, a second with a gene expression profile characteristic of activated B-cells and a third intermediate between the two (type 3). Lower panel shows number of samples in each of the three subgroups demonstrating *c-rel* amplification or presence of t(14;18) translocation, with restriction of these genomic abnormalities to the germinal centre B-cell like subgroup; red areas indicate increased expression, and green areas decreased expression (reproduced with permission from Rosenwald *et al*, N Engl J Med 2002, 346, 1937-1947).

Figure 5**Prediction of DLBCL subgroup using gene expression profiling**

A DLBCL subgroup predictor was used to calculate the probability that each sample was of either activated B-cell like or germinal centre B-cell like demonstrating the existence of a subset of cases for which neither subtype could be predicted with greater than 90% probability indicating heterogeneity within DLBCL beyond the two major subtypes (reproduced with permission from Wright *et al*, PNAS 2003, 100, 9991-9996).

Figure 6**Identification of DLBCL subtype by unsupervised consensus clustering**

Gene expression levels for the top 50 genes associated with each DLBCL cluster are shown for the three DLBCL clusters; each column is a sample, and each row is a gene with high expression indicated in red and low expression in blue with a red-blue scale for intermediate levels of expression. Selected genes characteristic of each of the clusters are detailed in the right hand panels with genes over expressed in cluster I (OxPhos), cluster III (host response) and cluster II (BCR / proliferation) shown from top to bottom (reproduced with permission from Monti *et al*, Blood 2005, 105, 1851-1861).

Figure 7a

Schematic of combined high resolution, genome-wide copy number analysis coupled with gene-expression profiling from which recurrently altered minimal common regions (MCRs) were identified (reproduced with permission from Lenz *et al*, PNAS 2008, 105, 13520-13525).

Figure 7b

Recurrently altered minimal common regions identified in different DLBCL subtypes, with putative target gene noted for each region (reproduced with permission from Lenz *et al*, PNAS 2008, 105, 13520-13525).

Figure 8

Hierarchical cluster analysis of DNA methylation data displayed alongside DLBCL subtype (determined by gene expression profiling), MYC and t(14;18) status, morphological subtype and "complexity" (representing the number of chromosomal imbalances in each case detected by array CGH as a measure of chromosomal complexity). This analysis demonstrated that DNA methylation patterns are not strictly associated with morphologic, genetic, or transcriptional features (reproduced with permission from Martin-Subero *et al*, Blood 2009, 113, 2488-2497).

Figure 9a**Lymphoma subtype prediction by a molecular classifier of Burkitt lymphoma in discrepant cases of aggressive B-cell lymphoma**

The expression of Burkitt lymphoma classifier genes in nine cases of discrepant lymphoma (in which pathological and molecular diagnoses did not agree) was compared with the average expression of these genes in Burkitt lymphoma and diffuse large-B-cell lymphoma from which the probability that each case was either of Burkitt or DLBCL subtype calculated (reproduced with permission from Dave *et al*, N Engl J Med 2006, 353, 2431-2442).

Figure 9b**Relative expression of gene-expression signatures in Burkitt's lymphoma and DLBCL**

The average gene expression of the genes in each four gene-expression signatures for Burkitt lymphoma (c-myc target genes, BL-high germinal centre B-cell genes, MHC class I genes and NF kappa B target genes) are shown by diamonds for each biopsy, demonstrating high expression of c-myc target genes in Burkitt lymphoma, low levels in DLBCL and intermediate levels in cases of discrepant lymphoma, whilst MHC class I genes and NF kappa B genes were high in DLBCL and low in Burkitt lymphoma (reproduced with permission from Dave et al, N Engl J Med 2006, 353, 2431-2442).

Figure 10

Decision trees for immunohistochemical determination of DLBCL subtype based on (a) Hans *et al*¹⁴ and (b) Parker *et al*³¹; Hans *et al* subtyped cases into germinal centre B-cell like (GC) or non-GC subtypes and Parker *et al* into GC or activated B-cell like (ABC) subtypes, though some immunohistochemical combinations were unspecified in Parker *et al* (denoted by "?").

Figure 11

Schematic of hypotheses for role of immune response signatures 1 and 2 in outcome in follicular lymphoma

In the "immune response" model the immune response 1 signature is characterized by immune cell attack of malignant cells, resulting in an anti-tumor immune response that is able to prolong the survival of these patients, but not eradicate the tumor. Conversely patients with the immune response 2 signature may have evolved mechanisms to evade an adaptive immune response with resultant evasion of attack by the malignant cells, possibly due to increased macrophage activity.

In the "immune dependence" model tumor-infiltrating immune cells provide trophic signals for survival and/or proliferation of the malignant cells. In patients with the immune response-1 signature the malignant cells may be dependent upon these signals, making it difficult for them to leave the lymph node with resultant favorable prognosis, whilst patients with the immune response-2 signature may have lost dependence on microenvironment signals with resultant spread and consequent relatively short survival (adapted from Staudt and Dave, *Advances in Immunology* 2005, 87, 163-208).

Figure 12

a) Principle of the ArrayPlate assay (reproduced from and reviewed in Byers R, *Blood* 2008, 112, 3006-3007)

b) Overall survival in patients with DLBCL treated with either CHOP or R-CHOP according to gene expression levels for HLA-DRB, BCL6 and MYC demonstrating equivalence of outcome prediction for each of these genes in CHOP and R-CHOP treated patients; *HLA-DRB* is cut above and below 25%; BCL6 and MYC are cut at median (reproduced from Rimsza *et al*, *Blood* 2008, 112, 3425-3433).

Figure 13

Conceptual problems in analysis of microarray gene expression data

Gene expression data can be analysed on the basis of outcome, biology, or on the basis of both biology and outcome, though each has conceptual problems. Outcome based analyses may be rendered redundant with advent of new treatments, biologically based analyses are dependent upon accurate and detailed biological pathways, which are likely to change with time whilst studies with an underlying biology and clinical phenotype though most compelling, are also prone to both these limitations. Finally, in comparing the results of GEP to conventional diagnostic biomarkers there is the problem of comparing a new predictor against a gold standard predictor that is neither as powerful as or relevant as the new marker.

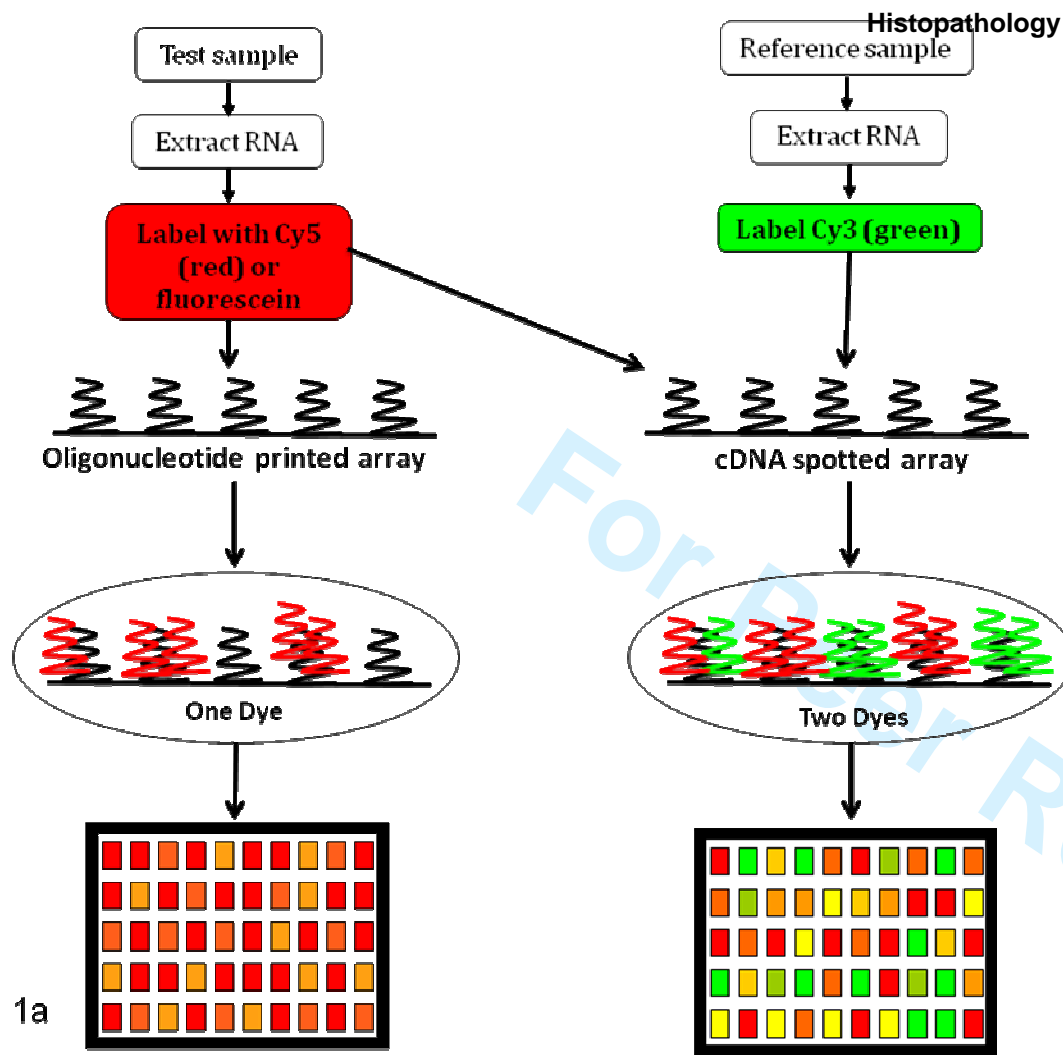


Figure 1a

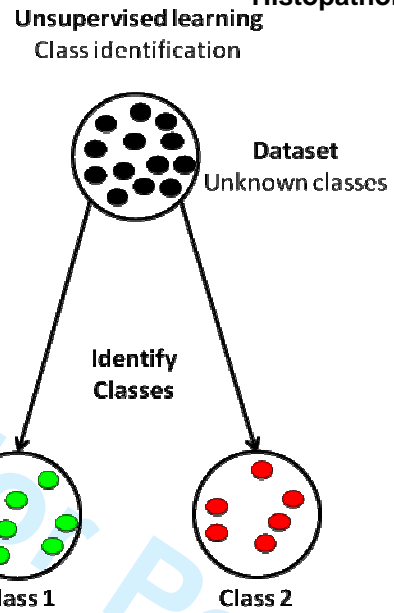
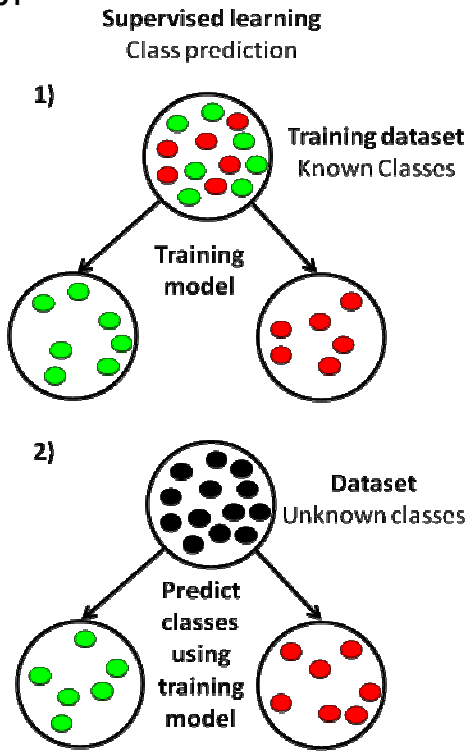


Figure 1b

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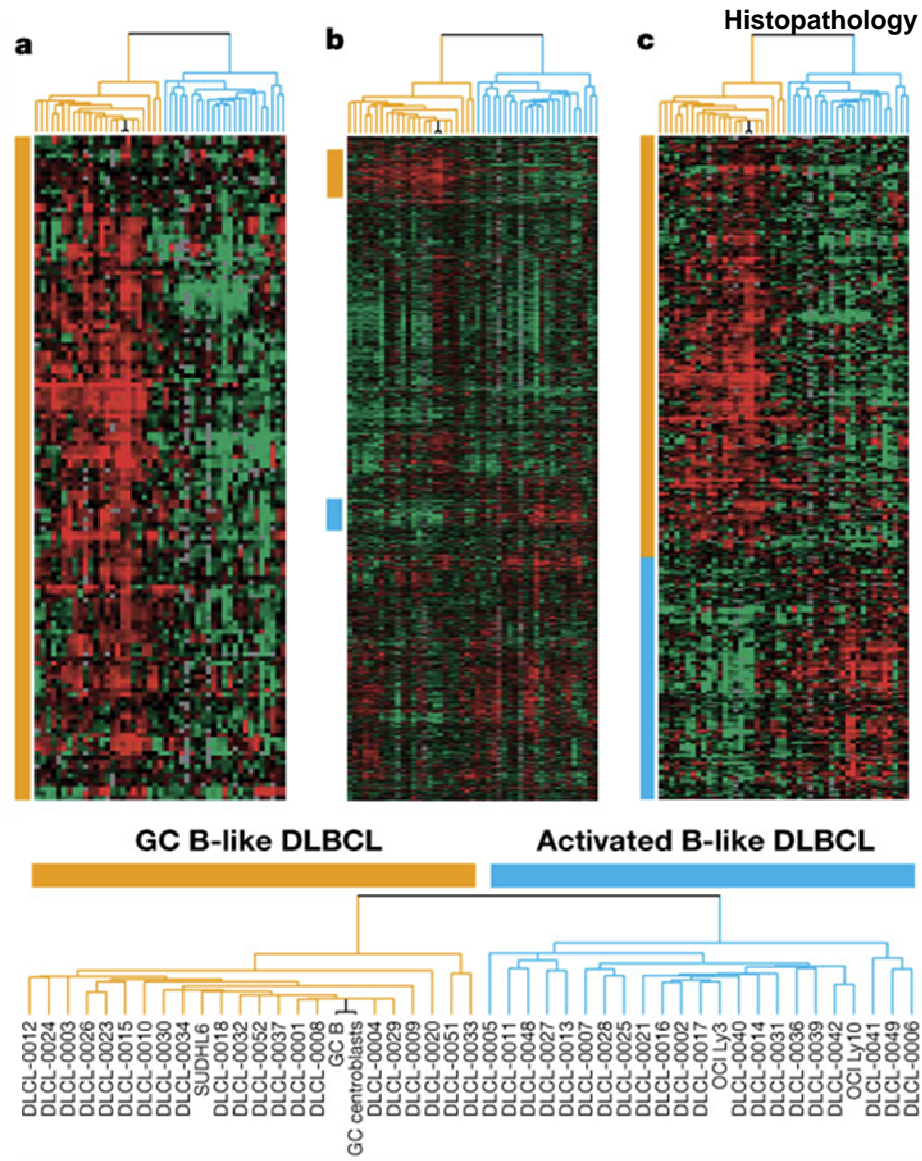


Figure 2a

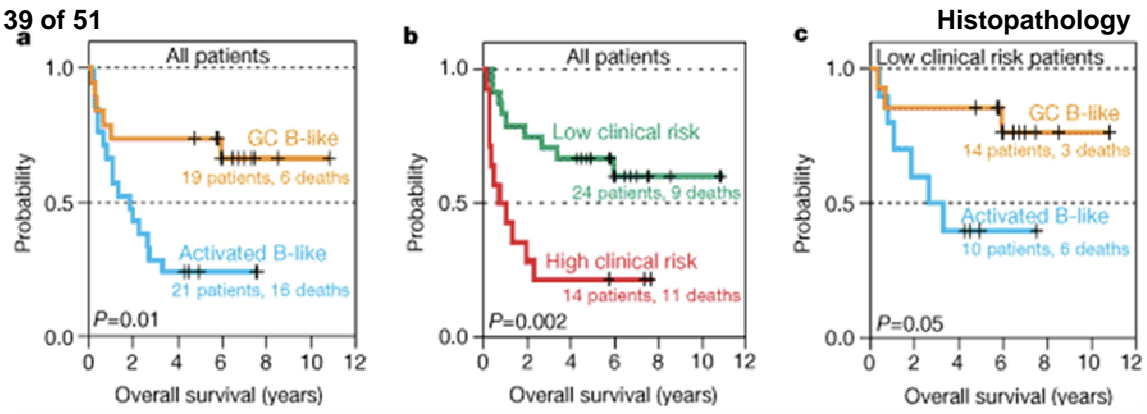


Figure 2b

For Peer Review

Curable

Fatal

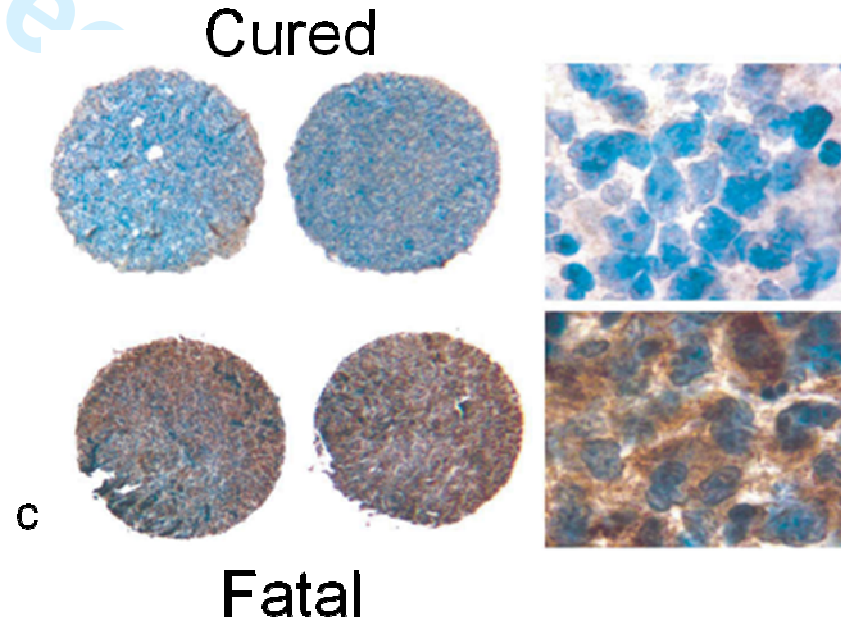
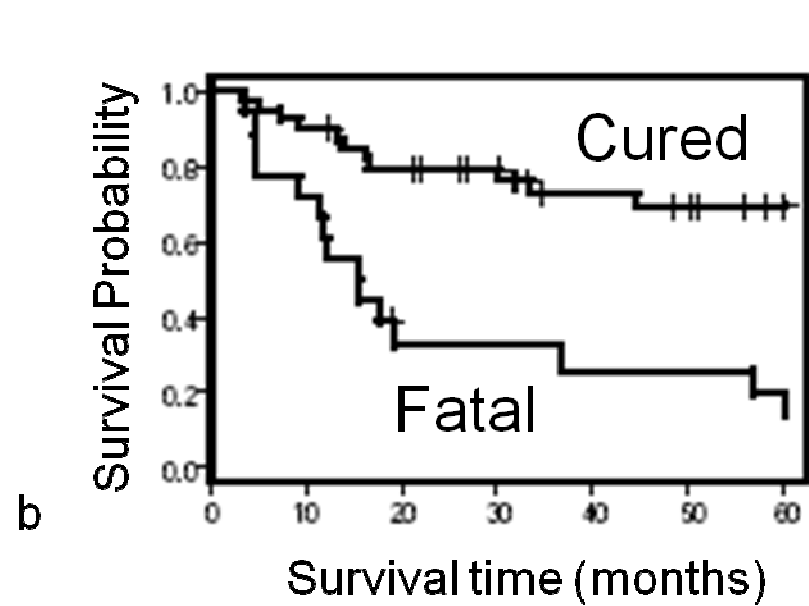
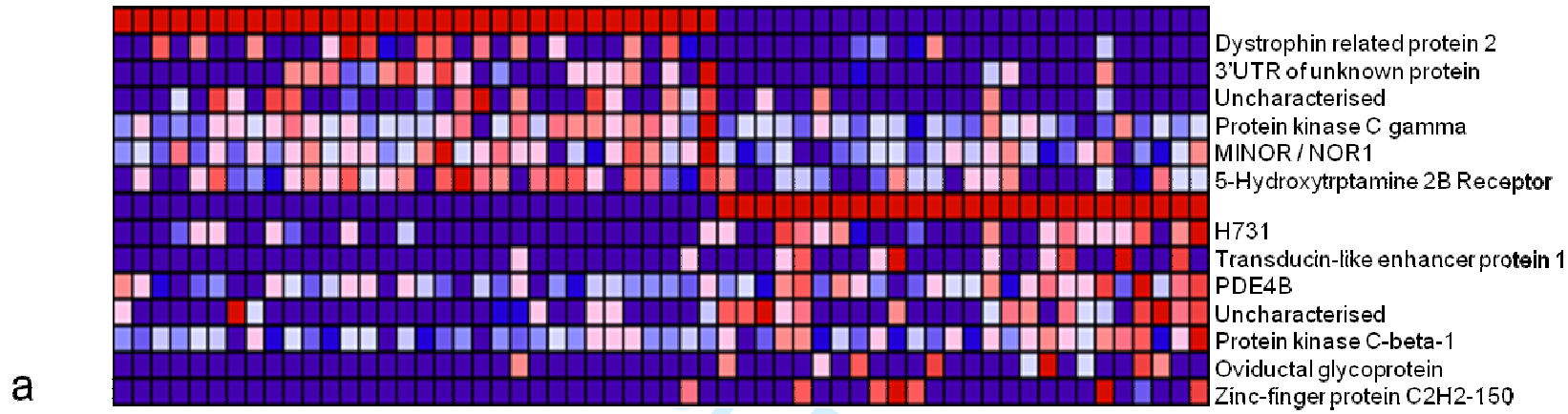
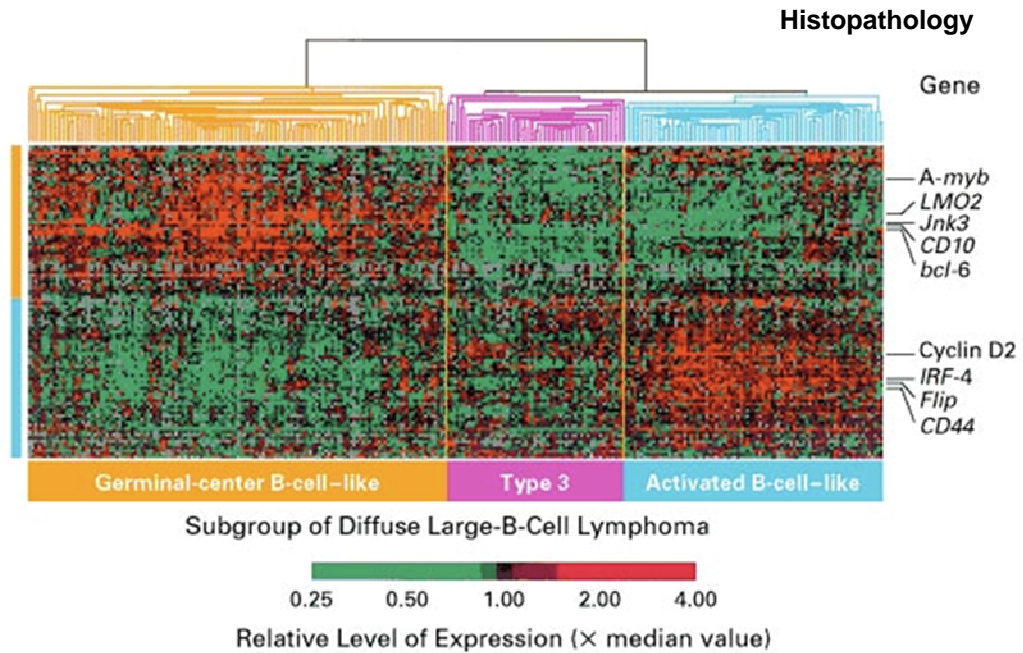


Figure 3



| Oncogenic Abnormality | Germinal-center B-cell-like | Type 3 | Activated B-cell-like |
|----------------------------|-----------------------------|--------|-----------------------|
| | no. of samples | | |
| <i>c-rel</i> amplification | 17 | 0 | 0 |
| <i>bcl-2</i> t(14;18) | 26 | 0 | 0 |

Figure 4

view

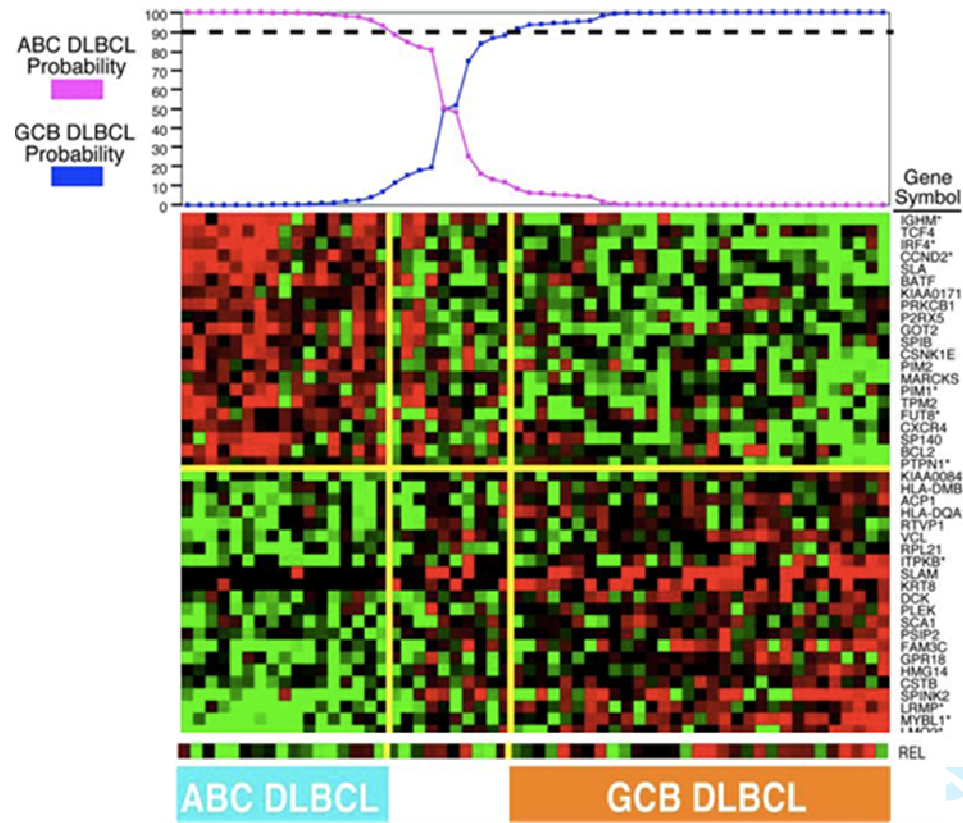


Figure 5

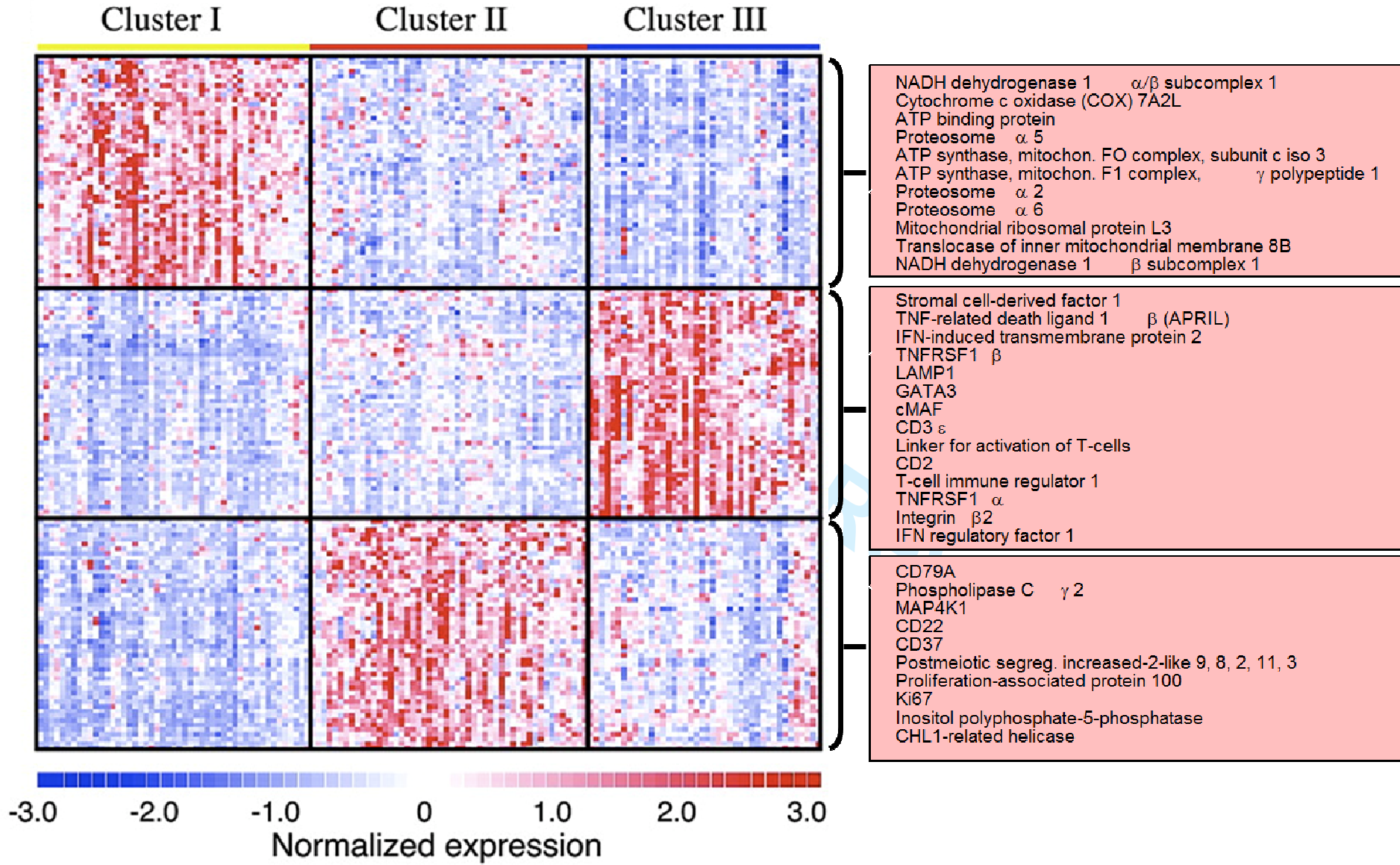


Figure 6

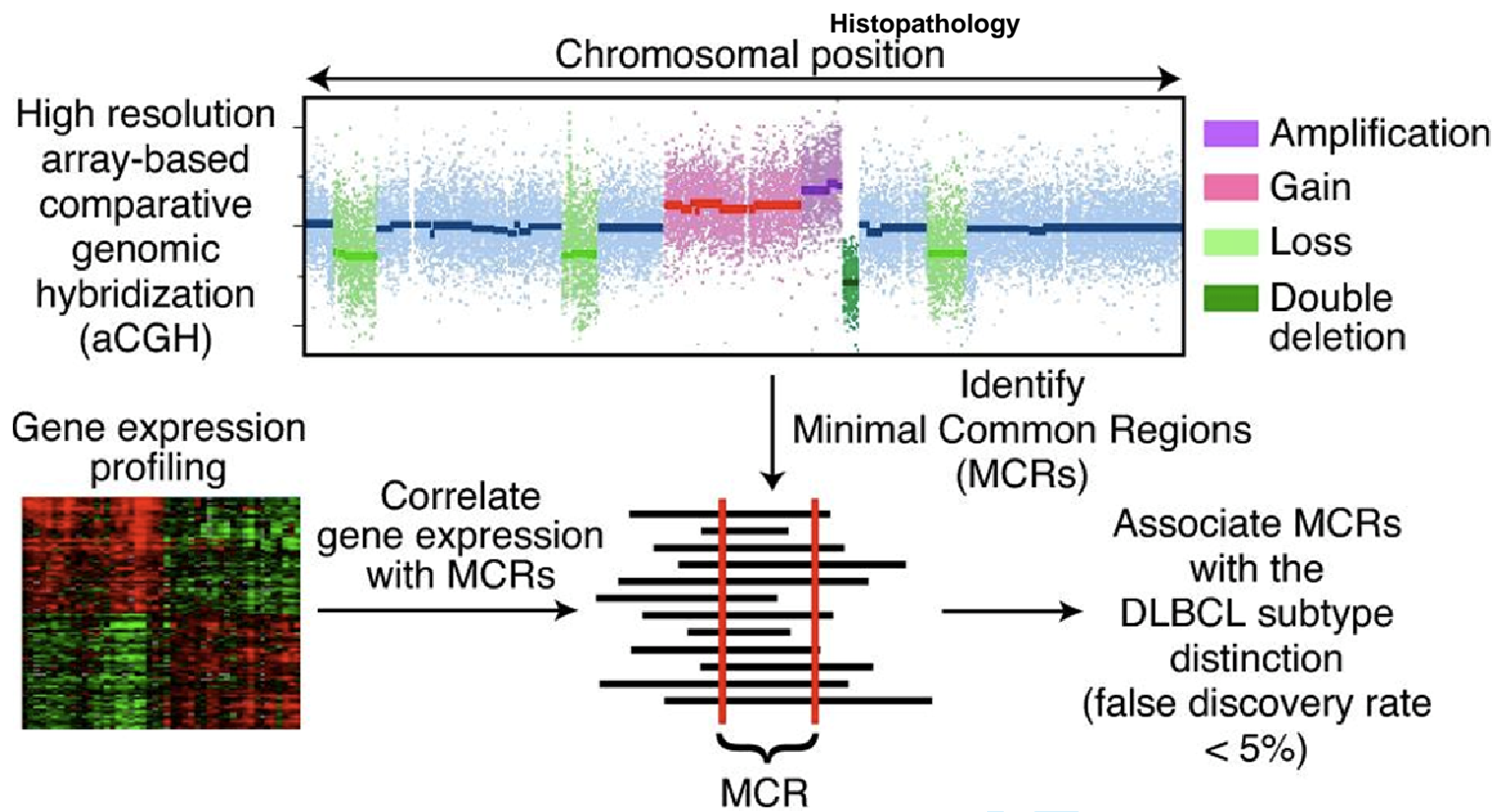
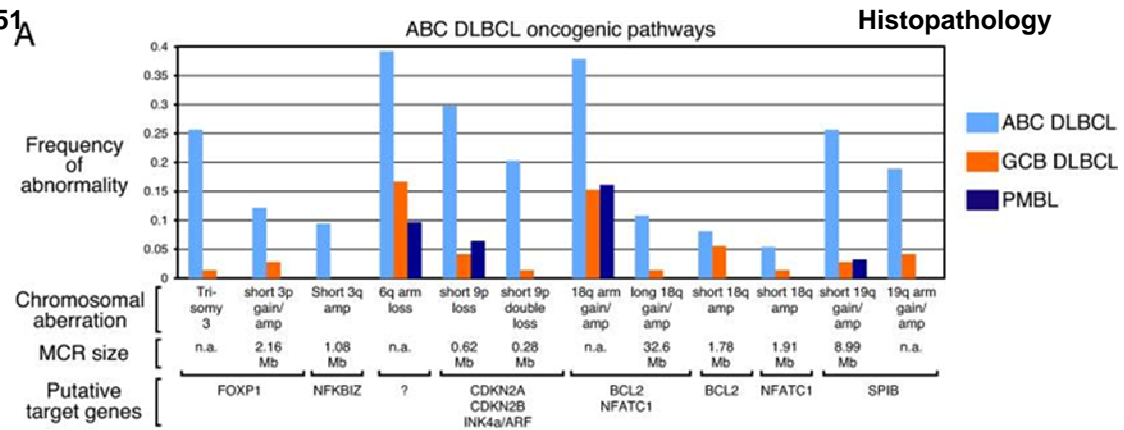
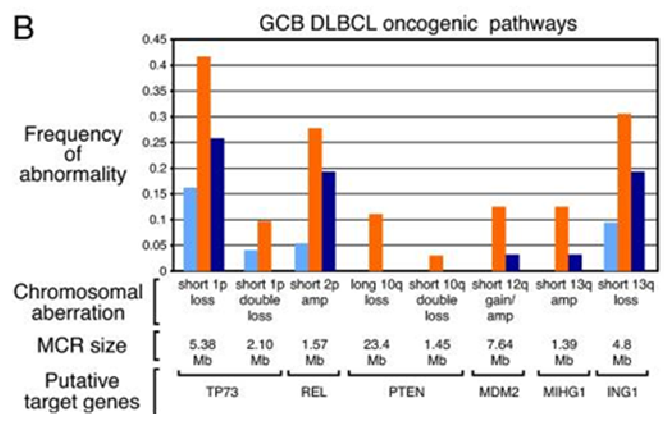


Figure 7a

A



B



C

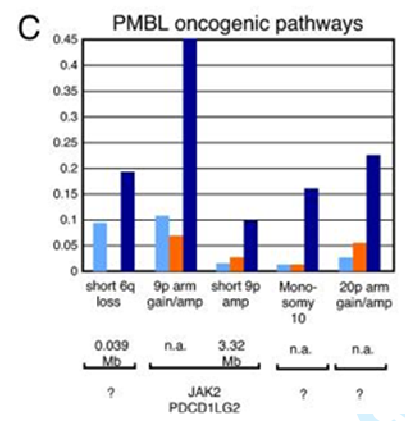


Figure 7b

Review

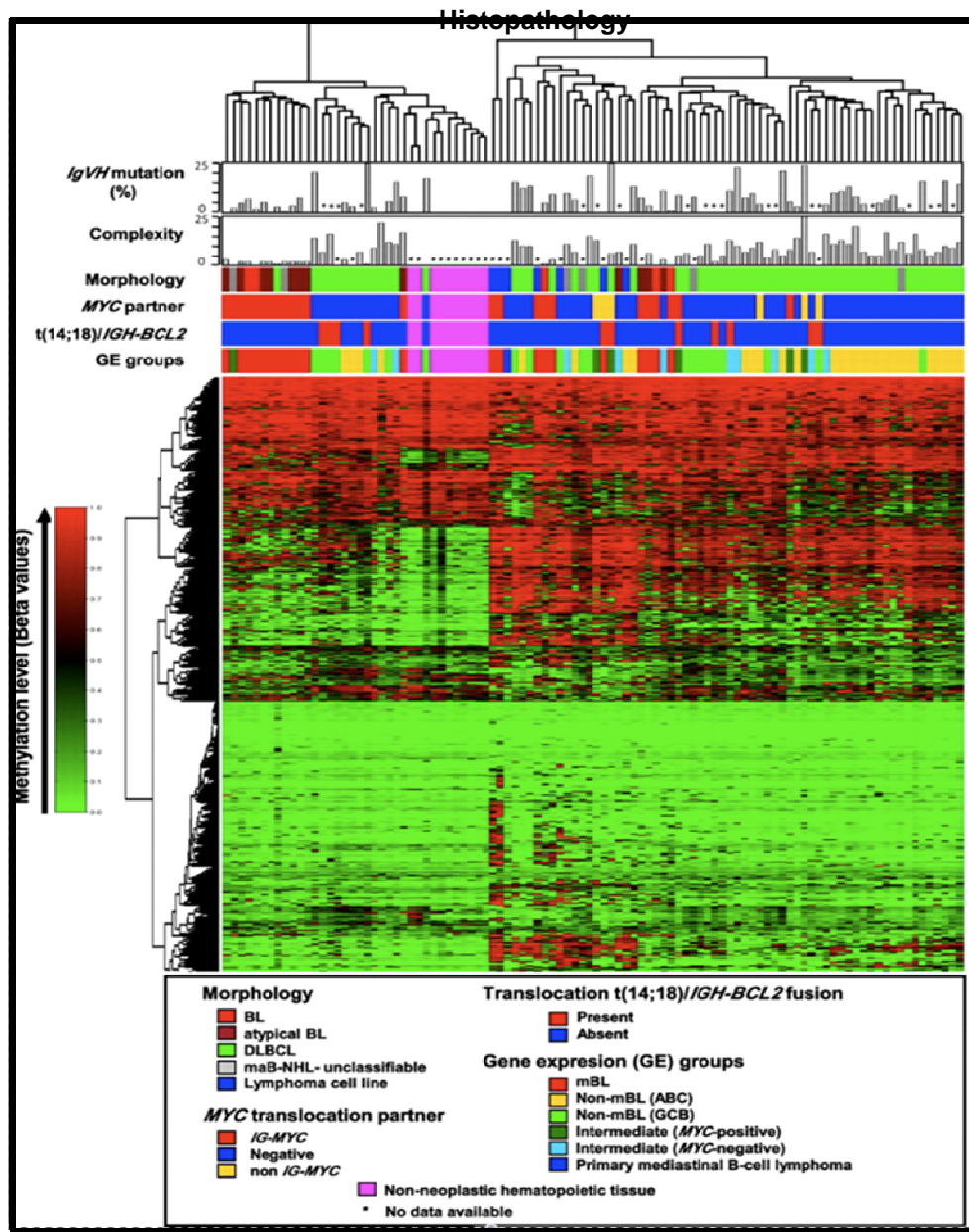


Figure 8

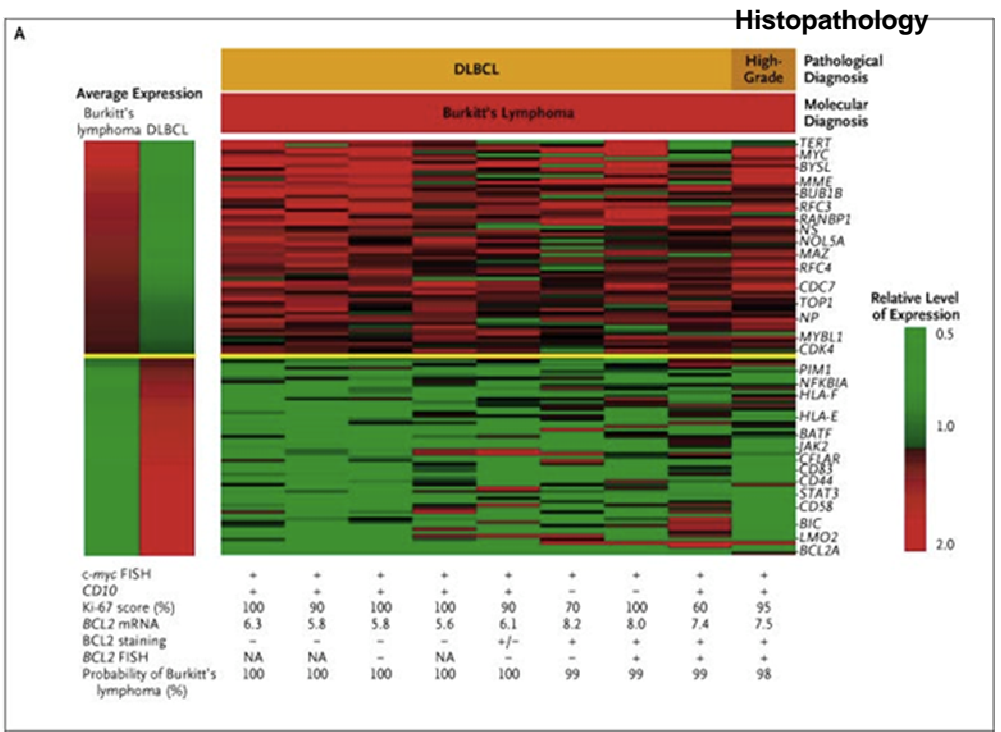


Figure 9a

Peer Review

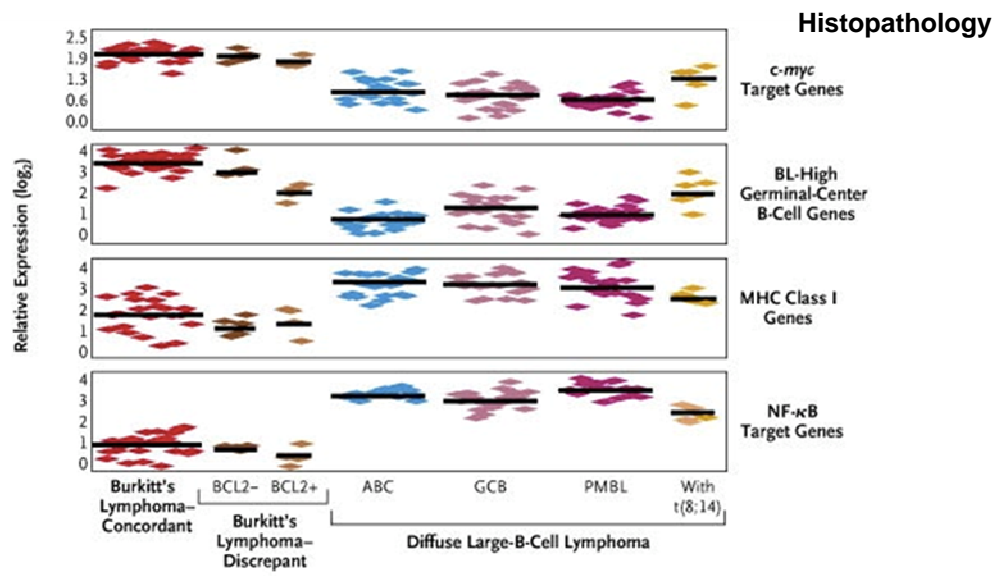


Figure 9b

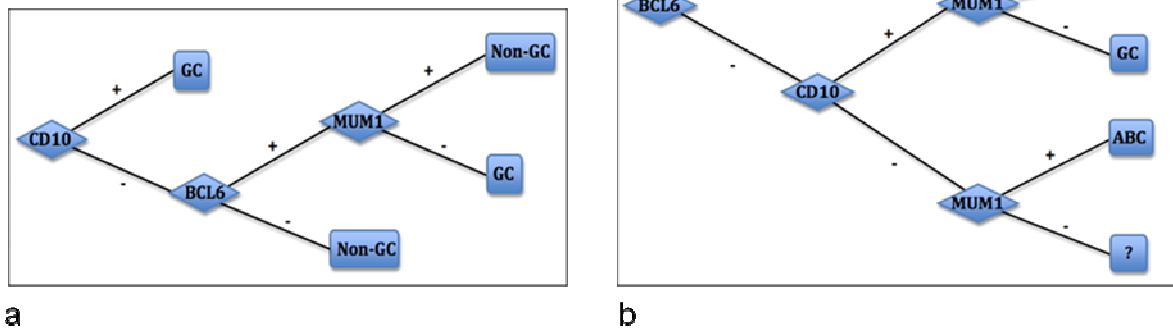


Figure 10

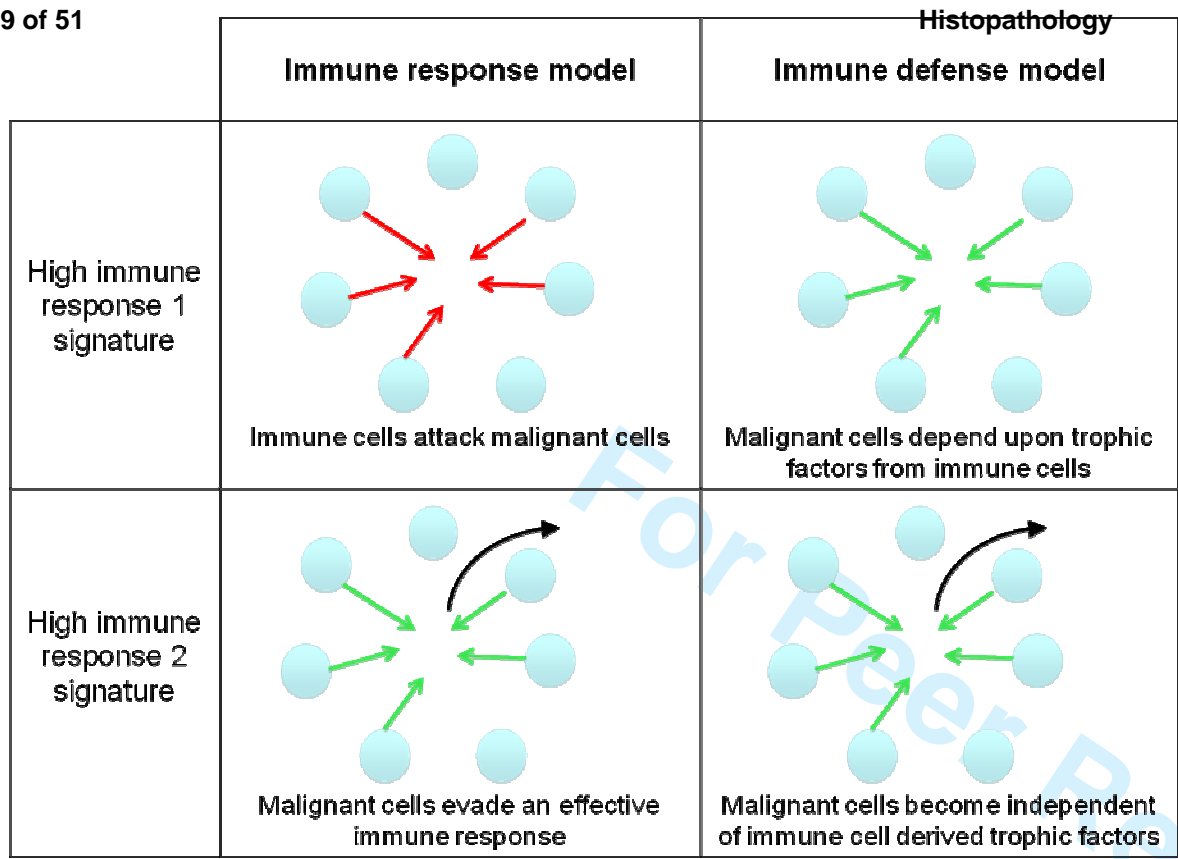
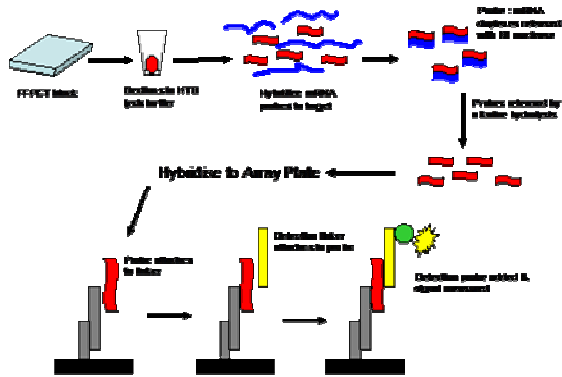
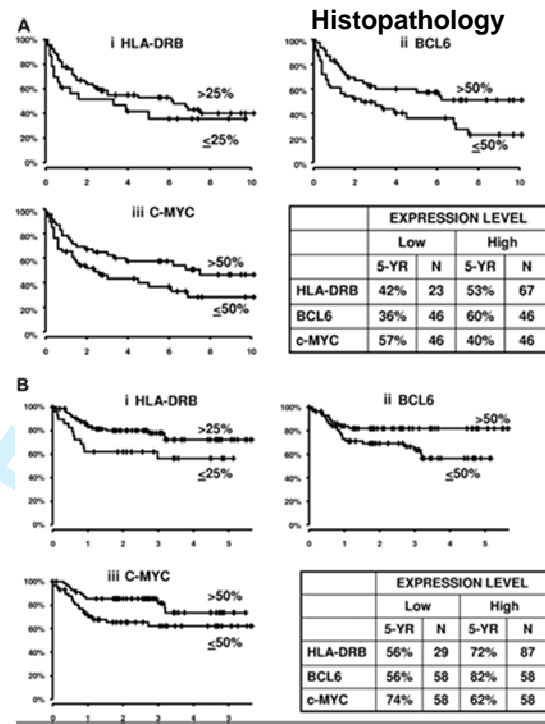


Figure 11



a



b

Figure 12

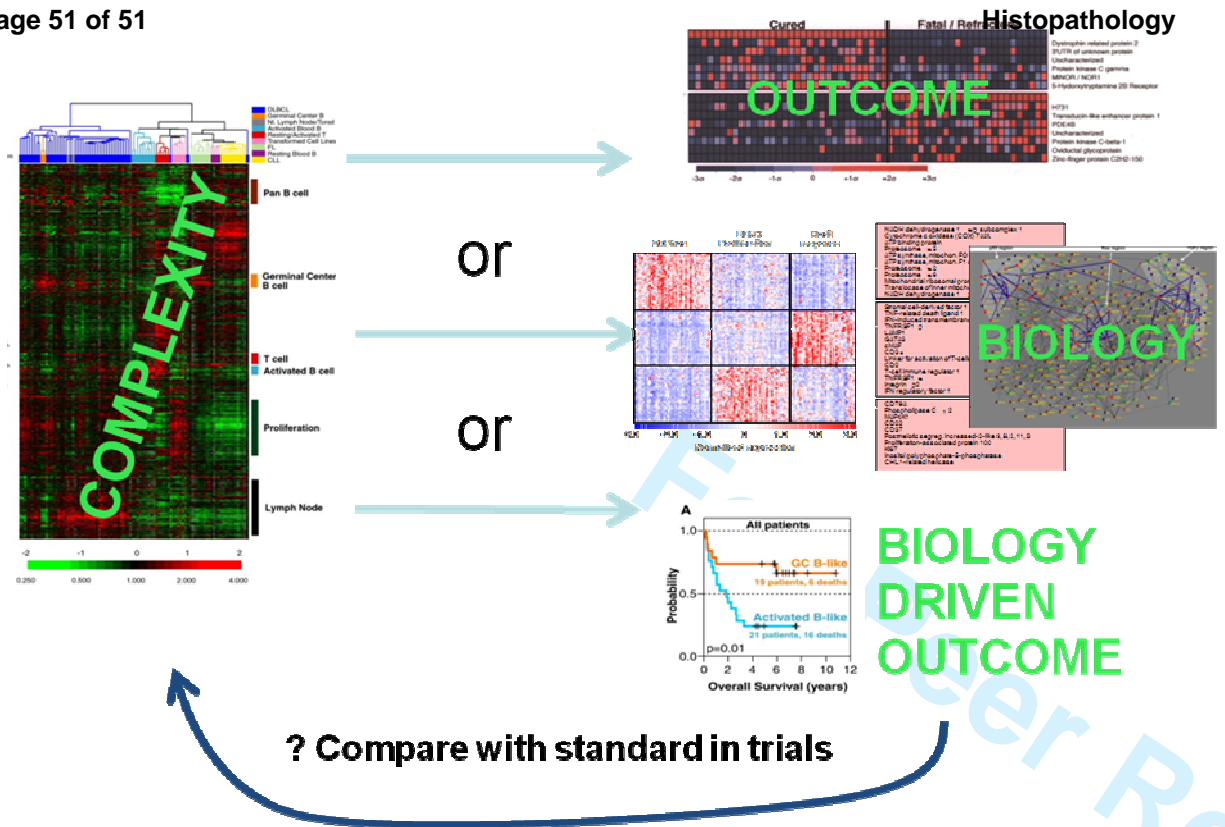


Figure 13