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Supplement Article

III. Applying molecular phenotyping in practice

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Introduction

Morphologic heterogeneity in diffuse large B-cell lymphoma (DLBL) was recognized under historic classification systems for lymphoma, and the application of cytogenetics with *in situ* hybridisation gave some indication of the variable molecular pathogenesis, but it was really only with gene expression profiling that a biologically plausible way was found to characterize the different entities. The examination of a very large number of different mRNA sequences led to the first demonstration of groups with different prognosis according to their apparent cell of origin [1]. Since then, there has been a progressive accumulation of information about the molecular characteristics in the two main groups, germinal centre B-cell (GCB) type and activated B-cell (ABC) type, with genomic analyses [2–4] and RNA knockdown library screens [5–7] adding to knowledge of the different pathogenic pathways, and the potential targets for intervention using selective inhibitors. Retrospective studies have confirmed the worse prognosis for patients with ABC type DLBL [8,9], although when multivariate analyses are used, it is clear that some of the difference may well be related to older age at diagnosis and other adverse features of their presentation [10].

The challenge now is to use this information to improve the results of treatment for patients. This requires two key elements: a reliable test and an effective intervention. To what extent are these currently available, or about to become so?

The proliferation of tests

Gene expression profiling was first used to subclassify DLBL over a decade ago, but there are still considerable technical challenges to its routine use. Originally, the arrays required large quantities of high quality mRNA, only available from fresh frozen biopsy material. As the majority of patients with DLBL are diagnosed using formalin-fixed biopsies, many of them in small needle cores obtained percutaneously, this considerably restricted the number and type of patients available for study, with the potential for sample bias. All the studies published to date have been retrospective, with the inherent difficulties this imposes for sampling and the lack influence on treatment selection. However, the findings seem to be consistent in some respects, in that GCB type DLBL carries a better prognosis in almost all the series reported, despite a variety of different classifiers. The relative number of unclassified (or in some series 'type 3') cases is apparently variable, and their prognostic significance is inconsistent, with some series showing outcomes as good as GCB and some as poor as ABC.

Recent developments in mRNA amplification and solid-phase chemistry have made it possible to obtain reliable information with much smaller samples from routine biopsies, and this promises to transform the practicality of the process. Reliable expression profiles can now be obtained from much smaller amounts of material, provided this has been promptly handled in the diagnostic laboratory, and adequately fixed.

The next challenge for application of molecular phenotyping is to develop analytic methods that can be applied prospectively to each individual sample, as opposed to the retrospective assignments of large numbers of cases, which have previously been reported. To make use of the information for clinical decisions, it needs to be possible to fit a specific sample's expression profile into a pre-existing framework and generate a reliable subtype, something which has not been attempted previously. The definitive algorithm for cell of origin developed by the NCI group used 27 features from an Affymetrix Lymphochip custom array to assign cases treated with CHOP chemotherapy to ABC, GCB or type III/unclassified, using a Bayesian predictor based on a training set distribution [11]. Since then, further studies have progressively expanded the classifier gene set, seeking to distinguish DLBL from, respectively, either Burkitt lymphoma or primary mediastinal lymphoma, and to predict outcomes in groups treated with rituximab in addition to chemotherapy. These increased the number of genes

studied to over 180. Other analyses used shortened forms of the original algorithm to identify subtypes based on signatures from the B-cell receptor, host responses and oxidative phosphorylation, and at the extreme a two gene signature based simply on the levels of one B-cell gene (*LMO2*) and a stromal response marker (*TNFRSF9*) [12]. The overall effect has been to generate significant uncertainty over the technology, with no two publications using the same approach, and no widely accepted means to allocate an individual case to a molecular subtype.

A recent meta-analysis of all the existing classifiers which were used to predict survival appears to move this field forward. Using balanced voting from four different machine learning classifiers, Care *et al*. were able to derive a robust 20-gene signature that carries excellent predictive power for survival, independent of the technology platform or method of sample processing [13]. The genes used were a subset of the original Wright classifier, but appeared to perform as well as or better than any of the other models used, even those drawing on information from much larger numbers of genes. This 'Diffuse large B-cell lymphoma automatic classifier' is available as open source software: http://www.bioinformatics.leeds.ac.uk/~bgy7mc/DAC/

The difficulty of using mRNA expression profiles has led to extensive efforts to define algorithms based upon immunostaining instead. This would be attractive on a practical level, being more readily applicable in routine hemato-oncology diagnostic laboratories. The first such algorithm used stains for CD10, Mum-1 and Bcl-6 [14]. It has been widely tested, but suffers from two key drawbacks. Firstly, one of the critical stains, for Bcl-6, shows extremely poor reproducibility, even in the hands of experienced hematopathologists, and secondly, it does not appear to distinguish groups of significantly differing prognosis in almost all the series examined, when patients were treated with rituximab-containing regimens. Refinements of the approach have led to the incorporation of further markers such as Fox-P1, GCET 1 and LMO2, and in some series, the inclusion of cytogenetic markers by fluorescent *in situ* hybridisation (FISH), but none has convincingly shown a robust capacity to predict the outcome of treatment. Most recently, stains for a stromal response marker and microvessel density have been added to the stains for GCB type with apparently improved prognostic significance [15], but this has not been evaluated in other series. For the time being, gene expression profiling remains the standard.

One distinct finding in DLBL has been the extremely poor prognosis for those lymphomas with chromosomal breaks targeting the *MYC* gene with rearrangement of other genes such as *BCL2* or *BCL6*, previously identified using FISH. The poor results of treatment in this setting have not been improved by conventional approaches such as treatment intensification and this group, comprising around 5% of DLBL, is one for which new approaches are urgently needed. They most often fall into the GCB type on the basis of GEP, but a recent study using antibodies that stain MYC has shown that co-expression of MYC and BCL2 is more common in ABC cases classified by GEP, which appear to contribute significantly to the worse prognosis of this group as well [16]. It is likely that the overexpression of MYC protein is more common than the large chromosomal rearrangements detected by FISH, and this appears to cut across the cell of origin classification.

The proliferation of targets

As understanding of the key pathogenic processes has built up, so has a picture of the potential targets for intervention. The ABC subtype is particularly linked to activation of the NF-κB pathway, with functionally relevant mutations found in many of the signalling intermediaries between the B-cell receptor and the nucleus [2]. These include activating mutations in *CD79A/B*, *CARD11*, *MYD88*, *TRAF2*, *TRAF5*, *MAP3K7* and *RANK*, and loss of function mutations in *TNFAIP3* (*A20*). The convergent functional effect of these is the upregulation of nuclear NF-κB activity, and ABC cell lines show particular sensitivity to its inhibition by dominant negative Iκ-B protein expression [6]. In those ABC lymphomas without somatic mutations affecting this pathway, there appear to be alternative mechanisms for persistent signalling, and addiction to activation of this pathway is a broad feature of the subtype.

There are a number of points at which this signalling may be interrupted using selective inhibitors. These include fostamatinib inhibiting Syk; ibrutinib targeting Bruton tyrosine kinase; enzastaurin, sotrastaurin and GS1101 blocking different protein kinase C species; everolimus inhibiting mTOR, and broad downregulation of NF-κB by blockade of proteosomal degradation of the inhibitory Iκ-B using bortezomib. All of these are currently being tested in the treatment of recurrent or refractory DLBL with varying levels of response, as is lenalidomide, whose mechanism of action is unclear, but may involve inhibition of NF-κB targets such as *IRF-4* through increased production of interferon-β. The latter may be particularly attractive in those cases where *CARD11* or *MYD88* mutations render them resistant to upstream inhibitors such as ibrutinib.

The GCB type of DLBL is less clearly dependent upon dysregulation of a particular pathway, but there is a characteristic pattern of mutational changes, in particular among epigenetic regulators such as *EZH2*, *CREBBP* and *EP300*. These lymphomas share this characteristic with other germinal centre origin malignancies such as follicular lymphoma, and inhibitors of EZH2 have shown striking efficacy in preclinical models of GCB lymphoma [17].

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It is important to note that the molecular changes described do not appear to be exclusive to any one subtype, although they do occur preferentially in one or the other. It is still too early to determine the relationship between clinical responses and specific molecular markers, although this data is starting to emerge. A small pilot study of bortezomib with chemotherapy in recurrent and refractory DLBL suggested a much higher response rate in ABC lymphoma defined by GEP [18], but these findings await confirmation in larger first line trials.

Thus, it appears that ibrutinib, bortezomib and lenalidomide may all be more likely to produce a response in ABC type DLBL, whilst the use of epigenetic modifiers is most logical in the GCB type, and those lymphomas with amplified *MYC* may require different approaches again.

Applying the knowledge

The design of clinical trials in DLBL is becoming more complex as a result of these developments. Because there is not an absolute correlation between molecular type and responsiveness to targeted agents, it may be necessary to test a novel drug across a range of molecular subtypes to clearly define its efficacy, and if possible link it to the gene expression or immunophenotyic signature in retrospect. There is a lack of specificity both in the biology of the lymphomas and in the action of the drugs that makes it risky to assume too much in designing the clinical trials. For example, there are a number of GCB lymphomas that exhibit upregulation of the NF-κB pathway, and similarly, there are examples of responses to ibrutinib and among GCB lymphomas, albeit less frequent than the responses among the ABC type.

For this reason, the first study to prospectively test the addition of bortezomib to R-CHOP in lymphomas defined by GEP is initially randomizing both ABC and GCB types, but with planned safety and futility analyses to determine whether there is a differential response rate according to the molecular phenotype: http://clinicaltrials.gov/show/NCT01324596. In this way, the study is initially agnostic of the relationship between the targeted agent and the subtype, but with opportunities to close randomization to one type if there is no evidence of benefit as the study proceeds. Other agents, for which the mechanism of action is more precisely defined, may be studied in subgroups defined by either immunostaining or GEP, and here, it may be an advantage to incorporate them into larger programmes of work in which treatment can be stratified according to molecular type, to conduct the most efficient studies. It is likely that in future we will conduct trials with portfolios of different targeted agents, selectively testing them in the sub-population with the highest chance of benefit according to mechanistic understanding.

Conclusions

The management of DLBL is becoming more sophisticated as the result of improved knowledge about pathogenesis, and a major expansion in the number of targeted agents that may prove effective in treatment. This progress will challenge traditional diagnostic methods and traditional means of conducting clinical trials, requiring new processes for patient selection and the increasing use of adaptive trial designs, relating responses to molecular subtypes.

Conflicts of interest

PJ receives research funding from J&J pharmaceuticals and has participated in paid advisory boards for J&J, Pfizer and Lilly. AD has participated in paid advisory boards for Roche and Novartis.

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