

ORIGINAL ARTICLE

High frequency of germinal centre derivation in diffuse large B cell lymphoma from Asian patients

A K H Shia, G-G Gan, S Jairaman, S-C Peh

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See end of article for authors' affiliations

Correspondence to:
Professor S C Peh,
Department of Pathology,
Faculty of Medicine,
University of Malaya,
50603 Kuala Lumpur,
Malaysia; pehsc@ummc.edu.my

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Background: Recent reports have divided diffuse large B cell lymphoma (DLBCL) into germinal centre B cell-like and activated B cell-like subgroups with implicated differences in prognosis.

Aims: To delineate the germinal centre B cell derivation group from an Asian series of DLBCLs.

Methods: Fifty four cases were analysed by polymerase chain reaction to detect the t(14;18) translocation and immunohistochemistry for BCL2, CD10, BCL6, and E2F1 expression.

Results: Eighteen of 54 cases had *bcl2* gene rearrangement, 36 of 52 expressed BCL2, 29 of 52 expressed BCL6, 20 of 53 expressed CD10, and 18 of 53 expressed E2F1. There was a significant association between *bcl2* gene rearrangement and the expression of both BCL2 and CD10. Using the minimally acceptable criteria of t(14;18) rearrangement and/or CD10 expression, 26 of 54 cases were probably germinal centre derived, in agreement with other reports. A higher proportion of cases had t(14;18) translocation, suggesting that they may be derived from transformed follicular lymphomas. E2F1 positivity did not correlate with the known germinal centre markers, even though it has recently been suggested that it may be a new GC marker.

Conclusions: It may be possible to stratify patients for treatment using markers for specific lineages of B cell differentiation.

Diffuse large B cell lymphoma (DLBCL) is a clinically and morphologically heterogeneous group of lymphomas. It constitutes approximately 30–40% of all lymphomas diagnosed in the West, and accounts for an even higher proportion in developing countries.^{1,2} Previous attempts at subclassification based on morphology failed because of unacceptable interobserver and intraobserver discrepancies.³ Hence, the new World Health Organisation classification has grouped “diffuse proliferation of large neoplastic B lymphoid cells with nuclear size equal to or exceeding normal macrophage nuclei” under the umbrella term of DLBCL.⁴ However, grouping biologically heterogeneous entities artificially as a single disease can result in poor understanding of prognostic factors, making it difficult to improve the survival of patients. Following recent advances in microarray technology, two subgroups—the germinal centre (GC) B cell-like and the activated B cell-like—gene expression signatures have been identified.⁵ The identification of these subgroups by Alizadeh *et al* (2000) has increased the interest in defining specific markers for each.⁵ However, it is expected that there will be more subgroups within DLBCL than has already been described.⁶

“Grouping biologically heterogeneous entities artificially as a single disease can result in poor understanding of prognostic factors, making it difficult to improve the survival of patients”

The t(14;18) chromosomal translocation is reported to occur in more than 80% of follicular lymphomas, and 7–38% of DLBCLs.^{7–9} Diffuse lymphomas showing this trademark translocation between the *bcl2* gene and the immunoglobulin heavy chain (IgH) locus are believed to have progressed from the more indolent follicular lymphoma.¹⁰ The translocation to the IgH promoter deregulates the *bcl2* gene, causing overexpression of the protein. Most breakpoints are clustered at the 3' untranslated region of exon 3, known as the major

breakpoint region (MBR) of the *bcl2* gene.¹¹ However, translocation of the *bcl2* gene does not correlate significantly with overexpression of the BCL2 protein in DLBCL.¹²

Both CD10 and BCL6 have been hailed as reliable GC markers.^{13,14} CD10 is a single chain 100 kDa type II cell surface glycoprotein with a sequence identical to neural endopeptidase. Its expression in lymphoid cells is strictly confined to the follicles of GCs.¹⁵ The expression of CD10 has been used to distinguish follicular lymphoma from other low grade non-Hodgkin lymphomas.¹⁶

The *bcl6* gene is a newly discovered putative protooncogene that encodes the BCL6 protein, which is expressed exclusively in the follicular centre B cells of reactive lymphoid tissue.^{15,17} It is presumed that translocation of the *bcl6* gene, found in 30–40% of DLBCLs, upregulates the protein by virtue of promoter substitution.¹⁰ Mutations at the 5' non-coding region of the gene are also thought to play a role in regulating the expression of the protein.¹⁸ A recently elucidated mechanism proposes that the BCL6 protein is negatively autoregulated under normal circumstances, so that mutations in the autoregulatory region would lead to increased protein expression.¹⁹ It is possible that overexpression of BCL6 inhibits the terminal differentiation of GC B cells, and facilitates the transformation of cells.¹⁹

It has been proposed that expression of both CD10 and BCL6 be used to determine the GC derivation of tumour cells.^{13,14} CD10 and BCL6 have been reported as markers of both improved survival and adverse prognosis.^{20–22} However, recent studies noted that these proteins are associated with GC derived DLBCL, implying an association with improved survival.^{5,6}

The transcription factor E2F1 is one of a group of proteins that mediate cell cycle progression from the G1 to S phase.²³

Abbreviations: DLBCL, diffuse large B cell lymphoma; GC, germinal centre; IgH, immunoglobulin heavy chain; JH, joining region; MBR, major breakpoint region; MCR, minor cluster region; PCR, polymerase chain reaction

In resting cells, E2F1 is sequestered by being bound to the hypophosphorylated retinoblastoma protein. When the retinoblastoma protein is phosphorylated, E2F1 protein is freed and becomes biologically active. Active E2F1, bound to its partner DP1, is then able to bind to the promoter regions of genes involved in DNA synthesis, such as those encoding cyclin E, cyclin A, cdc2, cyclin dependent kinase 2, thymidine kinase, and DNA polymerase α , and promote cell cycle progression. It is thought that the protein is the ultimate effector of G1 to S cycle progression. Study into the function of this protein is relatively new, and the pattern of staining has only recently been described.²⁴ Hence, its possible role in lymphomagenesis needs further investigation and evaluation.

In a previous study of a series of Asian adult non-Hodgkin lymphomas from a single medical centre, DLBCL was found to constitute a large proportion (58%) of the cases.² Therefore, it is important to gain a better understanding of this group of tumours and be able to delineate tumours with GC properties from other subtypes.

MATERIAL AND METHODS

Patients' material

Cases diagnosed as DLBCL from four consecutive years (1996–1999) were retrieved from the archives of the department of pathology, University of Malaya Medical Centre, Malaysia for our study. These cases were reviewed and reconfirmed as DLBCL according to the criteria of the new World Health Organisation classification. Cases with insufficient material, missing blocks, or with a doubtful diagnosis after the review were excluded from further analysis. In total, 54 cases were included in our study. The patients' demographic data were obtained from the pathology request forms provided by the supervising clinician.

Immunohistochemical stain

Serial 4 μ m thick sections were mounted on silanised slides, dewaxed in xylene, and cleared in alcohol, followed by a microwave or pressure cooking antigen retrieval step. Staining was performed according to a standard three step immunoperoxidase method. Table 1 summarises the panel of antibodies used and the methods applied for the respective antibodies. Semiquantitative evaluation of expression was performed by a pathologist (SCP). The tumour was considered to be BCL2 or CD10 positive if more than 75% of the tumour cells were positive, whereas for BCL6 and E2F1 this figure was 10%. Reactive lymphoid tissues were used as positive controls for the staining procedure. The cutoff points for the different stains were established from background staining of hyperplastic lymphoid tissue to determine over-expression resulting from the tumour.

PCR detection of t(14;18) translocation

DNA was extracted from formalin fixed, paraffin wax embedded tissues using proteinase K (200 μ g/ml) digestion

Table 2 Sequence of primers used for nested PCR amplification of MBR/JH and MCR/JH

Chromosome regions	Primer sequence
<i>IGH</i> JH external	5' ACC TGA GGA GAC GGT GAC 3'
<i>IGH</i> JH internal	5' ACC AGG GTC CCT TGG CCC CA 3'
<i>bcl2</i> MBR external	5' CAG CCT TGA AAC ATT GAT GG 3'
<i>bcl2</i> MBR internal	5' TAT GGT GGT TTG ACC TTT AG 3'
<i>bcl2</i> MCR external	5' CGT GCT GGT ACC ACT CCT G 3'
<i>bcl2</i> MCR internal	5' GGA CCT TCC TTG GTG TGT TG 3'

IGH, immunoglobulin heavy chain gene; JH, J region; MBR, major breakpoint region; MCR, minor cluster region; PCR, polymerase chain reaction.

as described by Zhang *et al.*²⁵ The supernatant containing DNA was used directly for polymerase chain reaction (PCR) amplification. A nested PCR method previously described by Gribben *et al* was used to amplify the MBR and minor cluster region (MCR) of the *bcl2* gene.²⁶ PCR was performed in a final volume of 50 μ l using 5 μ l of the template DNA, 0.5 μ M of oligonucleotide primers (table 2), 0.4mM of each dNTP (dATP, dGTP, dCTP, dTTP) (Fermentas, Vilnius, Lithuania), 3.0mM magnesium chloride, 2.0 U HotStarTaq DNA polymerase (Qiagen, Hilden, Germany), and 1 \times PCR buffer (Qiagen). The initial PCR amplification was performed with 15 minutes of preheating at 95°C to activate the HotStarTaq DNA polymerase, followed by 30 cycles of one minute of denaturation at 95°C, one minute of annealing at 55°C for MBR or 58°C for MCR, and one minute of extension at 72°C. After 30 cycles, the samples were incubated for a final extension step at 72°C for two minutes to complete the reaction. Nested PCR using 5 μ l aliquots of the first PCR reaction product was performed for 30 cycles using primers internal to the first primer set (table 2). The same cycling conditions as in the first PCR were used for the nested PCR. Contamination was tested by incorporating negative controls without DNA template for both the first and nested PCR reactions. The size of the products ranged from 100 to 300 bp for MBR/JH and 500 to 1000 bp for MCR/JH rearrangement. Cell lines RL-7 and SU-DHL-16 (provided by Dr J W Chan, University of Nebraska Medical Centre, USA) were used as positive controls for MBR and MCR, respectively. The products were separated on a 2% agarose gel (Hispanlab, Alcobendas, Spain) containing 5 μ g/ml ethidium bromide (Gibco BRL, Gaithersburg, Maryland, USA) and visualised on an ultraviolet illuminator.

Criteria for GC derivation

Cases were considered to be GC derived if they showed minimally acceptable criteria of *bcl2* translocation or CD10 expression. These criteria were determined based on the reports of Huang *et al* and McCluggage *et al*, who found *bcl2* translocated cases to represent a subset of GC derived cases,

Table 1 Antibodies, sources, clones, antigen retrieval methods, dilutions, and detection methods used

Antibody	Source	Clone	Antigen retrieval	Dilution	Detection
CD20	Dako, Glostrup, Denmark	L26; monoclonal	MW, CB pH 6.0	1/500	ABC
CD3	Dako	CD3; polyclonal	MW, CB pH 6.0	1/100	ABC
BCL6	Dako	PG-B6p; monoclonal	PC, EDTA pH 8.0	1/25	StreptABC
BCL2	Dako	124; monoclonal	MW, CB pH 6.0	1/50	Envision
CD10	Novocastra, Newcastle, UK	NCL-CD10-270; monoclonal	MW, Dako high pH retrieval solution	1/50	Envision
E2F1	Santa Cruz Technology, Santa Cruz, California, USA	KH95; monoclonal	MW, EDTA pH 8.0	1/20	StreptABC

ABC, avidin–biotin complex; CB, citrate buffer; Envision, Dako EnVision+™ mouse peroxidase; EDTA, 0.1mM EDTA; MW, microwave; PC, pressure cooker; StreptABC, streptavidin–biotin complex.

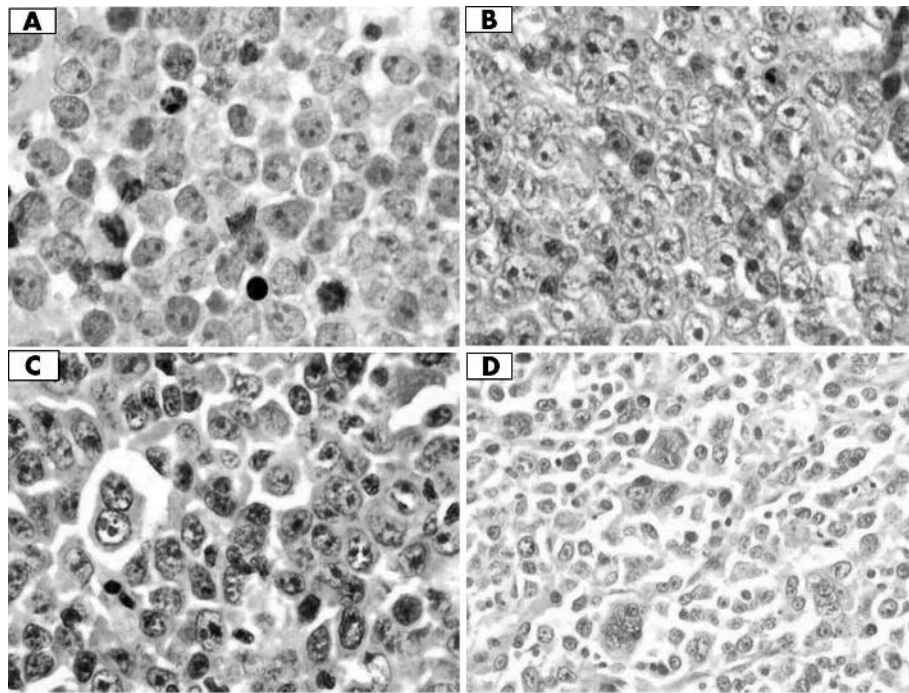


Figure 1 Haematoxylin and eosin stains showing the wide range of pleomorphism in our cases of diffuse large B cell lymphoma; original magnification, $\times 600$. (A) Centroblastic morphology, (B) immunoblastic morphology, (C) plasmablastic morphology, and (D) polylobated cells.

and CD10 to be a more specific marker for their groups of GC derived tumours when compared with BCL6.^{27 28}

Statistical analysis

Statistical analysis for immunophenotype was carried out using the χ^2 test and SPSS version 10.0 software. A p value of less than 0.05 was considered significant.

RESULTS

Demography

There were 33 male and 21 female patients, with a male to female ratio of 1.6 : 1. There were 27 Chinese, 22 Malays, and five Indians. The ages of the patients ranged from 9 to 86 years, with a mean of 49, and a median of 52. Of the 54 cases, there were 11 with extranodal presentation. The extranodal sites were the testicles (three), retroperitoneum (one), small bowel (one), and the soft tissue of the thigh (one), bone (one), skin (two), rectum (one), and nose (one).

Morphological appearance

All cases were composed of large lymphoid cells of B cell phenotype, with vesicular and hyperchromatic nuclei, prominent nucleoli, and varying amounts of cytoplasm. The cellular morphology was heterogeneous, with some being centroblast-like, others immunoblastic or mixed, and still others with a range of pleomorphism of nuclear sizes including multinucleation (fig 1).

Immunophenotypic expression

Figure 2 shows the staining pattern for each antibody. The staining patterns of CD10, BCL6, and BCL2 agreed with previous reports, but E2F1 showed stronger nuclear membrane staining in our series. Some of these cases were either non-reactive or showed non-specific staining, and they were excluded from further analysis. These involved two cases each for BCL6 and BCL2, and one each for CD10 and E2F1 staining. Of the well stained cases, 29 of 52 showed positive BCL6 expression, 20 of 53 expressed CD10, 36 of 52 expressed

BCL2, and 18 of 53 expressed E2F1. The expression of these proteins was not significantly associated with one another.

Bcl2 gene rearrangement

Molecular analysis for *bcl2* rearrangement was performed in all cases. Figure 3 shows the positive bands in the PCR analysis. Eighteen of 54 cases showed rearrangement involving MBR/JH, but none showed MCR/JH rearrangements. There was a significantly association between *bcl2* gene rearrangement and CD10 expression ($p = 0.018$), and also between *bcl2* gene rearrangement and BCL2 protein expression ($p = 0.039$; table 3). However, only 15 of 36 BCL2 expressing cases showed *bcl2* gene rearrangement.

These cases were subclassified into eight groups according to the presence or absence of *bcl2* gene rearrangement and the expression status of CD10 and BCL6 (table 4). Using the GC defining criteria already described, 26 of the 54 cases showed a GC derivation.

DISCUSSION

Recent data that subgrouped DLBCL into GC B-like DLBCL and activated B-like DLBCL, showed that GC-like DLBCL had a much better overall survival, at 76% and 16%, respectively, after five years.⁵ This increased interest in studying follicular centre cell markers to determine GC derivation within the heterogeneous group of DLBCLs. Studies have shown that CD10, BCL6, and BCL2 expression and t(14;18) rearrangement are reliable characteristics of follicular centre cell lymphomas^{9 13}; in addition, a recent report implicated E2F1 as a prognostic marker.²⁹

About a third of our series of DLBCLs showed *bcl2* gene rearrangements (table 3), which falls within the reported range of 7–38%.^{7–9 30} We were unable to detect MCR rearrangements; this could partially be explained by the large PCR product (> 600 bp), because amplification of larger products is difficult using paraffin wax embedded sections. The rate of expression of BCL2 (36 of 52; 69%; table 3) also agrees with the reported range of 40–81%.^{7 9 30}

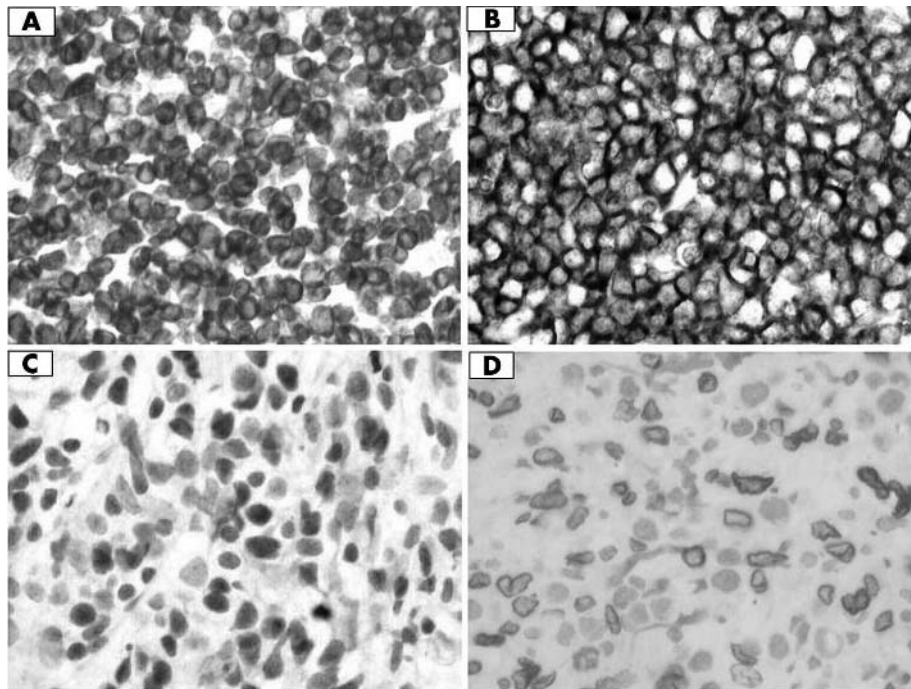


Figure 2 Immunohistochemical staining of diffuse large B cell lymphoma; original magnification, $\times 600$. (A) BCL2 expression, (B) CD10 expression, (C) BCL6 expression, and (D) E2F1 expression.

Although t(14;18) translocation was found to be significantly associated with BCL2 protein expression, it has often been reported that protein expression is independent of gene rearrangement.^{7,8} This was supported by the t(14;18) translocation being present in only a proportion (15 of 36) of BCL2 positive DLBCL cases. Hence, a large number of BCL2 expressing cases (21/36) do not have the t(14;18) translocation, which agrees with the hypothesis that other genetic alterations may be involved in upregulation of the gene.³¹ Rantanen *et al* suggested that gene amplification may be more important than gene rearrangement for BCL2 overexpression.³²

With only 29 of 52 (58%) of our series expressing BCL6, this falls within the lower end of the reported range of 57–95%.^{9,31} Although it has been reported that long exposure of cut sections to air, old blocks, or imperfect fixation could affect BCL6 staining,³¹ the lack of BCL6 expression could also result from *bcl6* gene rearrangements or somatic hypermutation at the 5' non-coding region.^{18,33} It is possible that

deregulation of BCL6 in the GC may result in the perturbation of proteins that control apoptosis, and the subsequent loss of control could lead to cells continually proliferating outside of the GC. In this way, the behaviour of cells overexpressing BCL6 is analogous to that of those overexpressing BCL2.

“We speculate that the lower frequency of follicular lymphoma detected in the Asian population may result from early transformation of follicular lymphoma to diffuse large B cell lymphoma”

E2F1 staining was present in 18 of 53 (34%) of our cases, lower than previous reports of 43–83%.^{24,29,34} It was included in the study as a possible new GC marker. However, it showed no significant association with other known GC markers. It is possible that in DLBCL the E2F1 protein may be lost or mutated, leading to a lower detection rate. Moller *et al* found that low expression of the E2F1 protein is related to treatment failure in DLBCL.²⁹ The E2F1 protein has both oncogenic and tumour suppressive abilities; too much or too

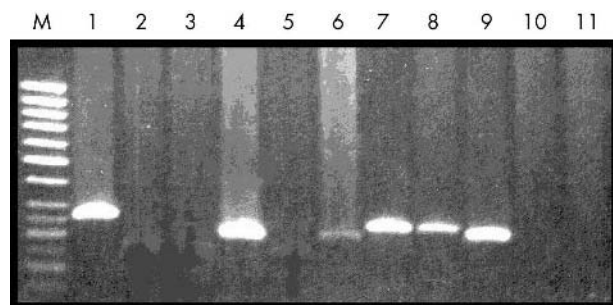


Figure 3 Polymerase chain reaction (PCR) amplification product bands of the major breakpoint region/joining region in diffuse large B cell lymphoma (DLBCL). M, 50 bp DNA marker; lane 1, positive control (RL-7; ~ 275 bp), lanes 2, 3, and 5, t(14;18) translocation negative DLBCL cases; lanes 4, 6, 7, 8, t(14;18) translocation positive DLBCL cases; lanes 10, 11, first and second PCR negative controls.

Table 3 The series of diffuse large B cell lymphomas showing *bcl2* gene rearrangement and their immunophenotypic expression

		t(14;18) translocation		p Value
		+	-	
BCL2	+	15	21	0.039
	-	2	14	
CD10	+	12	8	0.018
	-	6	27	
E2F1	+	6	12	0.597
	-	12	23	
BCL6	+	11	18	0.395
	-	7	16	

Table 4 Subclassification of our cohort of diffuse large B cell lymphomas

Group	Immunophenotype	No.
I	t(14;18)+, BCL6+, CD10+	7
II	t(14;18)+, BCL6+, CD10-	5
III	t(14;18)+, BCL6-, CD10+	4
IV	t(14;18)+, BCL6-, CD10-	2
V	t(14;18)-, BCL6+, CD10+	7
VI	t(14;18)-, BCL6-, CD10+	1
VII	t(14;18)-, BCL6+, CD10-	11
VIII	t(14;18)-, BCL6-, CD10-	17

little E2F1 protein can induce apoptosis.²³ Because the role of E2F1 appears to be cell type dependent, more data on the role of E2F1 in lymphoma cells are needed to confirm the hypothesis by Moller *et al* that E2F1 is a tumour suppressor gene.²⁹

Our study suggested that 26 of 52 of our DLBCL cases could be GC derived. This finding agrees with Alizadeh *et al* and McCluggage *et al*, who found that 21 of 42 and 15 of 34 of their cases were GC derived, respectively.^{3,35} We considered groups I–IV to be transformed from follicular lymphoma because they show the characteristic *bcl2* gene rearrangement,¹¹ and the t(14;18) translocation has been detected only at a very low frequency in benign hyperplastic lymph nodes and blood samples from normal donors, whereas this translocation is mostly restricted to follicular lymphoma and cases of DLBCL transformed from follicular lymphoma, and has not been detected in other lymphomas.¹¹ *Bcl2* gene rearrangements in other lymphomas often involve variant translocations. Hence, we conclude that we have a higher proportion (18 of 26; 69%) of cases that appear to have been transformed from follicular lymphoma compared with the series of Huang *et al* (seven of 20; 35%) and McCluggage *et al* (five of 15; 33%). We speculate that the lower frequency of follicular lymphoma detected in the Asian population may result from early transformation of follicular lymphoma to DLBCL.

The concept that the neoplastic cell will continue to express markers of its biological origin, and that these markers can therefore be used to define disease entities and predict the clinical outcome, is relatively new. Some markers may be more helpful than others in predicting the clinical outcome of

patients and, in the future, some of these biological markers might be used routinely to assist in the management of patients.

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Authors' affiliations

A K H Shia, G-G Gan, S Jairaman, S-C Peh, Department of Pathology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

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Take home messages

- There was a significant association between *bcl2* gene rearrangement and expression of both BCL2 and CD10 in our series of diffuse large B cell lymphomas (DLBCLs) from Asian patients
- Using the minimally acceptable criteria of t(14;18) rearrangement and/or CD10 expression, 26 of 54 cases were classed as germinal centre derived, a similar proportion to that reported previously
- A higher proportion of cases had t(14;18) translocation, suggesting that they may be derived from transformed follicular lymphomas
- E2F1 positivity did not correlate with known germinal centre markers, so that this marker may not be specific for germinal centre derived DLBCLs
- It may be possible to stratify patients for treatment using markers for specific lineages of B cell differentiation

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ECHO

Rules on organ donation are coherent



Please visit the *Journal of Clinical Pathology* website [www.jclinpath.com] for a link to the full text of this article.

Rules about consent to organ donation are being reviewed in the United Kingdom and New Zealand. In law, organs and tissues cannot be taken after death if the deceased have not consented. Medical practice is also not to proceed if a family refuses consent, even against the wishes of the dead donor. These arrangements—the so called double veto—may be right or wrong but at least they are coherent, and that is encouraging, says one commentator.

The ethical situation is complex: arguments for granting a veto to one side tend to undermine those for the granting it to the other. The double veto can be interpreted as coherent by adopting an argument that it has the best effects, or an alternative argument that the rights of the deceased and their families are negative rights—against interference—as opposed to positive rights—to obtaining help—and therefore limited to a veto.

The best effects argument contends that once all costs and benefits are taken into account, including the supply of organs or tissues, effect on recipients, their families, and the dead person, even extending to effects on medical care, a veto over consent leads to greatest net benefit. Showing this needs empirical research.

The argument based on rights—in the sense of moral claims that cannot be overridden—holds that if deceased and their families have only negative rights the double veto has the beginnings of a coherent plan. This scenario seems unlikely to our commentator; instead, he argues that consent from donors or families does not impose a correlative duty on doctors to comply.

▲ Wilkinson TM. *Journal of Medical Ethics* 2005;**31**:00–00.



High frequency of germinal centre derivation in diffuse large B cell lymphoma from Asian patients

A K H Shia, G-G Gan, S Jairaman, et al.

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