CD68+ cell numbers and dendritic cell numbers and phenotype fail to predict the presence of a MYC rearrangement in aggressive B-cell lymphomas

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Abstract MYC rearrangements are frequently associated with aggressive B-cell lymphomas, such as Burkitt's lymphoma. We sought surrogate markers of MYC rearrangements that might obviate the need for a complex and expensive technique such as fluorescent in situ hybridization (FISH). We show that numbers of CD68+ macrophages/dendritic cells (DCs), DC-SIGN+ myeloid DCs and CD123+ plasmacytoid DCs fail to correlate with MYC rearrangement, although a starry sky appearance shows positive correlation ($p=0.003$) when statistical analysis is performed on all three diagnostic groups together. We conclude that FISH is required to investigate the presence of a MYC rearrangement in cases of aggressive B-cell lymphoma, in order to guide the chemotherapeutic regime offered to the patient.

Keywords Burkitt's lymphoma · Diffuse large B-cell lymphoma · MYC · Translocation · Rearrangement · Histiocyte · Macrophage · Dendritic cell · CD68 · DC-SIGN · CD123

Introduction

The major types of aggressive B-cell lymphoma (BCL) are Burkitt's lymphoma (BL) and diffuse large B-cell lymphoma (DLBCL). BL has specific starry sky morphology, consequent upon macrophages taking up apoptotic debris. It is almost always associated with MYC rearrangement and often with Epstein–Barr virus (EBV), although a MYC rearrangement is not specific to BL. It has a high proliferation index (PI) and characteristic CD10+ bcl-2- TdT- immunophenotype [1]. DLBCL encompasses most other aggressive BCLs with a PI >40%. In general, DLBCL is less aggressive than BL. An intermediate category “B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and Burkitt lymphoma” (IBLDBCL) between the two exists. The majority of cases in this category have morphological features that are intermediate between DLBCL and BL with some cells that are smaller than typical DLBCL, resembling BL, and some cells that are larger than typical BL, resembling DLBCL, as well as a high proliferation fraction, starry sky pattern, and an immunophenotype consistent with BL. In addition to BL, a significant proportion of IBLDBCL and a smaller proportion of DLBCL carry a MYC translocation. These tumours usually have a high PI, often >90% [1]. BL lacking demonstrable MYC rearrangements and DLBCL with MYC rearrangements should not be included in the IBLDBCL diagnostic category, meaning that almost all cases will be assigned to this diagnostic category on the basis of a combination of morphological and immunohistochemical features [1]. Notwithstanding, a recent publication suggests a three-phase approach may be helpful in the separation of these categories. In this approach, phase 1 involves assessment of

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morphology with CD10 and BCL2 immunostains; phase 2 employs CD38, CD44, and Ki-67 immunostains and phase 3 entails fluorescent in situ hybridization (FISH) on paraffin sections for MYC, BCL2, BCL6 and immunoglobulin family genes [2]. However, this approach has yet to be adopted as the “gold standard” by the WHO [1]. Increasingly, MYC+ BCLs are treated with a specialised chemotherapeutic protocol, CODOX-M (cyclophosphamide, vincristine (Oncovin), doxorubicin and methotrexate), rather than the CHOP-R protocol (cyclophosphamide, doxorubicin (hydroxydaunorubicin), oncovin, prednisolone and rituximab) normally used for DLBCL [3]. MYC rearrangements can be demonstrated by FISH, a relatively expensive, specialised and time-consuming technique [3]. We investigated whether the numbers of macrophages or dendritic cells (DCs) of plasmacytoid (pDC) or myeloid (mDC) type could be used as a surrogate marker to predict the presence of MYC rearrangements.

Macrophages and the majority of DCs are CD68+ [4]. DCs are specialised antigen-presenting cells capable of initiating immune responses. The DC component in a tumour may be important in determining aggressiveness and prognosis [5]. As aggressive BCLs with a MYC rearrangement carry a poorer prognosis than those without [1], we wondered whether different complements of DCs might be found in BL and DLBCL. DCs may be divided into myeloid and plasmacytoid types. Outside secondary lymphoid tissue, such as lymph nodes, tonsil and spleen, DCs are generally immature, requiring particular signals to become competent to activate naïve T cells and induce immune responses. Relatively specific markers are available for immature mDC and pDC and here we use DC-SIGN (mDC) and CD123 (pDC) [4]. We fail to demonstrate any correlation between the presence of a MYC rearrangement and the number of cells positive for CD68, DC-SIGN or CD123, but demonstrate correlation between a starry sky appearance and MYC rearrangement.

Materials and methods

Tissue was obtained with full ethical approval from the National Research and Ethics Service (ref: C02.162) from the Department of Cellular Pathology, John Radcliffe Hospital, Oxford, from cases of CD10+ bcl-2- PI >90% Burkitt's lymphoma ($n=8$), CD10+ bcl-2+ PI >90% diffuse large B-cell lymphoma ($n=10$) and CD10+ bcl-2- PI >90%, IBLDBCL ($n=11$, Table 1). The IBLDBCL cases demonstrated an immunophenotype, but not a morphology, compatible with Burkitt lymphoma. No cases with Burkitt morphology and CD10+ bcl-2+ PI >90% phenotype (which would also count as IBLDBCL) were identified in our archives. To avoid selection bias, all cases in our diagnostic archive from the last 10 years that fitted the above criteria and where adequate tissue remained were used in this study.

All cases had been reviewed by two or three specialist consultant haematopathologists at the time of diagnosis, and only cases with full agreement between all reviewing pathologists were used in the study.

Haematoxylin and eosin staining was performed followed by immunostaining for CD68 (clone PGM1, a generous gift from Professor Kevin Gatter, Nuffield Department of Clinical Laboratory Sciences, Oxford University, UK) [6], DC-SIGN (clone DC28; R&D Systems, Abingdon, UK) [4], and CD123 (clone 107D2.08; Schering Plough, Welwyn Garden City, UK) [7] using the automated Bond-Max™ staining system (Leica Microsystems, Wetzlar, Germany), as per the manufacturers' instructions. For all three antibodies, antigen retrieval was performed using the high pH Epitope Retrieval 2 solution (Leica Microsystems, Wetzlar, Germany) at 100°C for 20 min. This was followed by a 5-min peroxidase block as per the manufacturers' kit and instructions (Leica Microsystems, Wetzlar, Germany). Numbers of positively immunostained cells were counted in five representative high power ($\times 400$) microscope fields in each tumour, and the intensity of staining was also recorded on a scale of 1–3 where 3 was most intense. The DC28 antibody cross-reacts with DC-SIGN and DC-SIGNR [4], both of which are expressed on lymph node sinus endothelial cells [8]. These were identified as a continuous line of cells and excluded from cell counts. CD123+ vascular endothelial cells [9] were similarly excluded from cell counts.

In situ hybridisation for EBV with the EBER probe (Leica Microsystems, Newcastle, UK) was performed using the BondMax™ staining system (Leica Microsystems, Wetzlar, Germany), as per the manufacturers' instructions. Fluorescent in situ hybridisation (FISH) was performed, using Vysis LSI MYC Dual Color Break Apart probe 30191096 (Abott Molecular, Maidenhead, UK), as per the manufacturers' instructions [10]. Slides were examined using a Zeiss Axioskop microscope with an excitation filter wheel (Carl Zeiss Ltd., Welwyn Garden City, UK) and a Hamamatsu Orca C4742-95-12NRB camera (Hamamatsu Photonics UK Ltd., Welwyn Garden City, U.K.). For each case, 100 nuclei were scored for the presence of a rearrangement and deemed positive if a rearrangement was identified in >20% cells [11]. Statistical analysis was performed using SPSS Statistics 17.0. (SPSS Inc., Chicago, IL, USA) and the non-parametric Kruskal–Wallis test or Fisher's exact test was used in the generation of all p values using a 95% confidence level.

Results

A starry sky appearance was present in 6 out of 7 BL cases (poorly preserved morphology made this difficult to judge

Table 1 Number of CD123+, DC-SIGN+ and CD68+ cells and their average staining intensity in a total of five microscopic fields, presence of a MYC rearrangement, evidence of EBV by in situ hybridisation and starry sky pattern for each case

Case no.	Diagnostic category		EBV		CD123		DC-SIGN		CD68		Pattern		MYC FISH	
			Bcl-2 expression	Status	Total	Intensity	Total	Intensity	Total	Intensity	Starry sky	Translocation		
	Overall morphology													
BL 1	BL	–	–	–	2	2	16	1	62	3	No ^a	Yes		
BL 2	BL	–	–	–	20	2	5	3	209	3	Yes	Yes		
BL 3	BL	–	–	–	0	–	0	1	262	3	Yes	Yes		
BL 4	BL	–	–	–	38	2	0	–	287	3	Yes	Yes		
BL 5	BL	–	–	+	25	1	15	1	274	3	Yes	Yes		
BL 6	BL	–	–	–	2	2	16	1	433	3	Yes	Yes		
BL 7	BL	–	–	–	16	2	96	2	338	3	Yes	No		
DLBCL 1	DLBCL	+	–	–	111	2	3	2	423	3	Yes	No		
DLBCL 2	DLBCL	+	–	–	27	1	1	2	434	3	No ^a	No		
DLBCL 3	DLBCL	+	+	–	15	1	66	2	445	3	No	No		
DLBCL 4	DLBCL	+	–	–	29	2	21	2	155	3	No	No		
DLBCL 5	DLBCL	+	–	–	46	2	0	–	245	3	No	Yes ^b		
DLBCL 6	DLBCL	+	–	–	0	–	4	1	220	3	No	No		
DLBCL 7	DLBCL	+	–	–	203	3	239	3	345	3	No	No		
DLBCL 8	DLBCL	+	–	–	28	1	19	2	358	3	No	Yes		
DLBCL 9	DLBCL	+	–	–	30	2	174	2	170	3	No	Yes		
DLBCL 10	DLBCL	+	–	–	12	1	0	–	295	3	No	No		
IBLDBCL 1	Intermediate	–	–	–	10	2	307	3	292	3	No	No		
IBLDBCL 2	Intermediate	–	–	–	18	1	0	–	327	3	No ^a	No		
IBLDBCL 3	Intermediate	–	–	–	178	2	4	2	389	3	No	No		
IBLDBCL 4	Intermediate	–	–	–	2	1	0	–	229	3	No	No		
IBLDBCL 5	Intermediate	–	–	–	2	1	7	1	281	3	Yes	No		
IBLDBCL 6	Intermediate	–	–	–	111	1	0	–	306	3	No ^a	No		
IBLDBCL 7	Intermediate	–	–	–	115	2	0	–	305	3	Yes	Yes		
IBLDBCL 8	Intermediate	–	–	–	68	2	0	–	682	3	No	No		
IBLDBCL 9	Intermediate	–	–	–	98	1	6	2	373	3	No	No		
IBLDBCL 10	Intermediate	–	–	–	7	1	1	1	274	3	No	Yes ^b		
IBLDBCL 11	Intermediate	–	–	–	4	1	14	1	366	3	Yes	Yes		
Statistical correlation with FISH					0.443	0.537	0.906	0.063	0.070	1.000	0.049	–		
Statistical correlation with diagnostic category					0.263	0.212	0.309	0.307	0.392	1.000	0.003	0.034		

The lowest two rows show *p* values for the statistical correlations between the parameters. Statistically significant correlations are shown in bold

^a Too crushed for accurate assessment of morphology

^b MYC amplification present in addition to translocation

in 1 out of 7), 0 out of 10 DLBCL cases and in 3 out of 11 IBLDBCL cases, although 2 further IBLDBCL cases had poorly preserved morphology (Table 1). Figure 1a

(CD68 immunostaining) demonstrates the presence of abundant large macrophages in BL. FISH using MYC break-apart probes demonstrated a translocation in 6 out

Fig. 1 Macrophage and dendritic cell markers (a–f) immunostained using the peroxidase technique (brown). CD68 immunostaining was strong on any positive cells (Fig. 1a, b). In BL, the vast majority of CD68+ cells were large round cells with macrophage morphology, containing apoptotic debris. CD68+ cells in DLBCL (b) and most IBLDBCL were smaller with either plasmacytoid or dendritic morphology, in keeping with a mixture of macrophages, pDC and mDC. In all three categories, the immature mDC marker, DC-SIGN, immunostained very variable numbers of cells with dendritic morphology relatively weakly, as demonstrated by the central light brown cell with dendritic morphology in c. DLBCL generally showed stronger immunostaining and, in this case, DCs are more abundant (d). The pDC marker, CD123, immunostained variable numbers of small- to

medium-sized round cells with weak or intermediate intensity, highlighting smaller numbers of cells in the BL (e) than the IBLDBCL or DLBCL groups (f). pDC are marked P and vascular CD123+ endothelial cells (not counted) are marked E. FISH using MYC break-apart probes shows two fused red-green (yellow) signals in normal cells, but allows separate red and green signals to be visualized if there is a translocation. There was a translocation in 6/7 BL cases, as in the case in g (good example shown with an arrow and magnified in top right hand corner). There was a MYC translocation in 3 out of 10 DLBCL cases, one of which (h) additionally demonstrated MYC amplification (good example shown with an arrow and magnified in top left hand corner). Scale bar in a (50 microns) refers to panels a–f. Scale bars in g and h are 20 and 40 microns, respectively

of 7 BL cases, but showed no abnormality in 1 out of 7 (Table 1, Fig. 1g). There was a MYC translocation in 3 out of 10 DLBCL cases, 1 additionally demonstrating MYC amplification (Table 1, Fig. 1h). Three out of 11 IBLDBCL cases demonstrated a MYC translocation, 1 with an additional MYC amplification (Table 1). EBV RNA expression was detected in only 1 out of 7 BL cases and 1 out of 10 DLBCL cases (Table 1).

CD68 immunostaining was strong on all positive cells (Fig. 1a, b). In BL, the vast majority of CD68+ cells were large round cells with macrophage morphology, containing apoptotic debris. CD68+ cells in DLBCL and most IBLDBCL were smaller with either plasmacytoid or dendritic morphology, in keeping with a mixture of macrophages, pDC and mDC. In all three categories, the immature mDC marker, DC-SIGN, immunostained very variable numbers of cells with dendritic morphology relatively weakly, although DLBCL generally showed stronger immunostaining (Fig. 1c, d). The pDC marker, CD123, immunostained variable numbers of cells with weak or intermediate intensity in the IBLDBCL and DLBCL groups, but generally immunostained only small numbers of cells in BL cases.

There was no statistically significant correlation between the numbers or staining intensity of CD68+, DC-SIGN+ or CD123+ cells and either the presence of a MYC rearrangement or the diagnostic category, with p values >0.05 (Table 1). In contrast, starry sky morphology correlated significantly with MYC rearrangement ($p=0.003$). The diagnostic category of lymphoma also correlated with MYC rearrangement, with BL predicting a rearrangement ($p=0.034$). These two expected statistical correlations validate the study and suggest that adequate numbers of cases were used to detect any strong statistical correlations between CD123+, CD68+ and DC-SIGN+ cell numbers/staining intensity and MYC rearrangement or diagnostic category. However, it is possible that weaker correlations could only be detected by using a larger number of cases.

In light of the correlation between a starry sky morphological pattern and a MYC rearrangement when all the diagnostic categories were analysed together, we investigated whether a starry sky pattern alone could be used to predict the presence of a MYC rearrangement within the IBLDBCL diagnostic category. A starry sky morphological pattern alone did not demonstrate statistically significant correlation with the presence of a MYC rearrangement ($p=0.09$).

Discussion

We investigated whether the numbers of macrophages, pDCs or mDCs could be used as a surrogate marker to predict the presence of MYC rearrangements and showed

that they could not. However, we demonstrated statistical correlation between both starry sky morphology and the diagnostic category of lymphoma and the presence of a MYC rearrangement. Interestingly, narrowing the analysis to within the IBLDBCL diagnostic category, a starry sky morphological pattern alone did not demonstrate statistically significant correlation with the presence of a MYC rearrangement ($p=0.09$).

FISH studies demonstrated a MYC rearrangement in all but 1 out of 7 cases of BL. This is consistent with reports suggesting that in up to 10% BL cases, no MYC translocation is demonstrable by FISH, the precise explanation for which remains uncertain [1, 12, 13]. A MYC rearrangement was identified in only in 3 out of 11 (27%) IBLDBCL cases, which, given the small size of the present study, is in keeping with other studies suggesting that 35–50% IBLDBCL cases carry a MYC rearrangement [1, 12–14].

A simple explanation for the correlation between the starry sky pattern and the presence of a MYC rearrangement is Myc's ability to trigger rapid apoptosis, as well as cell proliferation. Deregulated Myc expression indirectly induces accumulation and activation of the tumour suppressor p53. Increased p53 activity then transactivates genes encoding pro-apoptotic proteins, such as Bax, Bid, Noxa and Puma, and/or initiates release of cytochrome c from mitochondria to activate the apoptotic machinery [15]. Abundant macrophages take up apoptotic debris, giving typical starry sky morphology [1].

The fact that CD68 is expressed by DCs as well as macrophages may have obscured any correlation between the numbers of macrophages and the presence of a MYC rearrangement. It may, therefore, have been helpful to use a more macrophage-specific marker, such as CD14, although this can also be expressed by certain DC subsets [4]. Due to the degree of overlap between macrophage and DC phenotypes, we did not try to identify macrophages as a distinct category from DCs by immunostaining. The factors determining the numbers of DCs of particular phenotypes present in any tissue, including a tumour, are complex and may include the cytokine milieu and adhesion molecule expression by the tumour and its microenvironment [16], which may be entirely unrelated to the PI, rate of apoptosis or presence of a MYC rearrangement.

Although BL is a relatively homogeneous entity, the current DLBCL category is by no means homogeneous and may be divided into two or three major subgroups [17] by immunohistochemistry or gene expression profiling. However, given our selection of CD10+ DLBCLs, it is likely that, although still not an entirely homogeneous group, all the DLBCL and IBLDBCL cases would have fallen into the germinal centre B cell rather than the activated B-cell subgroup. While the cases in our IBLDBCL category were as homogeneous as possible on morphological and

immunohistochemical grounds, the demonstration of a MYC rearrangement in only 3 out of 11 cases suggests that this category is not particularly homogeneous. Some IBLDBCL cases that carry a MYC rearrangement may also carry a second chromosomal rearrangement (“double hit lymphoma”), usually of BCL2 [18]. Nevertheless, the IBLDBCL category is still useful for aggressive B-cell lymphomas with some features of Burkitt lymphoma, not least because all IBLDBCL cases should probably be investigated for MYC rearrangements. MYC rearranged IBLDBCL cases may then be treated with CODOX-M rather than CHOP-R.

This is a small and preliminary study, and if repeated on a larger patient cohort, it may demonstrate some weaker statistical correlations undetectable in the present study. Such a study would also allow analysis of the predictive ability of combinations of morphological, immunohistochemical and genetic features in the separation of these lymphomas into diagnostic groups. There are various difficulties obtaining larger numbers of cases. Firstly, the diagnosis of BL and IBLDBCL is relatively uncommon. Secondly, many diagnostic specimens in such cases are small core biopsies, particularly if patients are relatively unwell at diagnosis, for example precluding general anaesthetic. This frequently means that inadequate tissue remains after diagnosis for a study such as ours. Nevertheless, this study showed a lack of correlation between a MYC rearrangement and the types and numbers of DCs and macrophages present in the tumours, DCs and macrophages, suggesting that they cannot be used as surrogate markers for MYC rearrangement. Despite being a relatively expensive, technically complex and time-consuming technique, we would continue to advocate the use of FISH to look for the presence of a MYC rearrangement in CD10+ aggressive B-cell lymphomas with a high PI to ensure that patients are categorised appropriately for treatment purposes.

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Conflict of interest The authors state that they have no conflict of interest.

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