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Pediatric Diffuse Large B-cell Lymphoma Demonstrates A High Proliferation Index, Frequent c-Myc Protein Expression, and A High Incidence of Germinal Center Subtype: Report of the French-American-British (FAB) International Study Group

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Abstract

Background—Diffuse large B-cell lymphoma (DLBCL) makes up 10-20% of pediatric non-Hodgkin lymphoma, and these patients have a significantly better prognosis than adults with DLBCL. The difference in prognosis may be related to clinical, phenotypic, and/or biological differences between adult and pediatric DLBCL. In adult DLBCL, the germinal center (GC) phenotype is associated with a better prognosis than the activated B-cell (ABC) phenotype. However, a high proliferative index and expression of Bcl2 and c-Myc protein have all been associated with worse outcomes. While multiple studies have addressed the phenotype and expression patterns of adult DLBCL, relatively little is known about these biological variables in pediatric DLBCL. The goal of this study was to investigate the proliferative index, the relative frequencies of the GC and non-GC subtypes, and the expression of Bcl2 and c-Myc protein in a cohort of children with DLBCL treated in a uniform manner.

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Procedure—We performed immunohistochemistry (IHC) for MIB1, CD10, Bcl6, MUM1, Bcl2, and c-Myc on DLBCL tissue from children treated uniformly in the FAB LMB96 trial (SFOP LMB96/CCG5961/UKCCSG/NHL 9600).

Results—Compared to published adult DLBCL studies, pediatric DLBCL demonstrated moderate to high proliferation rates (83%), increased c-Myc protein expression (84%), decreased Bcl2 protein expression (28%), and an increased frequency of the GC phenotype (75%).

Conclusions—These findings suggest that there are significant biologic differences between pediatric and adult forms of DLBCL, which may contribute to the superior prognosis seen in the pediatric population relative to adult disease.

Keywords

Pediatric diffuse large B-cell lymphoma; germinal center phenotype; proliferation; c-Myc; Bcl2

Introduction

Non-Hodgkin lymphoma (NHL) comprises about 8% of childhood malignancies (1), and diffuse large B-cell lymphoma (DLBCL) makes up 10-20% of pediatric NHL (2,3). Pediatric DLBCL differs from the adult disease: there is a higher incidence of *c-myc* translocation (4) while t(14;18) is uncommon (5,6); extranodal disease is more frequent; the tumors are more likely to demonstrate centroblastic or immunoblastic morphologies (6,7); and finally, the prognosis is significantly better with survival rates of 85-95% (7-12) versus around 50% in adults (13) following multi-agent chemotherapy. The difference in prognosis may be related to clinical, phenotypic, and/or biological differences between adult and pediatric DLBCL.

In adult DLBCL, microarray studies have shown distinct subgroups of gene expression patterns designated germinal center-like (GC) and activated B-cell-like (ABC) (14). Further investigations have demonstrated that the GC subtype can also be identified by immunohistochemistry (IHC) for the expression patterns of CD10, B-cell lymphoma 6 (Bcl6), and Multiple Myeloma 1 (MUM1/Interferon Regulatory Factor [IRF] 4) proteins (15). While CD10 and Bcl6 expression in DLBCL have been associated with a better prognosis (15-18), expression of MUM1 correlates with a worse outcome (15,19). For subclassification of DLBCL, CD10+ DLBCL are designated GC, and CD10-, Bcl6- tumors are non-GC. In CD10-, Bcl6+ cases, MUM1+ tumors are classified as non-GC and the MUM1- tumors are GC. In adult DLBCL, GC-phenotype cases had a significantly better prognosis, while MUM1 staining correlated with a worse outcome. Therefore, adult patients with the GC subtype, whether identified by microarray or IHC, have a significantly better prognosis (14,15).

Additional markers related to the prognosis of DLBCL include MIB1, Bcl2 and c-Myc. MIB1 antibody recognizes the proliferation-associated antigen Ki-67, and the proliferative index is the percentage of tumor cells that stain with MIB1. Miller *et al.* found that a proliferative index of at least 80% was associated with a significantly worse overall survival in adult patients (20). High Bcl2 expression in DLBCL predicts a worse prognosis (21-24), and c-Myc expression in adult DLBCL has been associated with a more aggressive disease (25,26). 30-60% of adult DLBCL over-express Bcl2 protein, with most studies reporting expression in 50% of cases (13,21). Over-expression of *c-myc* can be induced by a variety of mechanisms, including cytogenetically identifiable translocations as identified in a small minority of adult DLBCL (27,28). c-Myc protein over-expression is much more frequent (up to 57% of adult DLBCL) than accounted for by identifiable translocations and correlates

with more aggressive clinical behavior and/or immunosuppression (25,26). This suggests c-Myc protein expression may be dysregulated by other genetic mechanisms.

In multiple studies of adult DLBCL, the germinal center phenotype is associated with a better prognosis, while a high proliferative index, expression of Bcl2, and expression of c-Myc have all been associated with worse outcomes; however, relatively little is known about these parameters in pediatric neoplasms. The goal of this study was to investigate the proliferative index, the frequency of the GC subtype, and the expression of Bcl2 and c-Myc protein using immunohistochemical methods on DLBCL from uniformly treated children.

Methods

Trial/study design, enrollment and pathology review

Cases were obtained from the FAB LMB96 (COG5961) study and diagnosis confirmed by central pathology review as previously described (10,12,29)(Supplementary Appendix I).

Further characterization of DLBCL by immunohistochemistry

Formalin fixed, paraffin embedded tissue sections were available for 81 cases of pediatric DLBCL uniformly treated on the trial for this study. Sections were stained on an automated immunostainer (ES, Ventana Medical Systems, Tucson, AZ) with commercially available antibodies to CD10, Bcl6, MUM1, MIB1, Bcl2, and c-Myc (Supplemental Table I).

IHC staining was evaluated independently by two pathologists (RRM and SLP) with appropriate negative and internal positive controls. CD10, Bcl6, and MUM1 staining was scored as strong if visible at 100× magnification and weak if visible only at 400×. Cases were classified as GC or non-GC phenotype based on the expression patterns of CD10, Bcl6, and MUM1 based on the strategy employed by Hans *et al.* (15). CD10-/Bcl6+ cases on which MUM1 staining could not be performed were designated indeterminate. Cases were described as having low, moderate, or high proliferation rates based on positive staining for MIB1 in ≤ 50%, 51-79%, or ≥ 80% of tumor cells, respectively. Bcl2 was considered positive if more than 25% of the tumor cells stained.

Statistical methods

Analysis was performed on a subset of 79 of the 81 DLBCL cases for whom full clinical information was available with a primary endpoint of Event-Free Survival (EFS) based on the unstratified or stratified logrank test (30), as appropriate, with stratification on initial risk classification (groups A, B, or C). Further details of statistical analysis are provided in Supplemental Appendix I.

Results

Demographics

Table I shows the clinical characteristics of the patients including risk group and tumor location for the GC, non-GC, and indeterminate subtypes (see below).

Most pediatric DLBCL cases showed moderate to high proliferation rate and high proportion of GC phenotypes

Immunostaining for MIB1 demonstrated that the majority of the cases (55/66; 83%) studied had a moderate or high proliferation index. 33/66 (50%) showed a high proliferative index, while 22/66 (33%) were moderate and 11/66 (17%) had lower proliferation.

58/79 cases (73%) were classified as GC subtype based on CD10 staining (positive for CD10 with or without Bcl6; Figure 1A-C). An additional GC case was identified among eight CD10-, Bcl6+ cases available for MUM1 staining that was MUM1-, for 59/79 (75%) GC phenotype (Table II). 11/79 (14%) of the cases were non-GC subtype (Figure 1D-F) (2 expressed neither CD10 nor Bcl6, 10/19 were Bcl6+, CD10- of which 9/10 expressed MUM1 and were classified as non-GC) (Figure 1G-I)). MUM1 staining could not be performed on the remaining nine CD10-, Bcl6+ cases, so these cases cannot be defined as GC or non-GC and are thus designated indeterminate (9/79, 11%).

There were no significant differences age, sex or risk group of the patients in the three subtypes ($p=0.18$), although none of the non-GC patients were group A localized, and 4 of the 5 Group C patients were GC phenotype. When infrequent combinations were grouped together (Group C combined with B and indeterminates combined either with GC or non-GC) the lack of non-GC patients in Group A was significant ($p<0.04$).

EFS by germinal center phenotype

Clinical outcomes were analyzed on a subset of 79 cases. The non-GC group showed a trend towards shorter EFS in the unadjusted analysis ($p=0.056$) (Figure 2), although this was not statistically significant after stratifying by prognostic group ($p=0.275$) and could be related to the presence of a higher fraction of GC cases in risk group A.

Mediastinal DLBCL

The distribution of tumor location differed between the 3 groups (Fisher exact test $p=0.02$). Clinical analysis showed the non-GC cases included a high proportion of mediastinal DLBCL cases (6/12 cases; Table I). 11/11 mediastinal DLBCL tested expressed MUM1. Seven cases had CD10-, Bcl6+ pattern (example in Figure 1).

c-Myc protein expression

Immunohistochemical staining for c-Myc was positive in 27/32 (84%) of cases available for evaluation (Supplemental Table II) including 21/24 (88%) GC cases, 2/2 non-GC cases (100%), and 4/6 (67%) indeterminate cases. All cases tested with a high proliferative index expressed c-Myc (14/14; Table III).

Bcl2 protein expression

Bcl2 protein was identified by immunohistochemical staining in 17/64 cases (29%; Supplemental Table III) including 12/41 (29%) GC cases, 3/11 non-GC cases (27%), and 2/8 (25%) of the indeterminate cases. Interestingly, of the four mediastinal cases that expressed CD10 and MUM1 (4/12), three expressed Bcl2. The expression of Bcl2 was not clearly related to the proliferative index, but only one case that expressed Bcl2 showed a low proliferative index (Table IV).

Discussion

A higher rate of proliferation has been associated with a worse outcome in adult patients (20,21), however not all studies have supported this relationship, particularly in the era of anti-CD20 (rituximab) augmentation of chemotherapeutic regimens (17,31). The majority of the pediatric DLBCL cases evaluated in this study demonstrated a moderate to high proliferative index as assessed by MIB1. Our cases showed both high proliferation and excellent EFS, failing to support a relationship between high proliferation indices and worse outcomes in pediatric DLBCL. Alternatively, the negative effects associated with higher proliferation may be counteracted by other phenotypic features of pediatric DLBCL.

The germinal center phenotype, characterized by CD10 and Bcl6 expression, occurs in 50-60% of adult cases and has been associated with a better prognosis (14-16,18). In our study, pediatric DLBCL cases showed a predominance of the GC phenotype by IHC staining. 59/79 (75%) demonstrated a GC phenotype, while 11/79 (14%) were classified as non-GC with only two of these cases showing a complete lack of germinal center markers. Our findings of a relatively high proportion of GC cases in pediatric DLBCL are in agreement with a recent study demonstrating 83% GC subtype (6). In our study, the non-GC cases showed a trend toward shorter EFS compared to the GC and indeterminate cases, but after adjustment on risk group, the trend disappeared ($p=0.275$), likely reflecting the dearth of non-GC Group A patients. The CD10 negative, Bcl6 positive staining pattern seen in 19 (24%) cases was associated with MUM1 expression in 9/10 cases where MUM1 was evaluated. MUM1 expression is associated with inferior outcome (15,19), and may be associated with the poor outcome seen in the non-GC cases in this study, even when Bcl6 is expressed. In addition, the CD10-, Bcl6+ cases included a higher proportion of extranodal cases (data not shown), a finding that has been reported previously (32).

Primary mediastinal DLBCL (PMBCL) is a subtype of DLBCL that carries a worse prognosis in the pediatric age group (Gerrard M, personal communication) (3,33) and a relatively good to intermediate prognosis in adults (34-36). Our study included 12 mediastinal DLBCL cases. In agreement with previous reports, this group included a higher proportion of female patients (3). While specific studies to define PMBCL were not performed, the tumors did demonstrate histologic features characteristic of PMBCL including weak expression of CD30 in most cases and compartmentalizing sclerosis in all cases. All of the mediastinal DLBCL cases tested expressed MUM1, regardless of GC or non-GC phenotype, which may relate to the poor prognosis reported and may be a more important phenotypic feature than GC/non-GC classification. The association of a GC phenotype with MUM1 staining was not addressed by Hans *et al.* (15), and this unusual phenotype suggests another possible immunophenotypic subcategory, perhaps related to their theoretical thymic B-cell derivation.

c-Myc expression is usually minimal or weak in non-neoplastic lymphoid tissues. Thus increased expression of c-MYC protein relative to normal lymphocytes appears to be due to dysregulation at a protein level that can be achieved by translocations or other molecular mechanisms. c-Myc protein was expressed in 27/32 (84%) cases of pediatric DLBCL, including 21/24 GC subtype. All 14 cases with high proliferation that were tested expressed c-Myc, but there were too few cases with low proliferation tested to determine if c-Myc staining correlated with a higher proliferation index as described previously (37). It is interesting to note that cytogenetic analysis of DLBCL from this cohort of patients demonstrated relatively high (34% of DLBCL studied) incidence of abnormalities at the 8q24 c-Myc locus (4). This was usually a component of a highly complex karyotype and not the sole abnormality typical of Burkitt lymphoma (BL). In contrast, adult DLBCL appears to have a much lower frequency of *c-myc* translocations (5-10%) (27,38). Unfortunately, the majority of cases in the present study do not have cytogenetics available for correlation of abnormalities at 8q24 and c-Myc protein over-expression.

Recent studies have highlighted the difficulty in distinguishing some cases of DLBCL from BL. A small number of cases of adult DLBCL were reported that demonstrate gene expression profiles more consistent with BL(39) that responded poorly to CHOP therapy, suggesting that a molecular approach identifies BL misclassified as DLBCL by conventional diagnostic approaches. Approximately 50% of the BL patients in the study were children, while the DLBCL cases were primarily adults. A separate report described a gene expression signature that, when trained on morphologically defined BL, could identify additional cases with similar expression profiles, some of which were morphologically

DLBCL (42). However, that study also described a large number of “indeterminate” cases that showed significant overlap in molecular, cytogenetic, and morphologic features. These studies highlight the limitations of the current classification of aggressive B-cell lymphomas. And, while this issue is critical in pediatric B-cell lymphomas, studies to date have included predominantly adult DLBCL patients. Further study will be required to determine if a molecular approach can reliably distinguish BL from pediatric DLBCL, particularly in light of the findings of our group and others suggesting that pediatric DLBCL differs from the adult disease and may have features, such as high proliferation and c-Myc over-expression with relatively lower levels of Bcl-2 protein expression, that are similar to the phenotype associated with BL (13,37).

We found Bcl2 protein expression in 28% of pediatric DLBCL, a lower rate than described in adult patients. In adult DLBCL, Bcl2 expression is associated with the t(14;18) translocation, which is more common in the GC subset (40). Other studies find that Bcl2 expression is independent of the translocation (32,41,42). The t(14;18) *bcl2* translocation is very uncommon (<1%) in pediatric DLBCL (6).

Our cohort of pediatric DLBCL had much higher survival compared to rates reported elsewhere for adults. However, survival rates must be compared with caution due to differences in therapy between adult and pediatric patients. The high incidence of GC phenotype may contribute to the excellent prognosis of pediatric DLBCL, but we were unable to demonstrate a statistically significant shortening of event-free survival in non-GC cases (perhaps reflecting small numbers). This raises the possibility that this approach to DLBCL subtyping, which was developed on adult patients, lacks relevance in the pediatric disease. Recent studies in adults have shown that the addition of rituximab to standard chemotherapy improves outcomes in non-GC patients and thus eliminates the outcome differences between GC and non-GC patients(43,44). Thus it is possible that the intensive, short term therapy used in pediatric patients may similarly abrogate the differences in prognosis. Future studies may define a role for disease subtyping in tailoring therapeutic regimens rather than predicting outcomes.

Increased proliferation and c-Myc expression are not associated with decreased survival, but may relate to differences in lymphomagenesis. The high frequency of c-Myc expression suggests that c-Myc could drive proliferation in pediatric DLBCL, in contrast to adult DLBCL, where Bcl2 protein expression with inhibition of apoptosis is a primary pathogenetic feature and associated with worse outcome. In this sense, pediatric DLBCL shows similarities with BL that highlight the differences between pediatric and adult DLBCL. Future studies, including gene-expression profiling, will be required to determine if the differences between adult and pediatric DLBCL in terms of proliferative index, c-Myc expression, Bcl2 expression, and proportion of GC subtype can explain the marked difference in prognosis between these groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

IHC of representative examples of phenotypic subtypes. The GC-subtype case (top row) shows strong expression of CD10 (A) and Bcl6 (B) without MUM1 (C). A non-GC subtype case (middle row) lacks CD10 (D) and Bcl6 (E) but expresses MUM1 (F). A representative example of a mediastinal DLBCL case (bottom row) lacks CD10 (G) and expresses Bcl6 (H) and MUM1 (I). Samples were stained for indicated target antigen by IHC and counterstained with hematoxylin; original magnification 400×.

Figure 2. Event-free survival from trial entry. The non-GC group showed a trend toward worse survival that did not reach statistical significance and disappeared after adjustment on risk group.

Table 1

Clinical information by GC, non-GC, and indeterminate subtype classification.

		GC	Non-GC	Indeterminate	Total
Number of cases		59	11	9	79
Age	0-9 yrs	19	1	3	23
	10-19yrs	40 (68%)	10 (91%)	6 (67%)	56 (71%)
Gender	male	40	5	4	49
	female	19 (32%)	6 (55%)	5 (56%)	30 (38%)
Clinical Stage	A	20 (34%)	0	2 (22%)	22 (28%)
	B	35 (59%)	10 (91%)	7 (78%)	52 (66%)
	C CNS+	2 (3%)	1 (9%)	0	3 (4%)
	C CNS-	2 (3%)	0	0	2 (2%)
Tumor Location	Mediastinal	5 (8%)	6 (55%)	1 (11%)	12 (15%)
	Abdominal/Retroperitoneal	27 (46%)	2 (18%)	4 (44%)	33 (42%)
	Head/Neck	8 (14%)	1 (9%)	0	9 (11%)
	Peripheral	9 (15%)	2 (18%)	3 (33%)	14 (18%)
	Other	10 (17%)	0	1 (11%)	11 (14%)

Table II

GC and non-GC classification of cases.

CD10	Bcl-6	MUM-1	GC Type	Frequency	Percent
+	+	NE	GC	26	32.9%
+	+	-	GC	5	6.3%
+	+	+	GC	9	11.4%
+	-	NE	GC	12	15.2%
+	NE	NE	GC	6	7.6%
-	+	-	GC	1	1.3%
-	+	+	Non-GC	9	11.4%
-	-	+	Non-GC	2	2.5%
-	+	NE	Indeterminate	9	11.4%

NE – Not evaluated due to limited tissue.

Table III

c-Myc protein expression by proliferation.

Proliferation:	Low	Moderate	High
c-Myc +	2 (67%)	9 (75%)	14 (100%)
c-Myc -	1	3	0
Total	3	12	14

Table IV

Bcl2 protein expression by proliferation.

Proliferation:	Low	Moderate	High
Bcl2 +	1 (11%)	9 (47%)	6 (21%)
Bcl2 -	8	10	22
Total	9	19	28