Oncology Research, Vol. 23, pp. 137–146
Printed in the USA.

0965-0407/16 $90.00 + .00
DOI: http://dx.doi.org/10.3727/096504015X14500972666761
E-ISSN 1555-3906
www.cognizantcommunication.com

Flow Cytometric Evaluation of Double/Triple Hit Lymphoma

Christine G. Roth,* Amanda Gillespie-Twardy,† Stanley Marks,† Mounzer Agha,† Anastasios Raptis,† Jing-Zhou Hou,† Rafic Farah,† Yan Lin,§ Ying Qian,§ Liron Pantanowitz,‡ and Michael Boyiadzis†

*Department of Pathology, Division of Hematopathology, University of Pittsburgh Medical Center, Pittsburgh, PA, USA
†Department of Medicine, Division of Hematology/Oncology, University of Pittsburgh Medical Center, Pittsburgh, PA, USA
‡Department of Pathology, Division of Cytopathology, University of Pittsburgh Medical Center, Pittsburgh, PA, USA
§Department of Biostatistics, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA, USA

"Double" or "triple" hit lymphomas (D/THL) with recurrent translocations involving *MYC*/8q24 and *BCL2*/18q21 and/or *BCL6*/3q27 are characterized by a poor prognosis, but their identification is hampered by the clinicopathologic overlap with other disease categories. Cases with circulating blastic-appearing cells may initially cause concern for lymphoblastic leukemia a diagnostic dilemma, which has not been well studied. There is only limited literature regarding the flow cytometric (FC) D/THL phenotype and its clinical correlates. The FC features of 20 D/THL (11 $BCL2^+/MYC^+$, 5 $BCL6^+/MYC^+$, 4 $BCL2^+/BCL6^+/MYC^+$) were evaluated, compared to 20 B-lymphoblastic leukemias (B-LBL), and correlated with overall survival. Most (89%, 17/19) D/THL were $CD10^+$, 47% (9/19) lacked surface light chain, and a significant subset underexpressed CD45 (47%, 9/19), CD20 (42% 8/19), and/or CD19 (39%, 7/18), which did not vary by genetic subgroup. Compared to B-LBL, D/THL less frequently underexpressed CD45 ($p=0.0001$) and CD20 ($p=0.0004$). Lower levels of BCL2 expression were noted in the $BCL6^+/MYC^+$ and $BCL2^+/BCL6^+/MYC^+$ subgroups versus $BCL2^+/MYC^+$ cases ($p=0.0014$). Of the flow cytometric parameters assessed, dim CD45 expression correlated with inferior survival ($p=0.01$). Although there is some overlap with B-LBL, D/THL demonstrates a characteristic immunophenotype which may have prognostic significance and warrants further investigation.

Key words: Double hit lymphoma; MYC; BCL2; BCL6

INTRODUCTION

Aggressive B-cell lymphomas with recurrent translocations involving *MYC*/8q24 and *BCL2*/18q21 and/or *BCL6*/3q27 have been colloquially known as "double hit" or "triple hit" lymphomas (D/THL) (1–8). These tumors are characterized by the synergy of a *MYC*-driven proliferation signal and the activation of the antiapoptotic functions of *BCL2* and/or *BCL6*. Together with their high genomic complexity, these features contribute to an unusually aggressive clinical course and poor prognosis in spite of current therapeutic approaches, including high-intensity chemotherapy (9).

Despite the heightened clinical interest in D/THL identification, they are uncommon, and lack distinctive clinical features or a unifying morphologic appearance (10). Although most D/THL are subclassified within the 2008 WHO category of "B cell lymphoma, unclassifiable, with features intermediate between diffuse large B cell lymphoma and Burkitt lymphoma (BCLU)," not all BCLU cases will harbor *MYC*, *BCL2*, and/or *BCL6* gene rearrangements, (11) and in some cases, D/THL may fulfill the diagnostic criteria for diffuse large B-cell lymphoma, NOS, B-lymphoblastic leukemia/lymphoma (B-LBL), or other B-lymphoid neoplasms including composite lymphomas (3–9,12). These issues represent significant challenges in the initial and timely identification of D/THL.

Most D/THL show a germinal center (GC) phenotype as per the Han's algorithm (2,13,14); however, this phenotype is not specific and overlaps with other disease categories including DLBCL, NOS, Burkitt lymphoma, follicular lymphoma, and precursor lymphoid neoplasms. Although most D/THL are phenotypically mature, D/THL cells may show blastic morphologic features and raise the possibility of lymphoblastic leukemia when presenting as circulating blast-like cells (7,15,16). Multiparameter flow

Address correspondence to Christine G. Roth, M.D., UPMC-PUH G300, 200 Lothrop Street, Pittsburgh, PA 15213, USA.
E-mail: garciac@upmc.edu

cytometric studies enable rapid, comprehensive immunophenotyping and are more sensitive in detecting aberrancies in antigenic expression compared to paraffin section immunohistochemical studies. As the majority of the studies characterizing the D/THL phenotype have been based on immunohistochemical studies, there is only limited literature regarding the flow cytometric phenotype of D/THL (17–20). In addition, the clinical correlates of aberrant flow cytometric phenotypic expression in D/THL have not been previously investigated.

The aims of this study were to characterize the flow cytometric features of D/THL, compare the immunophenotypic aberrancies with B-lymphoblastic leukemia, and to identify flow cytometric parameters associated with survival.

MATERIALS AND METHODS

This study was conducted in accordance with the guidelines of the Institutional Review Board of the University of Pittsburgh. Between January 2008 and July 2013, 20 cases of B-cell lymphoma associated with *MYC* and *BCL2*, *MYC* and *BCL6*, or *MYC*, *BCL2*, and *BCL6* rearrangements documented with fluorescence in situ hybridization (FISH) studies were identified. Eleven cases were *BCL2*$^+$/*MYC*$^+$, five were *BCL6*$^+$/*MYC*$^+$, and four were *BCL2*$^+$/*BCL6*$^+$/*MYC*$^+$. The cases included 10 bone marrow biopsies, eight surgical biopsies, and two cytology specimens. Morphologic, immunophenotypic, and cytogenetic data were reviewed to confirm diagnosis and classify according to 2008 World Health Organization (12). One case been previously been reported by Pillai et al. (14). In addition, 20 cases of B-lymphoblastic leukemia diagnosed at our institution were identified; pathologic and cytogenetic data were reviewed for all cases to confirm the diagnosis and the lack of rearrangements of *MYC*, *BCL2*, and *BCL6*.

Corresponding medical records were reviewed to obtain clinical information including WBC at diagnosis, LDH at diagnosis, prior history of lymphoma, International Prognostic Index, Ann Arbor Stage, treatment regimens, response to therapy, and overall survival. The patient's responses to treatment were assessed using the International Working Group's Response Criteria for non-Hodgkin's lymphoma (21). A complete response (CR) required complete disappearance of all detectable clinical and radiographic evidence of disease and disappearance of all disease-related symptoms and biochemical abnormalities. In addition, all lymph nodes must have returned to normal size, the spleen must have regressed in size, and any bone marrow involvement must have cleared. A partial response (PR) required ≥50% decrease in the size of lymph nodes or lymph node masses, no increase in the size of other nodes/spleen/liver, a 50% decrease in splenic or liver nodules, and no new sites of disease. Stable disease (SD) was defined as less than a PR but not progressive disease. Finally, progressive disease (PD) required a ≥50% increase in lymph node size or the appearance of any new lesions.

Flow Cytometric Immunophenotypic Studies

Multiparameter flow cytometric studies were performed on BD FACSCanto II instrument and analyzed using FACSDiva software (BD Biosciences, San Jose, CA, USA). During the study period, the clinical flow cytometric testing include the following fluorochrome-labeled monoclonal antibody combinations: κ-FITC, λ-PE, CD5-PerCP-Cy5.5, CD19-PE-Cy7, CD10-APC, CD38 APC-H7, CD20-V450, CD45-V500; κ-FITC, λ-PE, CD19-PerCPCy5.5, CD5-APC; CD38-FITC, CD22-PE, CD20-PerCP-Cy5.5, CD10-APC; κ-FITC, λ-PE, CD20-PerCP-Cy5.5, CD10-APC; cyto TdT-FITC, cyto MPO-PE, cyto CD3- PerCP-Cy5.5, cyto CD34-APC; κ-FITC, λ-PE, CD5-PerCP-Cy5.5, CD19-PE-Cy7, CD10-APC, CD45 APC-H7, CD20 Pacific Blue; κ-FITC, λ-PE, CD5-PerCP-Cy5.5, CD19-PE-Cy7, CD10-APC, CD14 APC-Alexa Fluor 750, CD20-V450, CD45-V500, BCL2-FITC, CD10-PE, CD20_PerCP-Cy5.5. Most antibodies were obtained from BD Biosciences except for TdT (Supertech, Bethesda, MD, USA) and CD14-APC-Alexa Fluor 750 (Beckman Coulter, Brea, CA, USA). Dot plots from these antibody combinations were reviewed, and antigen expression on the neoplastic B-cell population was assessed relative to the germinal center B cells, similar to reported previously (17). When an internal control was not available in the sample, a comparison was made with the expected level of antigen expression based on observations of other clinical samples with that particular fluorochrome combination. In addition, significant variable expression of CD19 or CD20 was also noted, defined as a greater than log spread by visual inspection of the dot plots. BCL2 positivity was based on the criteria of Cook et al. (22).

Statistical Methods

Descriptive analysis was applied for age at diagnosis and variables in relation to the mutation group. Kaplan–Meier estimators were applied for time to event outcomes. ANOVA (or Kruskal–Wallis tests) and Fisher exact tests were used to compare continuous variables across different mutation groups. Univariate cox regression was applied to assess the association between continuous covariates and overall survival (OS) and the likelihood ratio test *p* values were reported. Exact log-rank tests (implemented in the R package coin) were used to test the association between categorical variables and OS. Statistical analysis was performed, and the figures were produced using SAS (SAS Institute Inc., Cary, NC, USA).

RESULTS

Clinical Characteristics

The study cohort consisted of 20 patients (9 males, 11 females); the median age was 66.5 years (range,

42–89 years). Patient demographics and baseline characteristics by genetic subtype are presented in Table 1. Fourteen (70%) patients were diagnosed with de novo disease, whereas in six (30%), there was a prior history of non-Hodgkin lymphoma [DLBCL (four patients), follicular lymphoma (one patient), CLL/SLL (one patient)]. Seventeen patients (85%) presented with advanced stage disease (Ann Arbor stage IV), and over half of the patients (13/20, 65%) had high IPI scores. Circulating blast-like lymphoma cells were noted in 12 cases (60%). No significant differences in the patient age, de novo versus transformed status, Ann Arbor stage, IPI score, bone marrow involvement, or peripheral blood hematologic values were found between the genetic subtypes.

Pathologic Features of D/THL

Among the 20 D/THL cases identified, the pathologic diagnosis was BCLU in 15 cases, DLBCL in three cases, B-lymphoblastic lymphoma in one case, and large B-cell lymphoma in one case. Cases categorized as BCLU showed sheets of intermediate to large-sized lymphoid cells, with moderately to finely dispersed chromatin both on smear preparations and histologic sections (Fig. 1A–C). On histologic sections, mitoses and apoptotic debris were often found in the background and was focally prominent with a "starry sky" pattern in some cases. Cases with a predominance of large, pleomorphic lymphoid cells were classified as DLBCL, NOS (Fig. 1D). The B-LBL involving a mandibular mass had a concurrent lymph node specimen, which showed grade 2 follicular lymphoma. One *BCL2*$^+$*/MYC*$^+$ cytology case lacked a core biopsy, limiting the initial diagnosis to "large B-cell lymphoma." In the fine needle aspiration samples, the cytomorphology revealed a mixed pattern of lymphocytes including several large atypical lymphoma cells within a necrotic background. Unstained fine needle aspiration smears obtained in the cytology cases facilitated FISH studies on intact cells, which were critical in establishing the presence of the recurrent translocations.

Flow Cytometric Phenotypic Features of D/THL

A characteristic flow cytometric phenotype was identified, which was seen across all genetic subgroups (Table 2) (Fig. 2). Eighty-nine percent (17/19) were CD10$^+$ and nearly half (47%, 9/19) lacked surface light chain. Forty-seven percent (9/19) were dim CD45$^+$, 39% (7/18) were dim CD19$^+$, and 42% (8/19) were dim CD20$^+$. Twenty-eight percent (5/18) showed concomitant dim CD19 and dim CD20 expression, and variable expression was noted in 39% (7/18) cases for CD19 and 39% (7/18) for CD20.

Table 1. Patient Characteristics by Genetic Subgroup

Characteristic	All Patients ($n=20$)	*BCL2*$^+$*/MYC*$^+$ ($n=11$)	*BCL6*$^+$*/MYC*$^+$ ($n=5$)	*BCL2*$^+$*/BCL6*$^+$*/MYC*$^+$ ($n=4$)
Median age [years (range)]	66.5 (42–89)	64 (42–83)	69 (61–89)	71 (52–83)
Classification				
De novo	14 (70%)	7 (64%)	5 (100%)	2 (50%)
Transformed	6 (30%)	4 (36%)	0	2 (50%)
Ann Arbor Stage				
IV	17 (85%)	8 (73%)	5 (100%)	4 (100%)
III	2 (10%)	2 (18%)	0	0
II	1 (5%)	1 (9%)	0	0
IPI score				
5	2 (10%)	1 (9%)	1 (20%)	0
4	6 (30%)	2 (18%)	1 (20%)	3 (75%)
3	5 (25%)	3 (27%)	1 (20%)	1 (25%)
2	7 (35%)	5 (45%)	2 (40%)	0
Bone marrow involvement at diagnosis				
Present	10/14 (71%)	4/7 (57%)	3/4 (75%)	3/3 (100%)
BM biopsy not performed	6	4	1	1
Median WBC count at diagnosis × 10^9/l (range)	7.5 (0.1–107)	7.7 (4.3–48.8)	6.9 (4.5–20.1)	11.1 (0.1–107.0)
Median % blastic cells in peripheral blood at diagnosis	1 (0–85%)	7 (0–61%)	1 (0–48%)	13 (0–85%)
Median hemoglobin at diagnosis × g/dl (range)	12.1 (9–15.3)	12.3 (9.6–14.3)	11.9 (9.8–13.6)	12.4 (9.0–15.3)
Median platelets at diagnosis	174 (28–407)	240 (28–388)	135 (72–407)	140.5 (55–162)
Median LDH at diagnosis	801 (170–6248)	980	801	855

IPI, International Prognostic Index; WBC, white blood cell.

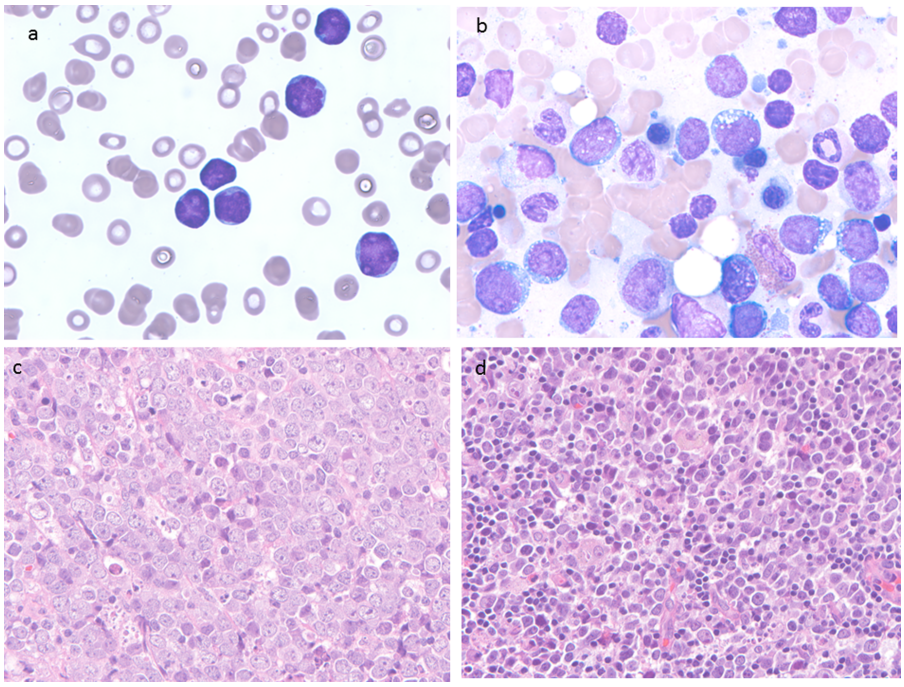

Figure 1. The heterogeneous morphology of double/triple hit lymphoma (D/THL). (a) Circulating *MYC*$^+$*/BCL2*$^+$*/BCL6*$^+$ triple hit lymphoma (THL) cells with moderately to finely dispersed chromatin, irregular nuclear contours, and occasional nucleoli, mimicking an acute leukemia (100×). (b) *MYC*$^+$*/BCL2*$^+$ lymphoma cells on bone marrow aspirate smears show fine chromatin, nucleoli, and cytoplasmic vacuolization (100×). (c) Histologic section of *MYC*$^+$*/BCL2*$^+$ BCLU case showing a monotonous proliferation of intermediate to large-sized cells with fine chromatin (400×). (d) Sheets of large lymphoid cells with marked nuclear pleomorphism in *MYC*$^+$*/BCL6*$^+$ DLBCL case (400×).

BCL2 was more frequently positive in *BCL2*$^+$*/MYC*$^+$ cases ($p=0.0014$) (Fig. 3); no other differences among the genetic subtypes were noted. The CD10$^+$ B cells in the *BCL6*-rearranged cases demonstrated levels of BCL2 expression, which overlapped with CD10$^-$ B cells. Given that a "negative" result requires a level of expression lower than the CD10$^-$ B cells, these cases were considered "indeterminate"; however, the possibility of nonspecific staining cannot be excluded.

Comparison of D/THL With B-Lymphoblastic Leukemias

The flow cytometric features of the 20 D/THL were compared with 20 B-lymphoblastic leukemias (B-LBL). Although both D/THL and B-LBL showed underexpression of CD45, CD19, and CD20, and variability in CD19 and CD20 expression (Table 3), underexpression of CD45 and CD20 was more frequently seen in B-LBL compared to D/THL ($p=0.0001$ and 0.0004, respectively). In addition, several qualitative differences were

Table 2. Flow Cytometric Features by Genetic Rearrangement

Flow Cytometric Parameter	BCL2$^+$/MYC$^+$	BCL6$^+$/MYC$^+$	BCL2$^+$/BCL6$^+$/MYC$^+$
CD10$^+$	10/11 (91%)	3/4 (75%)	4/4 (100%)
Surface immunoglobulin (–)	5/11 (45%)	2/4 (50%)	2/4 (50%)
Dim CD45	3/11 (27%)	3/5 (60%)	3/4 (75%)
Dim CD20	4/11 (36%)	2/4 (50%)	2/4 (50%)
Dim CD19	5/11 (45%)	1/3 (33%)	1/4 (25%)
Variable CD19	5/11 (45%)	1/3 (33%)	1/4 (25%)
Variable CD20	3/11 (27%)	1/3 (33%)	3/4 (75%)
BCL2			
Positive	9/9 (100%)	0/2 (0%)	0/2 (0%)
Indeterminate	0/9 (0%)	2/2 (100%)	2/2 (100%)
Negative	0/9 (0%)	0/2 (0%)	0/2 (0%)

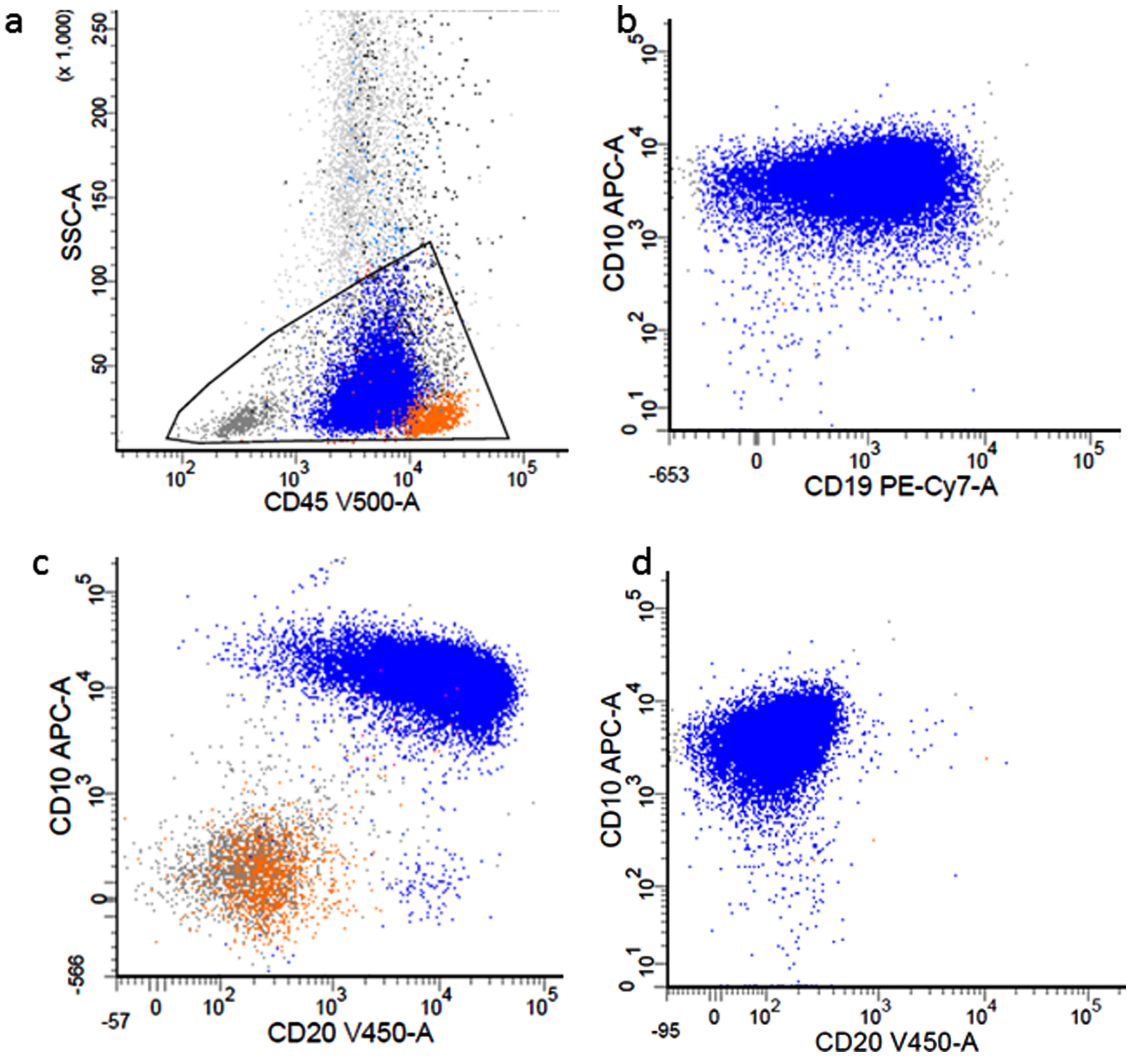

Figure 2. Characteristic immunophenotype of D/THL (a). D/THL (blue) shows dim CD45 expression compared to background T cells (orange) (b), dim and variable CD19 expression, and variable (c) or marked underexpression of CD20 (d).

observed, with B-LBL more frequently showing a greater degree of underexpression of these two antigens, overlapping with negative in 18/20 cases for CD45, and 19/20 cases for CD20 (Fig. 4). Although some D/THL cases showed significant underexpression of CD20, significant underexpression of CD45 that overlapped with negative was not seen. Concomitant dim expression of both CD19 and CD20 was seen slightly more frequently in D/THL (28% D/THL vs. 10% B-LBL), but was not statistically significant ($p=0.2224$). All 20 B-LBL were TdT positive by flow cytometry. TdT was negative in 100% (6/6) D/THL cases assessed by flow cytometry. One *BCL2*$^{+}$/*MYC*$^{+}$ DHL case did not have TdT assessed by flow cytometry, but was positive by immunohistochemistry. TdT was negative in the 15 other D/THL cases assessed by immunohistochemistry.

Response to Treatment and Survival

Among the 20 patients, 16 (80%) were treated with systemic chemotherapy, three patients had a rapid clinical decline and died before chemotherapy could be administered, and one patient was treated with steroids. Among the patients offered systemic chemotherapy, nine received rituximab (R) combined with CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) or EPOCH (dose adjusted etoposide, cyclophosphamide, doxorubicin, vincristine, prednisone), four received rituximab with HyperCVAD (cyclophosphamide, vincristine, doxorubicin), and one patient, respectively, received a combination of bendamustine–rituximab, gemcitabine–oxaliplatin–rituximab, and cyclophosphamide–vincristine–dexamethasone. Five (25%) patients achieved complete remission (CR) and four (20%) patients partial response (PR). No response or progressive disease

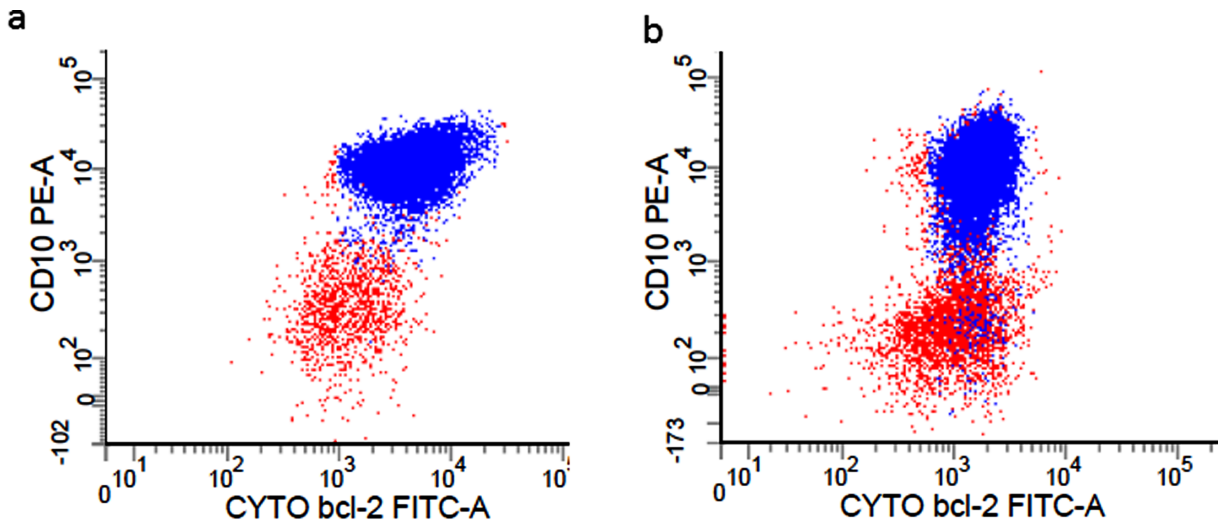

Figure 3. (a) A *BCL2*$^+$*/MYC*$^+$ DHL (blue) is CD10$^+$ and strongly positive for BCL2 by flow cytometric studies. (b) A *BCL6*$^+$*/MYC*$^+$ DHL (blue) is CD10$^+$ with weaker, "indeterminate" expression of BCL2 by flow cytometric studies.

(PD) was documented in three (15%) patients. Eight patients were not evaluable for response. Among the five CRs, three patients received HyperCVAD, and two patients received R+EPOCH. Three patients with persistent disease after their initial chemotherapy received additional chemotherapy regimens; two patients were treated with RICE (rituximab, ifosfamide, carboplatin, etoposide) and one with Hyper CVAD; one patient achieved a PR and two had resistant disease. Three patients that achieved CR and one patient that achieved PR after chemotherapy underwent autologous hematopoietic cell transplantation.

Fifteen of the 20 patients had died by the time of analysis. The median overall survival for all patients was 6.09 months (95% CI 5.8, 23.19) (Fig. 5A). The median overall survival for patients with *BCL2*$^+$*/MYC*$^+$ was 16.1 months (95% CI 1.2,24.80), 2.6 months (95% CI 0.6,23.1) for patients with *BCL6*$^+$*/MYC*$^+$, and 4.3 months (95% CI 0.3,8.85) for patients with *BCL2*$^+$*/BCL6*$^+$*/MYC*$^+$. No significant difference was found in survival among the three groups (*BCL2*$^+$*/MYC*$^+$ vs. *BCL6*$^+$*/MYC*$^+$, $p=0.09$; *BCL2*$^+$*/MYC*$^+$ vs. *BCL2*$^+$*/BCL6*$^+$*/MYC*$^+$, $p=0.1$; *BCL6*$^+$*/MYC*$^+$ vs. *BCL2*$^+$*/BCL6*$^+$*/MYC*$^+$, $p=0.9$)

Dim CD45 expression was associated with inferior overall survival ($p=0.01$) (Fig. 5B). Flow cytometric BCL2 positivity showed a trend for better overall survival ($p=0.1$). In addition, involvement of the bone marrow by lymphoma showed a trend toward poor overall survival ($p=0.08$). Leukemic involvement in the peripheral blood (>25% blastic-appearing cells in the peripheral blood), IPI score, and Ann Arbor Stage were not significantly correlated with OS.

DISCUSSION

Over recent years, several studies have highlighted the importance of recognizing D/THL given their aggressive clinical features and chemoresistance (5). However, these cases are uncommon, and the presenting pathologic features overlap with other disease categories, including B-LBL, especially when presenting in the peripheral blood and bone marrow (16,23). Multiparametric flow cytometric immunophenotypic studies are commonly employed in the evaluation of a suspected acute leukemia; however, there is limited literature related to the flow cytometric features of D/THL (17–19).

Table 3. Flow Cytometric Features of Double/Triple Hit Lymphomas Compared to B Lymphoblastic Leukemia

Flow Cytometric Parameter	D/THL	B-LBL	*p* Value
Dim CD45	9/20 (45%)	20/20 (100%)	0.0001
Dim CD19	7/18 (39%)	2/20 (10%)	0.0577
Dim CD20	8/19 (42%)	19/20 (95%)	0.0004
Variable CD19	7/18 (39%)	5/20 (25%)	0.4889
Variable CD20	7/18 (39%)	14/20 (70%)	0.1014
Dim CD19 + dim CD20	5/18 (28%)	2/20 (10%)	0.2224

D/THL, double/triple hit lymphomas; B-LBL, B lymphoblastic leukemia.

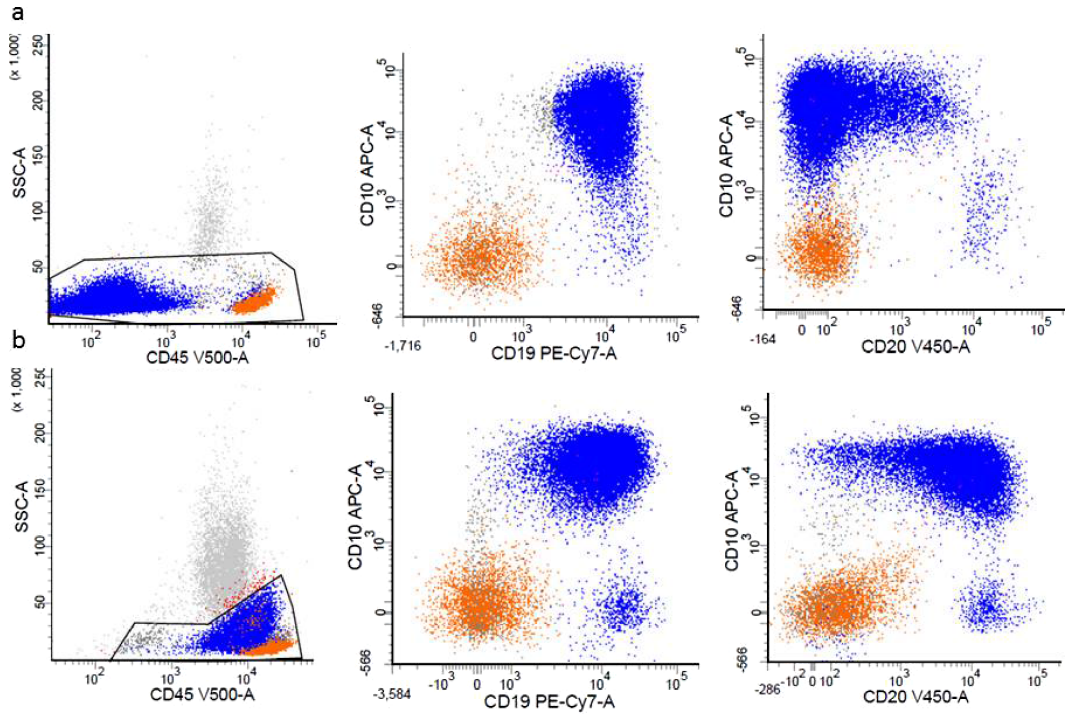

Figure 4. B-lymphoblastic leukemia (B-LBL) may show overlapping immunophenotypic aberrancies with D/THL. (a) B-LBL with CD45 expression that is dim to negative, with non-dim CD19 expression, but variable underexpression of CD20. (b) B-LBL with dim CD45 expression overlapping with what is typically seen in D/THL, with non-dim CD19 expression and variable CD20 expression.

In the current study, we confirm a characteristic flow cytometric immunophenotypic phenotype of D/THL. Nearly all cases were CD10$^+$ and approximately half lacked surface light chain. BCL2 was frequently positive by flow cytometry in D/THL, but was most strongly expressed within the *BCL2*$^+$*/MYC*$^+$ cases compared to the *MYC*$^+$*/BCL6*$^+$ and *MYC*$^+$*/BCL2*$^+$*/BCL6*$^+$ cases. This finding confirms the prior observations that *MYC*$^+$*/BCL6*$^+$ cases are less frequently BCL2 positive by immunohistochemistry (8,14), and that strong flow cytometric expression of BCL2 may be helpful in the correct clinicopathologic context to identify cases to assess for a BCL2 gene rearrangement (20). In addition, we confirm that aberrant underexpression of CD45, CD19, and/or CD20 is seen in a subset of D/THL, which generally falls within the spectrum previously reported in the literature (Table 4) and does not significantly vary by genetic subtype.

Sixty percent (60%) of our patients had circulating lymphoma cells, which often showed blast-like morphologic features. The D/THL phenotype we report overlaps with the dim CD45 and aberrant intensity of CD19 and CD20 often seen with B-lymphoblastic leukemia (24). Although most B-LBL are TdT positive at diagnosis, a subset (nearly 10%) may lack expression of this antigen, and therefore, one cannot entirely rely on the presence of this phenotypic feature to establish the diagnosis (24). Prior D/THL flow cytometric studies have focused comparisons between D/THL and other mature B-cell lymphoid neoplasms such as DLBCL, BCLU, and/or Burkitt lymphoma and have not evaluated the D/THL flow cytometric phenotype in the context of this differential diagnosis (19,25).

When comparing for the first time D/THL and B-LBL, we found overlapping phenotypic aberrancies; however, underexpression of CD45 and CD20 were less frequently found in D/THL. In addition, we note additional qualitative differences that might be helpful to distinguish between the two, as significant dim CD45 expression that overlapped with negative was not observed in D/THL. Although concurrent dim expression of CD19 and CD20 has been touted as highly specific for D/THL when compared with mature aggressive B-lymphoid neoplasms (19), in our series we found overlap with B-LBL and conclude that this is not a distinguishing feature in this differential diagnosis. In fact, the one *MYC*$^+$*/BCL2*$^+$ B-LBL in our series was dim CD45$^+$, but did not show dim expression for either CD19 or CD20. Awareness of these D/THL flow cytometric features and the potential diagnostic pitfall with B-LBL will be useful in the initial evaluation of D/THL with a leukemic presentation and circulating blast-like cells.

True D/THL B-LBL have been typically *MYC/BCL2* rearranged and usually occur in the context of a history

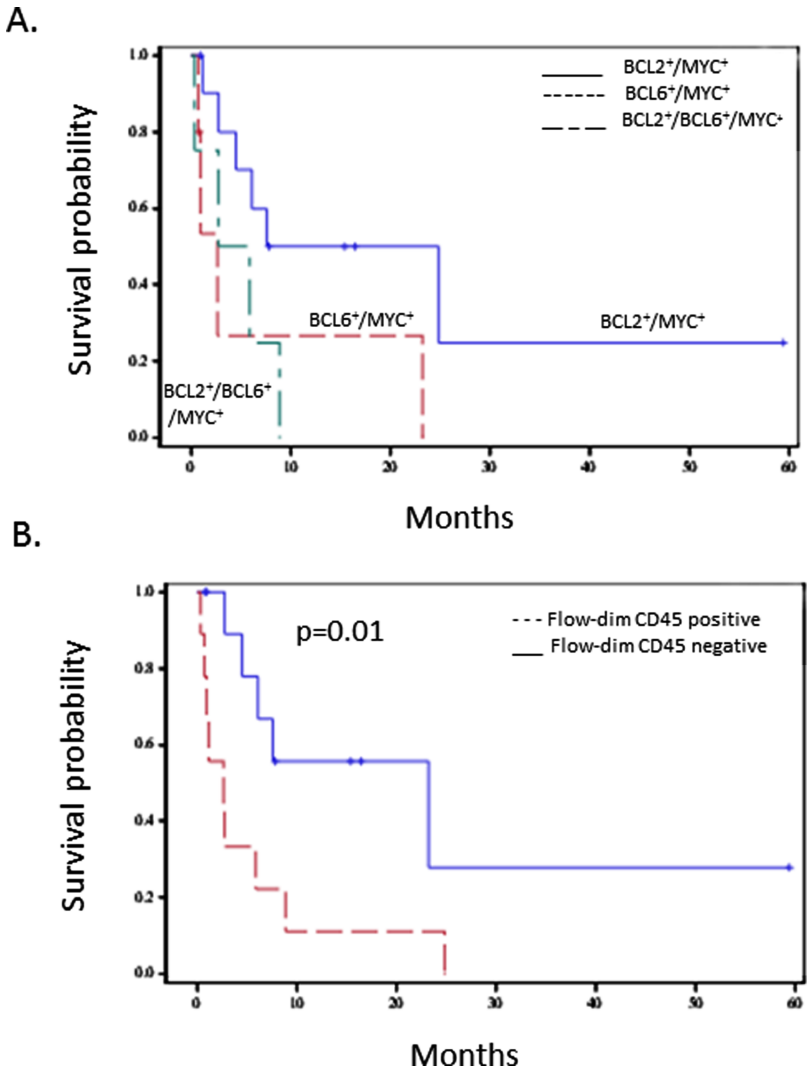

Figure 5. (A) Comparison of overall survival between the three groups. No significant difference was found in survival among the three groups. (B) Comparison in overall survival between cases with and without dim CD45 expression ($p = 0.01$).

of follicular lymphoma, as was seen with the one D/THL B-LBL in our series (26–29). In a study with detailed molecular analysis, the same *BCL2-J*$_H$ translocation was noted in both the follicular lymphoma and lymphoblastic leukemia, which would support a clonal relationship and transformation of the follicular lymphoma (28). There is evidence that this transformation represents dedifferentiation and stems from germinal center genetic mechanisms rather than a pre-germinal center subclone (30). However, not all *BCL2*$^+$*/MYC*$^+$ B-LBL arise from a preexisting follicular lymphoma; neither the D/THL B-LBL noted in the series reported by Snuderl et al. (3) nor the composite D/THL DLBCL/B-LBL described by Nanua et al. (31) reported a prior history of lymphoma. Indeed, the majority of D/THL cases overall (80–85%) occur in patients with no prior history of lymphoma,

Table 4. Frequency of Flow Cytometric Aberrancies in D/THL

Flow Cytometric Parameter	Decreased/Dim CD45	Decreased/Dim CD19	Decreased/Dim CD20
Wu et al. (17)	3/10 (30%)	3/10 (30%)	7/10 (70%)
Harrington et al. (18)	N/A	6/9 (67%)	6/9 (67%)
Platt et al. (19)	1/25 (4%)	14/26 (54%)	8/22 (36%)

N/A, not available.

whereas the remaining 15–20% represent transformation of a previous indolent lymphoma (32). Therefore, although B-LBL occurring in the context of a history of follicular lymphoma should certainly prompt additional cytogenetic testing to exclude *MYC*, *BCL2*, and/or *BCL6* rearrangements, rare cases will occur outside of this clinical context.

The relation of the flow cytometric parameters to clinical outcomes in D/THL has not been previously investigated. Dim CD45 expression as an adverse prognostic factor in D/THL represents an intriguing novel finding of this study. CD45 is a key negative regulator of the JAK family of phosphokinases involved in cell proliferation, and studies have shown that loss of CD45 expression increases cytokine-induced JAK/STAT signaling and activation (33,34). Dim CD45 expression, in combination with bright CD38 expression, has also been shown to be highly specific (100%) for the presence of *MYC* rearrangement across different lymphoma subtypes, suggesting shared biologic mechanisms, including a possible role in influencing proliferation pathways (25). Further evidence for a homogenous biological profile among non-Burkitt *MYC*$^+$ lymphomas comes from a recent gene expression array-based study, which evaluated both "single hit" and D/THL and found shared molecular features (8). We also found a trend of flow cytometric BCL2 positivity associated with better survival, which warrants further study. The BCL2 result is especially relevant in the era of targeted therapy, as anti-BCL2 agents are currently being developed and have been shown to have efficacy in MYC-driven lymphoma murine models (35).

An inherent limitation of this retrospective study is the relatively small sample size and the variability in therapeutic regimens used to treat the patients with D/THL. Factors affecting response and survival (overall and relapse-free) are likely to differ among the regimens, although countervailing strengths of our study are the inclusion of all patients with data on key risk factors for response and survival and adjustment for these factors.

In conclusion, we confirm a distinctive D/THL flow cytometric phenotype and highlight the overlap with B-LBL, a potential diagnostic pitfall. In the future, flow cytometric parameters may contribute to overall prognostic risk stratification of D/THL, in order to inform the most appropriate therapy for a particular patient (36). Further investigation of the clinical implications of dim CD45 expression is warranted, especially within D/THL and other aggressive B-cell lymphomas.

ACKNOWLEDGMENTS: The authors would like to gratefully acknowledge the excellent administrative assistance provided by Stephen Ten Eyck and Hannah Anderson of the Division of Hematopathology.

REFERENCES

1. Pedersen, M. O.; Gang, A. O.; Poulsen, T. S.; Knudsen, H.; Lauritzen, A. F.; Nielsen, S. L.; Gang, U. O.; Norgaard, P. Double-hit BCL2/MYC translocations in a consecutive cohort of patients with large B-cell lymphoma—A single centre's experience. Eur. J. Haematol. 89:63–71; 2012.
2. Tomita, N.; Tokunaka, M.; Nakamura, N.; Takeuchi, K.; Koike, J.; Motomura, S.; Miyamoto, K.; Kikuchi, A.; Hyo, R.; Yakushijin, Y.; Masaki, Y.; Fujii S.; Hayashi, T.; Ishigatsubo, Y.; Miura, I. Clinicopathological features of lymphoma/leukemia patients carrying both BCL2 and MYC translocations. Haematologica 94:935–943; 2009.
3. Snuderl, M.; Kolman, O. K.; Chen, Y. B.; Hsu, J. J.; Ackerman, A. M.; Dal, Cin P.; Ferry, J. A.; Harris, N. L.; Hasserjian, R. P.; Zukerberg, L. R.; Abramson, J. S.; Hochberg E. P.; Lee H.; Lee A. I.; Toomey C. E.; Sohani A. R. B-cell lymphomas with concurrent IGH-BCL2 and MYC rearrangements are aggressive neoplasms with clinical and pathologic features distinct from Burkitt lymphoma and diffuse large B-cell lymphoma. Am. J. Surg. Pathol. 34:327–340; 2010.
4. Kanungo, A.; Medeiros, L. J.; Abruzzo, L. V.; Lin, P. Lymphoid neoplasms associated with concurrent t(14; 18) and 8q24/c-MYC translocation generally have a poor prognosis. Mod. Pathol. 19:25–33; 2006.
5. Johnson, N. A.; Savage, K. J.; Ludkovski, O.; Ben-Neriah, S.; Woods, R.; Steidl C.; Dyer, M. J.; Siebert, R.; Kuruvilla, J.; Klasa R.; Connors, J. M.; Gascoyne, R. D.; Horsman, D. E. Lymphomas with concurrent BCL2 and MYC translocations: The critical factors associated with survival. Blood 114:2273–2279; 2009.
6. Le Gouill, S.; Talmant, P.; Touzeau, C.; Moreau, A.; Garand, R.; Juge-Morineau, N.; Gaillard, F.; Gastinne, T.; Milpied, N.; Moreau, P.; Harousseau, J. L.; Avet-Loiseau, H. The clinical presentation and prognosis of diffuse large B-cell lymphoma with t(14; 18) and 8q24/c-MYC rearrangement. Haematologica 92:1335–1342; 2007.
7. Li, S.; Lin, P.; Fayad, L. E.; Lennon, P. A.; Miranda, R. N.; Yin, C. C.; Lin, E.; Medeiros, L. J. B-cell lymphomas with MYC/8q24 rearrangements and IGHBCL2/t(14; 18) (q32; q21): An aggressive disease with heterogeneous histology, germinal center B-cell immunophenotype and poor outcome. Mod. Pathol. 25:145–156; 2012.
8. Aukema, S. M.; Kreuz, M.; Kohler, C. W.; Rosolowski, M.; Hasenclever, D.; Hummel, M.; Kuppers, R.; Lenze, D.; Ott, G.; Pott, C.; Richter, J.; Rosenwald, A.; Szczepanowski, M.; Schwaenen, C.; Stein, H.; Trautmann, H.; Wessendorf, S.; Trumper, L.; Loeffler, M.; Spang, R.; Kluin, P. M.; Klapper, W.; Siebert, R.; Molecular Mechanisms in Malignant Lymphomas Network P. Biological characterization of adult MYC-translocation-positive mature B-cell lymphomas other than molecular Burkitt lymphoma. Haematologica 99:726–735; 2014.
9. Aukema, S. M.; Siebert, R.; Schuuring, E.; van Imhoff, G. W.; Kluin-Nelemans, H. C.; Boerma, E. J.; Kluin, P. M. Double-hit B-cell lymphomas. Blood 117:2319–2331; 2011.
10. Landsburg, D. J.; Nasta, S. D.; Svoboda, J.; Morrissette, J. J., Schuster, S. J. 'Double-Hit' cytogenetic status may not be predicted by baseline clinicopathological characteristics and is highly associated with overall survival in B cell lymphoma patients. Br. J. Haematol. 166:369–374; 2014.
11. Perry, A. M.; Crockett, D.; Dave, B. J.; Althof, P.; Winkler, L.; Smith, L. M.; Aoun, P.; Chan, W. C.; Fu, K.; Greiner, T. C.; Bierman, P.; Gregory Bociek, R.; Vose, J. M.; Armitage,

J. O.,Weisenburger, D. D. B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and burkitt lymphoma: Study of 39 cases. Br. J. Haematol. 162:40–49; 2013.
12. Swerdlow, S. H.; Campo, E.; Harris, N. L.; Jaffe, E. S.; Pileri, S. A.; Stein, H.; Thiele, J.; Vardiman, J. W. WHO classification of tumours of the haematopoietic and lymphoid tissues. Lyon: IARC; 2008.
13. Hans, C. P.; Weisenburger, D. D.; Greiner,T. C.; Gascoyne, R. D.; Delabie, J.; Ott, G.; Muller-Hermelink, H. K.; Campo, E.; Braziel, R. M.; Jaffe, E. S.; Pan, Z.; Farinha, P.; Smith, L. M.; Falini, B.; Banham, A. H.; Rosenwald, A.; Staudt, L. M.; Connors, J. M.; Armitage, J. O.; Chan, W. C. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood 103:275–282; 2004.
14. Pillai, R. K.; Sathanoori, M.; Van Oss, S. B.; Swerdlow, S. H. Double-hit B-cell lymphomas with BCL6 and MYC translocations are aggressive, frequently extranodal lymphomas distinct from BCL2 double-hit B-cell lymphomas. Am. J. Surg. Pathol. 37:323–332; 2013.
15. Chen, D.; Ketterling, R. P. A leukemic presentation of a "triple-hit" lymphoma. Blood 123:1126; 2014.
16. Rashidi, A.; Fisher, S. I. Double-hit lymphoma mimicking acute lymphoblastic leukemia at presentation. Eur. J. Haematol. 94:374–376; 2015.
17. Wu, D.; Wood ,B. L.; Dorer, R.,Fromm J. R. "Double-hit" mature B-cell lymphomas show a common immunophenotype by flow cytometry that includes decreased CD20 expression. Am. J. Clin. Pathol. 134:258–265; 2010.
18. Harrington, A. M.; Olteanu, H.; Kroft, S. H.; Eshoa, C. The unique immunophenotype of double-hit lymphomas. Am. J. Clin. Pathol. 135:649–650; 2011.
19. Platt, M. Y.; DeLelys, M. E.; Preffer, F. I.; Sohani, A. R. Flow cytometry is of limited utility in the early identification of "double-hit" B-cell lymphomas. Cytometry B Clin. Cytom. 84:143–148; 2013.
20. Cordoba, R.; Alvarez, B.; Masso, P.; Gonzalez, F.; Conejo, L.; Velasco, D.; Alonso, J. M.; Villarrubia, J.; Cava, F.; Llamas, P. The utility of multiparametric seven-color flow cytometry in the detection of double hit lymphoma in ascitic fluid samples. Cytometry B Clin. Cytom., in press; 2015.
21. Cheson, B. D.; Pfistner, B.; Juweid, M. E.; Gascoyne, R. D.; Specht, L.; Horning, S. J.; Coiffier, B.; Fisher, R. I.; Hagenbeek, A.; Zucca, E.; Rosen, S. T.; Stroobants, S.; Lister, T. A.; Hoppe, R. T.; Dreyling, M.; Tobinai, K.; Vose, J. M.; Connors, J. M.; Federico, M.; Diehl, V.; International Harmonization Project on L. Revised response criteria for malignant lymphoma. J. Clin. Oncol. 25:579–586; 2007.
22. Cook, J. R.; Craig, F. E.; Swerdlow, S. H. bcl-2 expression by multicolor flow cytometric analysis assists in the diagnosis of follicular lymphoma in lymph node and bone marrow. Am. J. Clin. Pathol. 119:145–151; 2003.
23. Benz, R.; Tchinda, J. Double hit, triple hit--Look for it. Blood 121:2383; 2013.
24. Chen, W.; Karandikar, N. J.; McKenna, R. W.; Kroft, S. H. Stability of leukemia-associated immunophenotypes in precursor B-lymphoblastic leukemia/lymphoma: a single institution experience. Am. J. Clin. Pathol. 127:39–46; 2007.
25. Mandelker, D. L.; Dorfman, D. M.; Li, B.; Pozdnyakova, O. Antigen expression patterns of MYC-rearranged versus non-MYC-rearranged B-cell lymphomas by flow cytometry. Leuk. Lymphoma. 55(11):2592–2596; 2014.
26. Ning, Y.; Foss, A.; Kimball, A. S.; Neill, N.; Matz, T.; Schultz, R. Characterization of a case of follicular lymphoma transformed into B-lymphoblastic leukemia. Mol. Cytogenet. 6:34; 2013.
27. Kishimoto, W.; Shirase, T.; Chihara, D.; Maeda, T.; Arimoto-Miyamoto, K.; Takeoka, T.; Tsuji, M.; Ohno, T.; Kita, K. Double-hit lymphoma with a feature of follicular lymphoma concurrent with clonally related B lymphoblastic leukemia : a preference of transformation for the bone marrow. J. Clin. Exp. Hematop. 52:113–119; 2012.
28. Young, K. H.; Xie, Q.; Zhou, G.; Eickhoff, J. C.; Sanger, W. G.; Aoun, P.; Chan, W. C. Transformation of follicular lymphoma to precursor B-cell lymphoblastic lymphoma with c-myc gene rearrangement as a critical event. Am. J. Clin. Pathol. 129:157–166; 2008.
29. Kaplan, A.; Samad, A.; Dolan, M. M.; Cioc, A. M.; Holman, C. J.; Schmechel, S. C.; Pambuccian, S. E. Follicular lymphoma transformed to "double-hit" B lymphoblastic lymphoma presenting in the peritoneal fluid. Diagn. Cytopathol. 41:986–990; 2013.
30. Kobrin, C.; Cha, S. C.; Qin, H.; Raffeld, M.; Fend, F.; Quintanilla-Martinez, L.; Grove, S.; Jaffe, E. S.; Kwak L. W. Molecular analysis of light-chain switch and acute lymphoblastic leukemia transformation in two follicular lymphomas: implications for lymphomagenesis. Leuk. Lymphoma 47:1523–1534; 2006.
31. Nanua, S.; Bartlett, N. L.; Hassan, A.; Robirds, D.; Branson, J.; Frater, J. L.; Nguyen T. T.; Kreisel, F. Composite diffuse large B-cell lymphoma and precursor B lymphoblastic lymphoma presenting as a double-hit lymphoma with MYC and BCL2 translocation. J. Clin. Pathol. 64:1032–1034; 2011.
32. Lindsley, R. C.; LaCasce, A. S. Biology of double-hit B-cell lymphomas. Curr. Opin. Hematol. 19:299–304; 2012.
33. Porcu, M.; Kleppe, M.; Gianfelici, V.; Geerdens, E.; De Keersmaecker, K.; Tartaglia, M.; Foa, R.; Soulier, J.; Cauwelier, B.; Uyttebroeck, A.; Macintyre, E.; Vandenberghe, P.; Asnafi, V.; Cools, J. Mutation of the receptor tyrosine phosphatase PTPRC (CD45) in T-cell acute lymphoblastic leukemia. Blood 119:4476–4479; 2012.
34. Irie-Sasaki, J.; Sasaki, T.; Matsumoto, W.; Opavsky, A.; Cheng, M.; Welstead, G.; Griffiths, E.; Krawczyk, C.; Richardson, C. D.; Aitken, K.; Iscove, N.; Koretzky, G.; Johnson, P.; Liu, P.; Rothstein, D. M.; Penninger, J. M. CD45 is a JAK phosphatase and negatively regulates cytokine receptor signalling. Nature 409:349–354; 2001.
35. Vandenberg, C. J.; Cory, S. ABT-199, a new Bcl-2-specific BH3 mimetic, has in vivo efficacy against aggressive Myc-driven mouse lymphomas without provoking thrombocytopenia. Blood 121:2285–2288; 2013.
36. Petrich, A. M.; Gandhi, M.; Jovanovic, B.; Castillo, J. J.; Rajguru, S.; Yang, D. T.; Shah, K. A.; Whyman, J. D.; Lansigan, F.; Hernandez-Ilizaliturri, F. J.; Lee, L. X.; Barta, S. K.; Melinamani, S.; Karmali, R.; Adeimy, C.; Smith, S.; Dalal, N.; Nabhan, C.; Peace, D.; Vose, J.; Evens, A. M.; Shah, N.; Fenske, T. S.; Zelenetz, A. D.; Landsburg, D. J.; Howlett, C.; Mato, A.; Jaglal, M.; Chavez, J. C.; Tsai, J. P.; Reddy, N.; Li, S.; Handler, C.; Flowers, C. R.; Cohen, J. B.; Blum, K. A.; Song, K.; Sun, H. L.; Press, O.; Cassaday, R.; Jaso, J.; Medeiros, L. J.; Sohani, A. R.; Abramson, J. S. Impact of induction regimen and stem cell transplantation on outcomes in double-hit lymphoma: A multicenter retrospective analysis. Blood 124:2354–2361; 2014.