Int J Mol Epidemiol Genet 2011;2(3):245-252
www.ijmeg.org /ISSN1948-1756/IJMEG1106001

Original Article

Immunostaining to identify molecular subtypes of diffuse large B-cell lymphoma in a population-based epidemiologic study in the pre-rituximab era

Lindsay M. Morton[1], James R. Cerhan[2], Patricia Hartge[1], Mohammad A. Vasef[3], Vishala T. Neppalli[4], Yasodha Natkunam[5], Ahmet Dogan[2], Bhavana J. Dave[6], Smrati Jain[6], Ronald Levy[7], Izidore S. Lossos[8], Wendy Cozen[9], Scott Davis[10], Mary Jean Schenk[11], Matthew J. Maurer[2], Charles F. Lynch[12], Nathaniel Rothman[1], Nilanjan Chatterjee[1], Kai Yu[1], Louis M. Staudt[13], Dennis D. Weisenburger[6], Sophia S. Wang[14]

[1]Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, DHHS, Rockville, MD; [2]Mayo Clinic College of Medicine, Rochester, MN; [3]Department of Pathology, University of New Mexico, Albuquerque, NM; [4]Department of Pathology, University of Iowa, Iowa City, IA; [5]Department of Pathology, Stanford University School of Medicine, Palo Alto, CA; [6]University of Nebraska Medical Center, Omaha, NE; [7]Division of Oncology, Stanford University School of Medicine, Palo Alto, CA; [8]Department of Medicine, Division of Hematology/Oncology, University of Miami/ Sylvester Comprehensive Cancer Center, Miami, FL; [9]Keck School of Medicine, University of Southern California, Los Angeles, CA; [10]Fred Hutchinson Cancer Research Center and University of Washington, Seattle, WA; [11]Barbara Ann Karmanos Cancer Institute, Wayne State University, Detroit, MI; [12]Department of Epidemiology, University of Iowa, Iowa City, IA; [13]Center for Cancer Research, National Cancer Institute, NIH, DHHS, Bethesda, MD; [14]Division of Etiology, Department of Population Sciences, City of Hope, Duarte, CA, USA.

Received June 1, 2011; accepted June 26, 2011; Epub July 5, 2011; published August 30, 2011

Abstract: Gene expression profiling studies have distinguished diffuse large B-cell lymphomas (DLBCLs) by cell of origin, with distinct pathogenetic mechanisms and prognosis. We attempted to identify DLBCL molecular subtypes in an epidemiologic study of 214 DLBCL patients diagnosed during 1998-2000 with archival tissues to investigate etiology. Immunohistochemical staining for CD10, BCL6, LMO2, MUM1/IRF4, and BCL2 and fluorescence *in situ* hybridization for t(14;18) were conducted, with ≥93% blinded duplicate agreement. CD10, LMO2, and BCL2 expression was similar to previous reports (32%, 44%, and 44% of DLBCLs, respectively), but BCL6 and MUM1/IRF4 expression was lower than expected (29% and 5%, respectively). We classified 112/214 (52%) cases as germinal center B-cell–like DLBCL (GCB-DLBCL; Hans *et al.*, *Blood* 2004; CD10+ or CD10-/BCL6+/MUM1-), with no difference in prognosis compared with non-GCB-DLBCL (Cox regression, P=0.48). Comparing other GCB correlates, LMO2 expression and t(14;18) were more common but not exclusive to GCB-DLBCL as defined in our study, whereas BCL2 expression did not differ between DLBCL molecular subtypes. We could not confidently identify patients with GCB-DLBCL using these immunohistochemistry-based markers on archival tissues.

Keywords: diffuse large B-cell lymphoma, germinal center, molecular epidemiology, immunohistochemistry

Introduction

Diffuse large B-cell lymphoma (DLBCL), the most common non-Hodgkin lymphoma (NHL) subtype, represents a heterogeneous group of malignancies [1]. Although DLBCL is potentially curable, with approximately 50% average five-year overall survival, the clinical course varies widely, and the International Prognostic Index (IPI) remains the best clinical prognostic predictor available [2-3].

Studies of gene expression profiles have distinguished DLBCL subtypes by cell of origin, demonstrating that patients with germinal center B-cell–like DLBCL (GCB-DLBCL) have a substantially better prognosis than patients with non-GCB-DLBCL [4]. Additional research has shown that the disease subtypes are characterized by distinct oncogenic events [e.g., t(14;18) in GCB-DLBCL and constitutive NFkB activation in non-GCB-DLBCL] [5-6], leading us to hypothesize

that the disease subtypes may also have distinct etiologies.

Identification of GCB- versus non-GCB DLBCL in epidemiologic studies to investigate etiology requires a biomarker that can be applied to fixed archival tissue specimens, since few epidemiologic studies collect fresh tissues. Using immunohistochemical (IHC) staining, Hans *et al*. distinguished DLBCL subtypes based on expression of three key markers of B-cell differentiation (CD10, BCL6, and MUM1/IRF4), by directly comparing results from IHC and gene expression for the same cases [7]. However, other IHC-based studies of protein expression and DLBCL prognosis using these or other combinations of markers have yielded inconsistent results [8-19], possibly due to small sample sizes, use of convenience clinical samples with variability in patient populations, methodological differences in IHC staining and interpretation, and varying ability of IHC to recapitulate the GEP signature [20-21].

In order to identify molecular subtypes of DLBCL in a population-based epidemiologic study with archival tissue, we systematically evaluated expression of CD10, BCL6, and MUM1/IRF4 as well as other GCB correlates [LMO2 and BCL2 expression, t(14;18)].

Materials and methods

Study population

The study population derived from the National Cancer Institute-Surveillance Epidemiology and End Results (NCI-SEER) NHL population-based case-control and prognosis studies, described in detail previously [22-23]. Briefly, the population-based case-control study included 1321 NHL cases (participation rate 76%) diagnosed during 1998-2000, aged 20-74 years, without known HIV infection, identified using rapid case ascertainment among residents of four SEER registries (Iowa, Detroit, Los Angeles, Seattle). Demographic data were obtained from patient interviews, and clinical data (date of diagnosis, stage, presence of B-symptoms, first course of therapy, date of last follow-up, and vital status) were obtained for 99% of cases from linkage to the SEER registries most recently in early 2008. Institutional Review Boards at the National Cancer Institute and each SEER center approved the study protocol. Participants provided written, informed consent prior to completing the interview.

Pathology

All 1321 NHL cases were initially histologically confirmed as NHL and coded according to the International Classification of Diseases for Oncology, 2nd Edition (ICD-O-2) by the local diagnosing pathologist. Updated ICD-O-2/ICD-O-3 codes based on local pathology review were received during the 2008 SEER record linkage. Pathology reports were obtained for 1215/1321 (92%) patients for review by an expert hematopathologist (Mohammad A. Vasef, University of New Mexico), who classified cases according to the World Health Organization classification for lymphoid neoplasms and assigned a confidence score to the subtype diagnosis (≥90% versus <90%). For all cases with low confidence in the NHL subtype classification as well as a 10% random sample of cases with high confidence in the NHL subtype classification, additional immunostaining was conducted to establish the NHL subtype for those patients with available pathology material (N=472). All cases were then assigned a final diagnosis based on the review of tumor specimens (N=472) or pathology reports (N=743), updated ICD-O-2 code from the SEER record linkage if pathology review data were not available (N=76), or original ICD-O-2 code from SEER at the time of case identification if updated data were not available (N=30).

Cases assigned a final diagnosis of DLBCL (ICD-O-2: 9680-84, 9688, 9712; ICD-O-3: 9678-80, 9684; N=417, of which 381 were confirmed by pathology report and/or specimen review) were eligible for this analysis. Sufficient archived, unstained slides (5-micron sections) from formalin-fixed, paraffin-embedded tumors were available for 240/417 (58%) DLBCL patients for laboratory analysis; tumor specimens were not available for the remaining patients (N=177, 42%).

Laboratory methods

Immunohistochemistry (IHC) staining was conducted for five markers (CD10, BCL6, MUM1/IRF4, LMO2, and BCL2) according to previously published methods [7, 16]. Samples were evaluated by expert hematopathologists, and those with ≥30% tumor cells stained were con-

sidered positive. Successful staining was achieved for ≥94% of slides for all markers. The final analytic study population included 214 DLBCL patients with IHC data for all five markers and available follow-up and clinical data. Duplicate slides from 28 randomly selected individuals were interspersed and blinded from the laboratory and hematopathologists, with ≥93% agreement for all markers. Additional information on the antibodies and approach to interpretation is available upon request.

We also identified tumors with the t(14;18) chromosomal translocation, the most common cytogenetic abnormality in NHL, using fluorescence *in situ* hybridization according to previously published methods (Bhavana J. Dave, Smrati Jain, University of Nebraska) [24].

Statistical Analysis

The prognostic significance of marker expression was evaluated using hazard ratios (HRs) and 95% confidence intervals (CIs) derived from multivariate Cox proportional hazards regression, adjusting for age, demographics (sex, race, study center, years of education), and clinical factors (stage, presence of B-symptoms, type of initial therapy) using a continuous risk score (SAS v9.1, SAS Institute, Inc., Cary, NC) [23]. Characteristics of the DLBCL molecular subtypes were compared using the Pearson chi-square statistic.

Results

The study population was predominantly non-Hispanic white (90%) and male (59%) with a median age at DLBCL diagnosis of 59 years (**Table 1**). Characteristics of the present DLBCL case subset were comparable to all DLBCL cases from the parent case-control study. Clinically, 28% of patients had B-symptoms, and 86% received initial chemotherapy (pre-rituximab era). During follow-up, 69 (32%) patients died. The median follow-up of living patients was 7.7 years (range, 2.3-9.0).

CD10, LMO2, and BCL2 were expressed in 32%, 44%, and 44% of DLBCLs, respectively, similar to previous reports, but expression of BCL6 and MUM1/IRF4 was lower than expected (29% and 5%, respectively). Of the five markers evaluated, the strongest individual predictors of overall survival were BCL2 (44% positive, HR=2.15, 95%CI 1.33-3.50, P=0.0019) and LMO2 (44% positive, HR=0.62, 95%CI 0.38-1.03, P=0.062; **Table 2**).

Based on the combination of markers recommended by Hans *et al.* [7], we classified 112 patients with GCB-DLBCL (CD10+ or CD10-/BCL6+/MUM1-), and while these patients had somewhat better overall survival than patients with non-GCB-DLBCL (HR=0.84, 95%CI 0.52-1.35; **Table 2**), this was not statistically significant (P=0.48). To further evaluate the molecular classification of the DLBCLs, we compared expression of other GCB correlates. LMO2 expression and the t(14;18) were more common in GCB- than non-GCB-DLBCL [LMO2: 61% versus 26%, P<0.0001; t(14;18): 49% versus 36%, P=0.06), whereas BCL2 expression did not differ between the DLBCL molecular subtypes (51% versus 44%, P=0.32). Other clinical and demographic characteristics also were similar among patients with GCB- and non-GCB-DLBCL (**Table 1**).

Discussion

We used IHC staining and fluorescence *in situ* hybridization for six key markers of B-cell differentiation to attempt to discriminate DLBCL molecular subtypes in a population-based epidemiologic study with archival tissues. Using Hans *et al.* [7], we classified 112 (52%) cases as GCB-DLBCL (CD10+ or CD10-/BCL6+/MUM1-), but patterns of additional molecular characteristics in our data suggested that we could not be confident in our classification. We observed the t(14;18) in 37% of non-GCB-DLBCL, whereas other series have reported a prevalence of <5% [6]. This observation suggests misclassification of the GCB-DLBCL subtype in our cases because of the high reliability of using fluorescence *in situ* hybridization to identify the t(14;18). Further supporting the likelihood of misclassification, expression of the GC marker LMO2 was absent in 39% of GCB-DLBCL and present in 26% of non-GCB-DLBCL, whereas expression of the activated B-cell marker BCL2 was present in 51% of GCB-DLBCL and absent in 56% of non-GCB-DLBCL. Although both LMO2 and BCL2 are expressed in a subset of lymphocytes at other stages of differentiation [16, 25], their lack of clear clustering with the DLBCL molecular subtypes in our data suggests that we cannot rule out the possibility of misclassification of the GCB-DLBCL subtype in our cases.

Table 1. Clinical and demographic characteristics among all DLBCL patients and by DLBCL molecular subtype*

		DLBCL patients in the present analysis		
	All DLBCL patients	Total	GCB-DLBCL	Non-GCB-DLBCL
Characteristic	N (%)	N (%)	N (%)	N (%)
Total	417 (100.0)	214 (100.0)	112 (100.0)	102 (100.0)
Age at DLBCL diagnosis				
<45 years	96 (23.0)	45 (21.0)	22 (19.6)	23 (22.5)
45-64	196 (47.0)	96 (44.9)	52 (46.4)	44 (43.1)
65+	125 (30.0)	73 (34.1)	38 (33.9)	35 (34.3)
Sex				
Male	236 (56.6)	125 (58.4)	68 (60.7)	57 (55.6)
Female	181 (43.4)	89 (41.6)	44 (39.3)	45 (44.1)
Race				
White	360 (86.3)	186 (86.9)	97 (86.6)	89 (87.3)
Non-white	57 (13.7)	28 (13.1)	15 (13.4)	13 (12.7)
Education				
<12 years	42 (10.1)	24 (11.2)	11 (9.8)	13 (12.7)
12-15	250 (60.1)	129 (60.3)	67 (59.8)	62 (60.8)
16+	124 (29.8)	61 (28.5)	34 (30.4)	27 (26.5)
Study center				
Detroit	100 (24.0)	37 (17.3)	23 (20.5)	14 (13.7)
Iowa	119 (28.5)	81 (37.9)	39 (34.8)	42 (41.2)
Los Angeles	99 (23.7)	43 (20.1)	24 (21.4)	19 (18.6)
Seattle	99 (23.7)	53 (24.8)	26 (23.2)	27 (26.5)
B-symptoms				
No	148 (35.5)	90 (42.1)	47 (42.0)	43 (42.2)
Yes	112 (26.9)	60 (28.0)	29 (25.9)	31 (30.4)
Unknown	157 (37.6)	64 (29.9)	36 (32.1)	28 (27.5)
Stage				
Local	137 (32.9)	70 (32.7)	39 (34.8)	31 (30.4)
Regional	96 (23.0)	56 (26.2)	27 (24.1)	29 (28.4)
Distant	164 (39.3)	83 (38.8)	44 (39.3)	39 (38.2)
Unknown	20 (4.8)	5 (2.3)	2 (1.8)	3 (2.9)
Initial chemotherapy				
No	55 (13.2)	29 (13.6)	18 (16.1)	11 (10.8)
Yes	355 (85.1)	185 (86.4)	94 (83.9)	91 (89.2)
Unknown	7 (1.7)	0 (0.0)	0 (0.0)	0 (0.0)
t(14;18) translocation				
Negative	120 (28.8)	120 (56.1)	56 (50.0)	64 (62.7)
Positive	92 (22.1)	92 (43.0)	55 (49.1)	28 (36.3)
Unknown	205 (49.2)	2 (0.9)	1 (0.9)	1 (1.0)

Abbreviations: diffuse large B-cell lymphoma (DLBCL), germinal center B-cell (GCB).
* DLBCL molecular subtype assigned using Hans *et al.*[7] (GCB-DLBCL: CD10+ or CD10-/BCL6+/MUM1-).

Our experience is illustrative of the promise and the challenges of molecular subtyping in epidemiologic studies. Our duplicate quality control samples indicated ≥93% agreement for all markers, supporting the internal consistency of our results. However, previous research has demonstrated that variability in IHC staining and interpretation can markedly affect results, particularly for BCL6 and MUM1/IRF4 [26]. This variability and degradation of the antigens on our stored slides may account for the lower than expected percentage of cases staining positive for each of these markers in our study and would contribute to misclassification of the DLBCL molecular subtypes. Specifically, a substantially larger proportion of our cases compared with Hans *et al*. [7] were classified as non-GCB-DLBCL (93% vs. 69%) due to a lack of BCL6 expression (**Figure 1**).

Although our study was population-based and used rapid reporting, we were unable to enroll cases with rapidly fatal disease. Observed survival in our study was therefore better than ex-

Table 2. Prognostic significance of immunohistochemical markers, individually and in combination, in 214 patients with histologically confirmed DLBCL from a population-based study

	N (%)	% Patients Deceased	HR (95% CI) Unadjusted	*P*	Adjusted*	*P*
Individual markers						
BCL6						
Negative	151 (70.6)	35.1	1.00 (referent)		1.00 (referent)	
Positive	63 (29.4)	25.4	0.70 (0.40-1.22)	0.20	0.77 (0.44-1.35)	0.36
CD10						
Negative	146 (68.2)	35.1	1.00 (referent)		1.00 (referent)	
Positive	68 (31.8)	33.8	1.15 (0.70-1.90)	0.58	1.12 (0.68-1.85)	0.65
LMO2						
Negative	119 (55.6)	37.8	1.00 (referent)		1.00 (referent)	
Positive	95 (44.4)	25.3	0.59 (0.36-0.97)	0.038	0.62 (0.38-1.03)	0.062
MUM1/IRF4						
Negative	204 (95.3)	31.9	1.00 (referent)		1.00 (referent)	
Positive	10 (4.7)	40	1.33 (0.48-3.65)	0.58	1.17 (0.42-3.23)	0.77
BCL2						
Negative	120 (56.1)	22.5	1.00 (referent)		1.00 (referent)	
Positive	94 (43.9)	44.7	2.26 (1.39-3.67)	0.0010	2.15 (1.33-3.50)	0.0019
Combinations of markers						
Hans *et al.*[7] †						
Non-GCB-DLBCL	102 (47.7)	36.3	1.00 (referent)		1.00 (referent)	
GCB-DLBCL	112 (52.3)	28.6	0.78 (0.49-1.26)	0.31	0.84 (0.52-1.35)	0.48

Abbreviations: confidence interval (CI), diffuse large B-cell lymphoma (DLBCL), germinal center B-cell (GCB), hazard ratio (HR). *Adjusted for age, demographic, and clinical factors. †GCB-DLBCL: CD10+ or CD10-/BCL6+/MUM1-, non-GCB-DLBCL: CD10-/BCL6- or CD10-/BCL6-/MUM1+

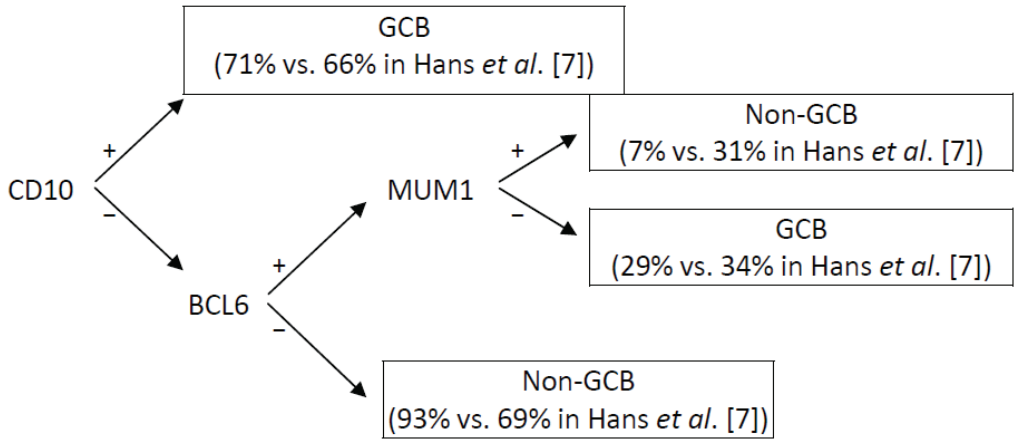

Figure 1. Comparison of the assignment of GCB- versus non-GCB DLBCL in our cases versus Hans *et al.*[7]

pected in the general population, but consistent with survival estimates for DLBCL patients surviving one year following diagnosis [23]. Exclusion of cases with rapidly fatal disease may have limited our ability to assess the prognostic significance of certain markers and decreased the proportion of non-GCB-DLBCLs, which have worse prognosis, in our population. Additionally, we were unable to obtain tumor tissue for all cases, though patient characteristics were similar for those with and without tissue. Detailed treatment and clinical data were not available, but our demographic and clinical risk score has a level of predictability similar to the IPI [5, 23].

Finally, we did not evaluate GCET1 and FOXP1, which were recently shown to modestly improve identification of GCB-DLBCL compared with Hans *et al*.[27].

Discrimination of DLBCL molecular subtypes in epidemiologic studies requires identification of valid and reliable markers that can be used on archival tissues, are robust to variability in patient populations (e.g., by treatment, race, or comorbid conditions such as AIDS),[12, 14-15, 17-19] and have high inter-laboratory agreement [26]. Establishment of such markers will also facilitate widespread adoption of DLBCL molecular subtyping in routine clinical care. Although IHC staining is a standard approach, methodological differences in IHC staining and interpretation for some of the current key B-cell markers suggest the need for new markers, further optimization of current markers for archival tissues, or the pursuit of alternative approaches [28].

Acknowledgements

We gratefully acknowledge the contributions of the staff and scientists at the SEER centers of Iowa, Los Angeles, Detroit, and Seattle for the conduct of the study's field effort; Freda Selk (University of Iowa) for coordination of tumor specimens; and Matthew Butcher, Mary McAdams, Pete Hui, Lonn Irish, and Michael Stagner (Information Management Services, Inc.) for programming support.

Disclosures: The authors have no conflicts of interest to disclose.

Address correspondence to: Lindsay M. Morton, Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, DHHS, 6120 Executive Blvd., EPS 7040, MSC#7238 / Rockville, MD 20852, Tel: 301-435-3972, Fax: 301-402-0207, E-mail: mortonli@mail.nih.gov

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