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Prognostic impact of concurrent *MYC* and *BCL6* rearrangements and expression in *de novo* diffuse large B-cell lymphoma

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ABSTRACT

Double-hit B-cell lymphoma is a common designation for a group of tumors characterized by concurrent translocations of *MYC* and *BCL2*, *BCL6*, or other genes. The prognosis of concurrent *MYC* and *BCL6* translocations is not well known. In this study, we assessed rearrangements and expression of *MYC*, *BCL2* and *BCL6* in 898 patients with *de novo* diffuse large B-cell lymphoma treated with standard chemotherapy (cyclophosphamide, doxorubicin, vincristine, and prednisone plus rituximab). Neither *BCL6* translocation alone (more frequent in activated B-cell like

diffuse large B-cell lymphoma) nor in combination with *MYC* translocation (observed in 2.0% of diffuse large B-cell lymphoma) predicted poorer survival in diffuse large B-cell lymphoma patients. Diffuse large B-cell lymphoma patients with *MYC/BCL6* co-expression did have significantly poorer survival, however, *MYC/BCL6* co-expression had no effect on prognosis in the absence of *MYC/BCL2* co-expression, and had no additive impact in *MYC⁺/BCL2⁺* cases. The isolated *MYC⁺/BCL6⁺/BCL2⁻* subset, more frequent in germinal center B-cell like diffuse large B-cell lymphoma, had significantly better survival compared with the isolated *MYC⁺/BCL2⁺/BCL6⁻* subset (more frequent in activated B-cell like diffuse large B-cell lymphoma). In summary, diffuse large B-cell lymphoma patients with either *MYC/BCL6* rearrangements or *MYC/BCL6* co-expression did not always have poorer prognosis; *MYC* expression levels should be evaluated simultaneously; and double-hit B-cell lymphoma needs to be refined based on the specific genetic abnormalities present in these tumors.

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma and has heterogeneous biologic features. Chromosomal rearrangements, the biologic and diagnostic hallmarks of some other types of B-cell lymphoma, also occur in DLBCL. The most common chromosomal rearrangements in DLBCL are those involving chromosomal gene loci 8q24/*MYC*, 18q21/*BCL2*, and 3q27/*BCL6* [1, 2]. *MYC* rearrangement, a disease-initiating event in Burkitt lymphoma (BL), can be observed in approximately 10% of *de novo* DLBCL and correlates with a poorer outcome [3-7]. However, *MYC* rearrangement alone may not explain the poor prognosis of patients with DLBCL that carry *MYC* rearrangement plus another chromosomal rearrangement. The designation double-hit lymphoma (DHL) has been used for a B-cell lymphoma carrying a *MYC/8q24* rearrangement in combination with a rearrangement involving either *BCL2*, *BCL6*, or rarely other known oncogenes [2, 8, 9].

By far, the most common and well-studied type of DHL is characterized by concurrent *MYC* and *BCL2* rearrangements (*MYC/BCL2* DHL), occurring in about 5% of all cases of DLBCL [10, 11]. As key regulators of cell proliferation and apoptosis, respectively, *MYC* and *BCL2* may act synergistically to drive the pathogenesis of *MYC/BCL2* DHL [12]. Clinically, patients with *MYC/BCL2* DHL often exhibit adverse prognostic factors, such as high serum lactate dehydrogenase (LDH) level, advanced stage of disease, extranodal involvement, and a high proliferation index with a median value of 90%. There is a general consensus that *MYC/BCL2* DHL represents a treatment-refractory subgroup with a median survival of approximately 8 months [13-20]. Despite the dismal outcome of patients with *MYC/BCL2* DHL, almost all of these tumors arise within the germinal center B cell-like (GCB) subtype, a generally favorable prognostic subtype, illustrating a discordance between clinical behavior and cell of origin (COO) subtypes [1, 15, 17, 21].

As an extension of the concept of *MYC/BCL2* DHL, the concept of “double protein lymphoma (DPL)”

has been developed in recent years referring to DLBCL with coexpression of *MYC* and *BCL2* detected by immunohistochemistry (IHC), regardless of activation mechanisms. *MYC/BCL2* DPL is more common than *MYC/BCL2* DHL and accounts for 18-44% of DLBCL cases and might result from gene amplification, transcriptional dysregulation or both [11, 22-26]. A series of studies have shown that patients with *MYC/BCL2* DPL have a significantly poorer outcome than patients who express only one or neither protein, with a 5-year progression-free survival (PFS) of 25% following R-CHOP treatment [11, 22]. Interestingly, unlike *MYC/BCL2* DHL which is mainly observed in the GCB subtype, *MYC/BCL2* DPL is more common in the activated-B cell-like (ABC) subtype and may largely contribute to inferior survival via NF- κ B pathway activation [23].

According to the concept of DHL used currently, another type of DHL is *MYC/BCL6* DHL with concurrent *MYC* and *BCL6* rearrangements [1, 2]. However, there are far less data available for *MYC/BCL6* DHL, in part because of its rarity. *BCL6* is a transcriptional suppressor required for germinal center formation with numerous transcriptional targets, including the cell cycle regulator *CCND2* and *MYC*, which explains downregulation of *MYC* in normal germinal center B-cells [27-29]. Studies by others have suggested that *BCL6* expression is associated with better survival of DLBCL patients [24, 30]. The frequency and the prognostic impact of concurrent *MYC/BCL6* rearrangements and *MYC/BCL6* protein coexpression in DLBCL remain unclear.

In this study, we assessed the frequency, clinicopathologic features, and the prognostic impact of concurrent *MYC/BCL6* rearrangements or *MYC/BCL6* coexpression in a large cohort of *de novo* DLBCL patients treated with R-CHOP, in comparison to *MYC/BCL2* rearrangements and *MYC/BCL2* coexpression. The study evaluated the role of each genetic translocation separately and in combinations, providing reliable conclusion and practical recommendations for diagnostic workup and prognostic prediction.

Table 1: Frequencies of MYC, BCL2 and BCL6 gene translocations and protein overexpression, and multivariate survival analysis

	Overall	GCB	ABC	GCB vs ABC	OS		PFS	
	n/n (%)	n/n (%)	n/n (%)	P value	HR (95% CI)	P value	HR (95% CI)	P value
MYC translocation	71/600 (11.8%)	51/314 (16.2%)	20/284 (7.0%)	.0005	1.36 (0.83-2.23)	.22	1.26 (0.79-2.01)	.34
BCL2 translocation	94/690 (13.6%)	85/360 (23.6%)	9/328 (2.7%)	<.0001	1.39 (0.86-2.24)	.17	1.34 (0.86-2.09)	.20
BCL6 translocation	145/628 (23.1%)	59/338 (17.5%)	86/286 (30.1%)	.0002	1.06 (0.70-1.61)	.77	1.13 (0.77-1.66)	.53
MYC ⁺ expression	249/825 (30.2%)	121/430 (28.1%)	127/390 (32.6%)	.17	1.89 (1.26-2.84)	.002	1.85 (1.26-2.71)	.002
BCL2 ⁺ expression	439/849 (51.7%)	194/439 (44.2%)	245/406 (60.3%)	<.0001	1.67 (1.14-2.46)	.009	1.70 (1.19-2.42)	.004
BCL6 ⁺ expression	555/887 (62.6%)	350/462 (75.8%)	204/424 (48.1%)	<.0001	0.67 (0.45-1.00)	.048	0.47 (0.40-0.89)	.016
MYC+/BCL2+ double-hit	20/710 (2.8%)	19/359 (5.3%)	1/349 (0.3%)	<.0001	3.38 (1.75-6.52)	<.0001	3.04 (1.63-5.67)	<.0001
MYC+/BCL6+ concurrent translocation	14/683 (2.0%)	9/356 (2.5%)	5/323 (1.5%)	.37	0.67 (0.23-1.97)	.47	0.85 (0.33-2.21)	.74
BCL2+/BCL6+ concurrent translocation	21/718 (2.9%)	18/371 (4.9%)	3/341 (0.9%)	.0045	0.47 (0.16-1.39)	.14	0.52 (0.20-1.33)	.17
MYC ⁺ /BCL2 ⁺ double-positive	146/831 (17.6%)	62/434 (14.3%)	84/390 (21.5%)	.0079	2.54 (1.65-3.94)	<.0001	2.92 (1.91-4.47)	<.0001
MYC ⁺ /BCL6 ⁺ co-expression	178/821 (21.7%)	97/434 (22.4%)	81/413 (19.6%)	.35	1.02 (0.65-1.58)	.94	0.85 (0.56-1.30)	.46
BCL2 ⁺ /BCL6 ⁺ co-expression	282/845 (33.4%)	151/443 (34.1%)	131/415 (31.6%)	.47	1.01 (0.68-1.49)	.97	0.90 (0.62-1.30)	.58
MYC ⁺ /BCL2 ⁺ /BCL6 ⁻	43/871 (4.9%)	13/460 (2.8%)	30/403 (7.4%)	.0019	1.60 (1.01-2.54)	.046	2.16 (1.39-3.34)	.001
MYC ⁺ /BCL6 ⁺ /BCL2 ⁻	75/850 (8.8%)	49/434 (11.3%)	26/412 (6.3%)	.011	0.87 (0.54-1.40)	.56	0.86 (0.58-1.34)	.51
MYC ⁺ /BCL2 ⁺ /BCL6 ⁺ (triple-positive)	101/829 (12.2%)	47/432 (10.9%)	54/390 (13.8%)	.20	2.57 (1.86-3.56)	<.0001	2.41 (1.77-3.28)	<.0001

Abbreviations: OS, overall survival; PFS, progression-free survival; HR, hazard ratio; CI, confidence interval.

Note: (1) Not all the patients had experiment results available for all three genes (*MYC/BCL2/BCL6*) or protein mostly due to tissue exhaustion. Total patient numbers for particular biomarkers are listed. For defined combination biomarkers (double-hit/concurrent translocation or double positive/co-expression), total case numbers are more than those for single markers because some cases had only one gene/protein (as a component of the defined biomarker combinations) data available and the results were negative. For example, cases known to have no *MYC* translocation but without *BCL2* translocation status known are included as non *MYC+/BCL2+* double-hit cases during frequency calculation.

(2) For multivariate analyses, four Cox models including different prognostic factors were used. The first model included 4 clinical parameters (International Prognostic Index, gender, B-symptoms, and tumor size), *MYC*, *BCL2*, and *BCL6* translocation status, and *MYC*, *BCL2*, and *BCL6* protein expression levels as variables (totally 10 factors) and the results are as shown in the top six rows. The second model included clinical parameters, and status for *MYC+/BCL2+*, *MYC+/BCL6+*, *BCL2+/BCL6+*, *MYC⁺/BCL2⁺*, *MYC⁺/BCL6⁺* and *BCL2⁺/BCL6⁺* (totally 10 factors) and the results are shown in the 7th-12th rows. The third models included clinical parameters, and status for “*MYC⁺/BCL2⁺/BCL6⁻* and *MYC⁺/BCL6⁺/BCL2⁻* (totally 6 factors) and the results are shown in the 13-14 rows. The fourth model included clinical parameters and *MYC⁺/BCL2⁺/BCL6⁺* as a factor and the results are shown in the last row.

RESULTS

Overall frequency and distribution

The median age of the study population was 64 years (range, 16-95). The median follow-up time was 58.9 months (range, 1-187 months). Among the 898 cases, 469 (52%) were GCB and 429 (48%) were determined to be GCB and ABC subtype, respectively. The complete response rate to R-CHOP therapy was 75%. As shown in Table 1, rearrangements of *MYC*, *BCL2*, and *BCL6* were detected in 71 (11.8%) of 600, 94 (13.6%) of 690, and 145 (23.1%) of 628 cases, respectively. *MYC* and *BCL2* rearrangements were detected predominantly in GCB subtype ($P = 0.0005$ and $P < 0.0001$, respectively) whereas *BCL6* rearrangement was more frequently observed in the ABC subtype ($P = 0.0002$). *MYC/BCL2*, *MYC/BCL6*, and *BCL2/BCL6* concurrent rearrangements were identified in 20 (2.8%), 14 (2%), and 21 (2.9%) patients, respectively. Both *MYC/BCL2* and *BCL2/BCL6* concurrent rearrangements were observed mostly in the GCB subtype (*MYC+/BCL2+*: $P < 0.0001$; *BCL2+/BCL6+*: $P = 0.0045$) whereas *MYC/BCL6* concurrent rearrangements were

observed in two COO subtypes (9 GCB, 5 ABC) without significantly difference in frequency ($P = 0.37$).

Using immunohistochemistry 249 (30.2%), 439 (51.7%), and 555 (62.6%) patients had high levels of *MYC* ($\geq 70\%$), *BCL2* ($\geq 70\%$), and *BCL6* ($> 50\%$) expression, respectively. *MYC* expression was similarly distributed between GCB and ABC subtypes ($P = 0.17$) whereas *BCL2* and *BCL6* expression were significantly more common in the ABC and GCB subtypes, respectively (both $P < 0.0001$), in contrast to the association of their gene translocations with GCB and ABC subtype respectively. *MYC⁺/BCL2⁺*, *MYC⁺/BCL6⁺*, and *BCL2⁺/BCL6⁺* coexpression were observed in 146 (17.6%), 178 (21.7%), and 282 (33.4%) cases, respectively. Unlike the predominance of *MYC⁺/BCL2⁺* coexpression in the ABC subtype ($P = 0.0079$), both *MYC⁺/BCL6⁺* and *BCL2⁺/BCL6⁺* coexpression were equally distributed between the two COO subtypes ($P = 0.35$ and $P = 0.47$, respectively). All *MYC* protein positive patients were further stratified into three subgroups: *MYC⁺/BCL2⁺/BCL6⁻* (*MYC⁺/BCL2⁺* coexpression, *BCL6⁻*), *MYC⁺/BCL6⁺/BCL2⁻* (*MYC⁺/BCL6⁺* coexpression, *BCL2⁻*) and *MYC⁺/BCL2⁺/BCL6⁺* coexpression. The *MYC⁺/BCL2⁺/BCL6⁻* subgroup was predominantly of ABC subtype ($P = 0.0019$), whereas the *MYC⁺/BCL6⁺/BCL2⁻* subgroup was more commonly of

GCB subtype ($P = 0.011$) (Table 1).

Clinicopathologic features of DLBCL with concurrent rearrangement and coexpression

The clinicopathologic features of patients in the study cohort with or without concurrent gene rearrangements and protein coexpression are listed in Table 2. DLBCL patients with *MYC/BCL2* rearrangements more frequently had large tumors ($P = 0.02$) and a lower complete response rate ($P = 0.0033$), and commonly the tumors were of GCB subtype ($P < 0.0001$). No clinicopathologic features were significantly different between DLBCL patients with concurrent *MYC/BCL6* rearrangements versus patients without *MYC/BCL6* concurrent rearrangement, although larger tumor size was of borderline significance ($P = 0.058$).

Patients with *MYC*⁺/*BCL2*⁺ coexpression were more often of older age ($P = 0.0016$) and more often had advanced disease stage ($P < 0.0001$), extranodal involvement ($P = 0.0026$), large tumor size ($P = 0.03$), International Prognostic Index score > 2 ($P < 0.0001$), low complete response rate ($P = 0.0071$), and high Ki-

67 ($P = 0.0002$), and the lymphoma was more often of ABC subtype ($P = 0.0079$). Patients with *MYC*⁺/*BCL6*⁺ coexpression were associated with advanced disease stage ($P = 0.015$), extranodal sites ($P = 0.011$), low complete response rate ($P = 0.0023$) and high Ki-67 index ($P = 0.0017$). When *MYC*⁺/*BCL2*⁺/*BCL6*⁻ and *MYC*⁺/*BCL6*⁺/*BCL2*⁻ subgroups were isolated from the *MYC*⁺/*BCL2*⁺ and *MYC*⁺/*BCL6*⁺ patients respectively, *MYC*⁺/*BCL2*⁺/*BCL6*⁻ tumors were more often of ABC subtype, whereas *MYC*⁺/*BCL6*⁺/*BCL2*⁻ tumors were more commonly of GCB subtype ($P = 0.0003$) (Table 2).

Prognostic impact of concurrent rearrangements of MYC, BCL2, and BCL6 in DLBCL

We first assessed the prognostic impact of rearrangements of *MYC*, *BCL2*, and *BCL6* in DLBCL (Figure 1 and Supplementary Figure S1). Both *MYC* and *BCL2* rearrangements correlated to a poorer survival in the whole cohort ($P = 0.003$ for *MYC*, Figure 1A; $P = 0.046$ for *BCL2*, Figure 1B) and in the GCB subtype ($P < 0.0001$ for *MYC*, Figure 1D; $P < 0.0009$ for *BCL2*, Figure 1E) but not in the ABC subtype (Figure 1G-H). *BCL6*

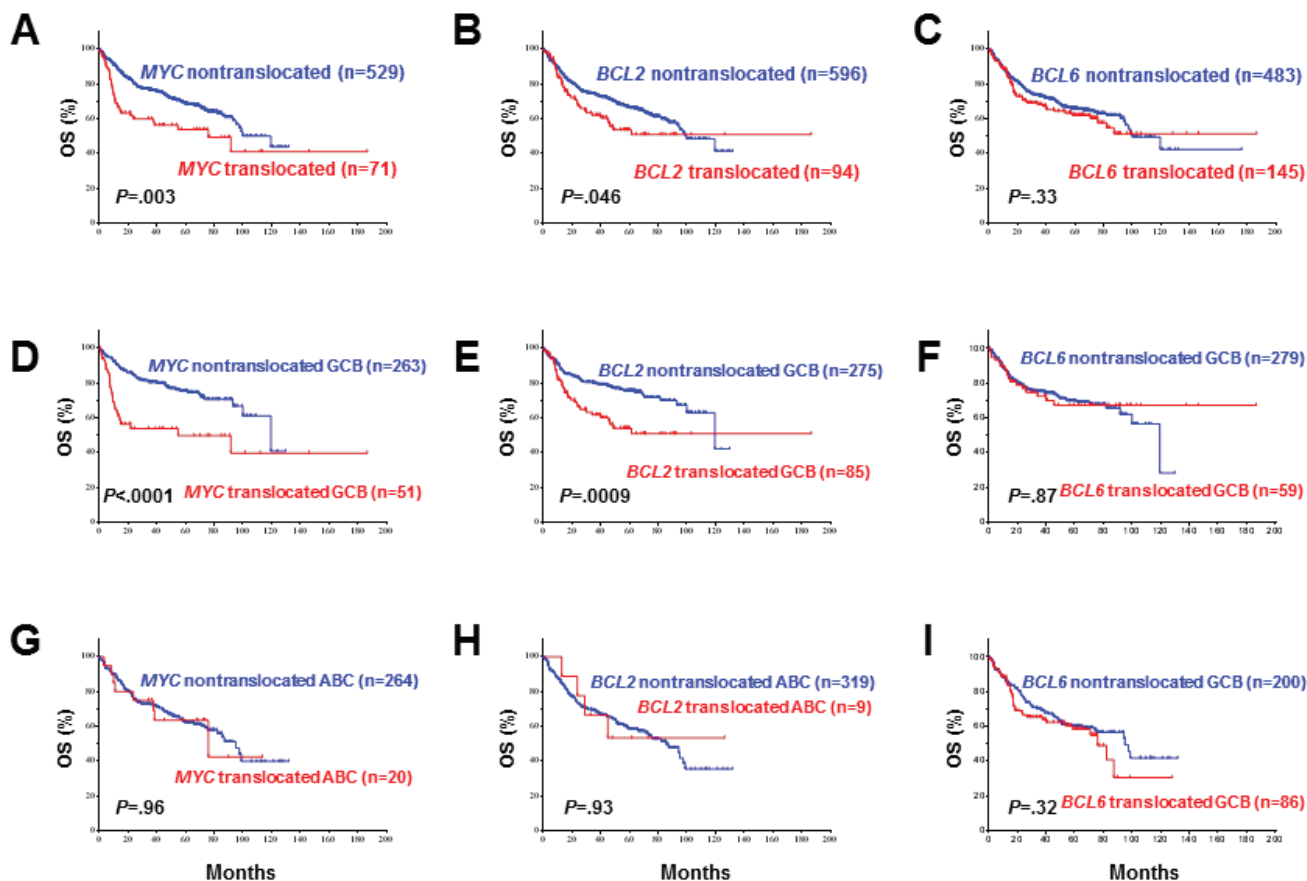


Figure 1: Univariate analysis for patients with DLBCL with *MYC*, *BCL2*, and *BCL6* rearrangements in the overall, GCB, and ABC groups. A.-B., D.-E., G.-H. *MYC* and *BCL2* rearrangements correlated with significantly poorer overall survival in overall and GCB- but not ABC-DLBCL. **C., F., I.** *BCL6* translocation did not correlate with poorer overall survival.

translocation, by contrast, did not correlate with poorer survival for either the whole cohort ($P = 0.33$, Figure 1C) or in the GCB ($P = 0.87$, Figure 1F) and ABC ($P = 0.32$, Figure 1I) subtypes.

We then assessed the prognostic impact of concurrent rearrangements in DLBCL. Patients with *MYC*/

BCL2 translocations had a worse survival than patients with *MYC* rearrangement alone (overall survival [OS]: $P = 0.025$; PFS: $P = 0.012$, Figure 2A-2B). However, no difference was observed in OS and PFS between DLBCL patients with *MYC/BCL6* rearrangement versus DLBCL patients with only *MYC* rearrangement (Figure 2C-2D).

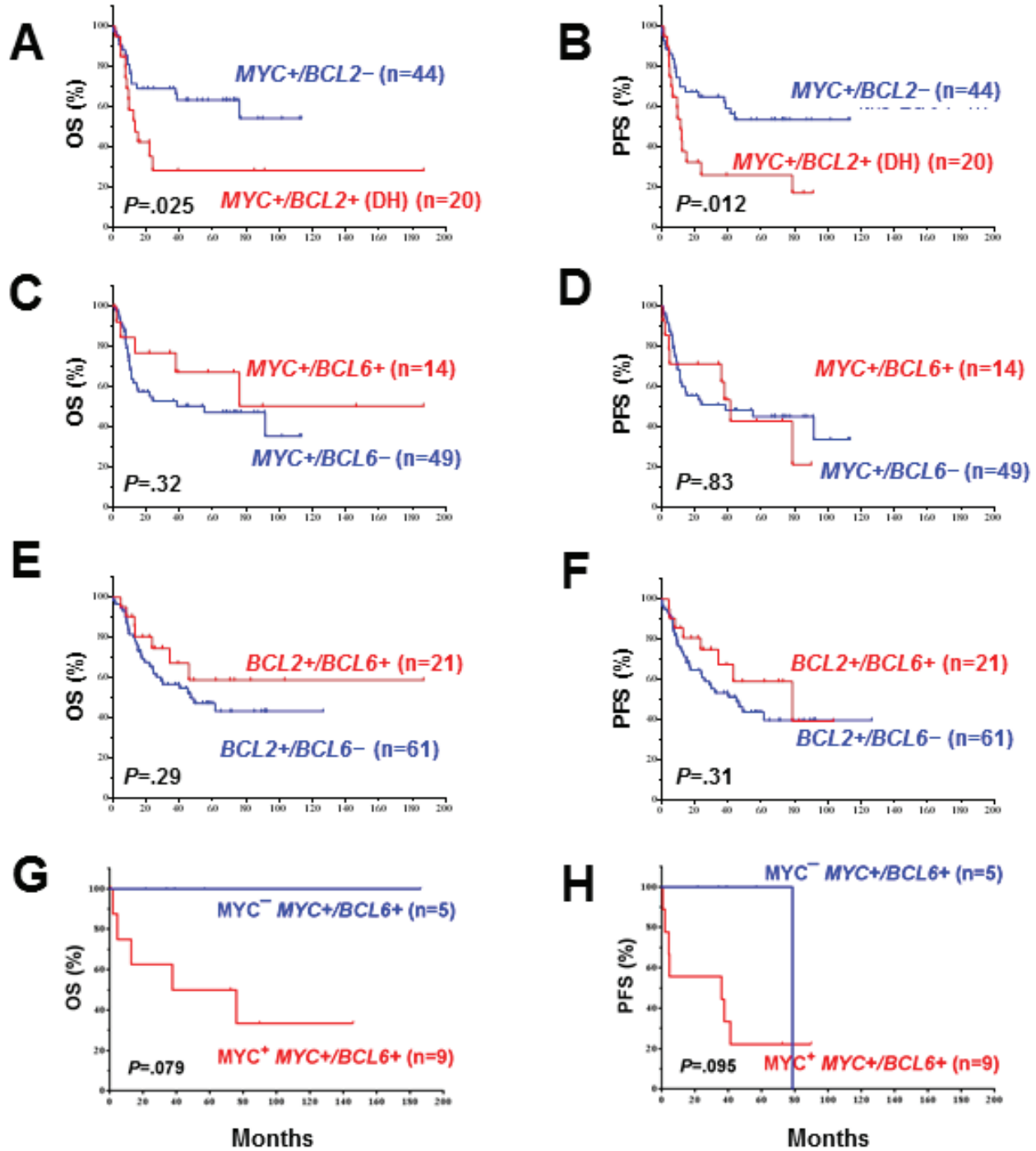


Figure 2: A.-B. The prognostic significance of *MYC* rearrangements in DLBCL depends on *BCL2* rearrangement. C.-D. *BCL6* rearrangement had no additive effect to *MYC* rearrangements. E.-F. *BCL6* translocation had no additive effect to *BCL2* rearrangements. G.-H. *MYC* expression levels appeared to impact the survival of *MYC+/BCL6+* rearranged DLBCL with marginal P values probably due to the small case numbers.

Table 2: Clinical characteristics of patients with concurrent MYC, BCL2 or BCL6 translocations and MYC, BCL2 or BCL6 protein co-expression

Parameters	MYC+/BCL2+ (DH)	Non-MYC+/BCL2+	P	MYC+/BCL6+	Non-MYC+/BCL6+	P	MYC+/BCL2+ (DP)	Non-MYC+/BCL2+	P	MYC+/BCL6+	Non-MYC+/BCL6+	P	MYC+/BCL2+, BCL6+	MYC+/BCL6+, BCL2+	P
	N (%)	N (%)		N (%)	N (%)		N (%)	N (%)		N (%)	N (%)		N (%)	N (%)	
Age															
<60	7 (35)	254 (39.9)	.73	4 (30.8)	253 (38.8)	.51	39 (28.7)	285 (43.2)	.0016	61 (36.5)	262 (40.4)	.36	14 (34.1)	36 (50)	.10
≥60	13 (65)	401 (60.1)		9 (69.2)	381 (61.2)		97 (71.3)	374 (56.8)		106 (63.5)	386 (59.6)		27 (65.9)	36 (50)	
Gender															
F	6 (30)	266 (39.6)	.35	7 (53.8)	252 (40.4)	.30	61 (44.5)	272 (41.2)	.47	74 (44)	266 (41)	.47	17 (40.5)	30 (41.1)	.95
M	14 (70)	392 (60.4)		6 (46.2)	385 (59.6)		76 (55.5)	388 (58.8)		94 (56)	383 (59)		25 (59.5)	43 (58.9)	
Stage															
I-II	5 (27.8)	292 (45.2)	.13	5 (41.7)	278 (45.8)	.81	40 (30.5)	318 (49.9)	<.0001	60 (37)	299 (47.7)	.015	16 (39)	36 (50)	.26
III-IV	13 (72.2)	345 (54.8)		7 (58.3)	337 (54.2)		91 (69.5)	319 (50.1)		102 (63)	328 (52.3)		25 (61)	36 (50)	
B-symptoms															
No	11 (61.1)	372 (61.3)	.98	6 (50)	357 (60.9)	.43	70 (55.6)	393 (63.9)	.078	96 (60.8)	377 (62.6)	.67	22 (53.7)	48 (66.7)	.17
Yes	7 (38.9)	239 (38.7)		6 (50)	225 (39.1)		56 (44.4)	222 (36.1)		62 (39.2)	225 (37.4)		19 (46.3)	24 (33.3)	
LDH															
Normal	5 (27.8)	244 (39.5)	.25	4 (30.8)	226 (41.1)	.52	44 (35.2)	243 (40.5)	.27	59 (37.6)	239 (40.6)	.48	12 (31.6)	27 (38.6)	.47
Elevated	13 (72.2)	349 (60.5)		9 (69.2)	346 (58.9)		81 (64.8)	357 (59.5)		98 (62.4)	349 (59.4)		26 (68.4)	43 (61.4)	
# of extranodal sites															
0-1	12 (63.2)	481 (77.4)	.16	7 (63.6)	468 (77)	.28	88 (67.7)	501 (79.8)	.0026	113 (70.2)	490 (79.5)	.011	28 (70)	53 (74.6)	.60
≥2	7 (36.8)	144 (22.6)		4 (36.4)	137 (23)		42 (32.3)	127 (20.2)		48 (29.8)	126 (20.5)		12 (30)	18 (25.4)	
ECOG															
0-1	14 (77.8)	471 (82.5)	.62	7 (63.6)	456 (82.3)	.11	93 (75)	486 (84.4)	.012	119 (76.8)	470 (83.3)	.06	29 (76.3)	56 (81.2)	.55
≥2	4 (22.2)	101 (17.5)		4 (36.4)	97 (17.7)		31 (25)	90 (15.6)		36 (23.2)	94 (16.7)		9 (23.7)	13 (18.8)	
Size of largest tumor															
<5cm	4 (25)	273 (54)	.02	2 (22.2)	259 (54.4)	.058	50 (45)	288 (56.4)	.03	67 (48.2)	268 (56.1)	.10	11 (31.4)	28 (45.2)	.19
≥5cm	12 (75)	229 (46)		7 (77.8)	221 (45.6)		61 (55)	223 (43.6)		72 (51.8)	210 (43.9)		24 (68.6)	34 (54.8)	
IPI score															
0-2	8 (44.4)	377 (59.8)	.17	6 (50)	355 (60.5)	.50	56 (42.7)	401 (65.1)	<.0001	81 (49.7)	381 (63.2)	.0018	19 (47.5)	44 (61.1)	.16
3-5	10 (55.6)	246 (40.2)		6 (50)	239 (39.5)		75 (57.3)	215 (34.9)		82 (50.3)	222 (36.8)		21 (52.5)	28 (37.9)	
Therapy response															
CR	10 (55)	487 (75.3)	.026	8 (66.7)	464 (76.3)	.48	79 (48.2)	501 (78.8)	<.0001	108 (65.9)	484 (77.4)	.0023	25 (61)	55 (77.5)	.06
PR	3	80		2	80		59	70		31	70		9	8	
SD	2	27		1	24		9	23		7	30		4	2	
PD	4	44		1	47		17	42		18	41		3	6	
Cell-of-origin															
GCB	19 (95)	340 (52.2)	<.0001	9 (64.3)	347 (49.4)	.43	62 (42.5)	372 (54.9)	.0079	97 (54.5)	337 (50.4)	.35	13 (30.2)	49 (65.3)	.0003
ABC	1 (5)	348 (47.8)		5 (35.7)	318 (50.6)		84 (57.5)	306 (45.1)		81 (45.5)	332 (49.6)		30 (69.8)	26 (34.7)	
Ki-67 index															
<70%	5 (45.5)	137 (32.5)	.52	3 (37.5)	125 (33.1)	.72	19 (19.4)	170 (39.2)	.0002	29 (23.8)	159 (39.6)	.0017	6 (19.4)	16 (28.6)	.44
≥70%	6 (54.5)	277 (67.5)		5 (62.5)	260 (66.9)		79 (80.6)	264 (60.8)		93 (76.2)	243 (60.4)		25 (80.6)	40 (71.4)	
TP53 mutation															
WT	8 (72.7)	301 (78.8)	.71	7 (100)	276 (78.2)	.35	62 (71.3)	311 (78.9)	.12	81 (75.7)	284 (78.0)	.60	20 (76.9)	40 (83.3)	.54
MUT	3 (27.3)	81 (21.2)		0 (0)	77 (21.8)		25 (28.7)	83 (21.1)		26 (24.3)	80 (22.0)		6 (23.1)	8 (16.7)	

Abbreviations: DH, double-hit; DP, double-positive; LDH, lactate dehydrogenase; ECOG, Eastern Cooperative Oncology Group performance status; IPI, International Prognostic Index; CR, complete remission; PR, partial response; SD, stable disease; PD, progressive disease; GCB, germinal center B-cell like; ABC, activated B-cell like; WT, wild type; MUT, mutant. For therapy response, we calculated *P* values as CR vs other responses. Some clinical features of certain cases were not available.

There was also no difference in OS and PFS between patients with DLBCL with *BCL2/BCL6* rearrangement versus DLBCL with only *BCL2* rearrangement (Figure 2E-2F). Notably the survival of patients with *MYC/BCL6* rearrangement appeared to be affected by *MYC* expression levels (Figure 2G-2H).

The prognostic differences between patients with concurrent rearrangements of *MYC/BCL2*, *MYC/BCL6*, *BCL2/BCL6* versus the remaining DLBCL patients are shown in Figure 3. Only concurrent *MYC/BCL2* rearrangements correlated with significantly poorer survival (Figure 3A-3B). Additional *BCL6* translocation (triple-hit, *n* = 5, 26% of 19 *MYC+/BCL2+* cases with *BCL6* translocation status available) had no synergistic effect with concurrent *MYC/BCL2* rearrangements and, on the contrary, attenuated the adverse impact of concurrent *MYC/BCL2* rearrangements (Figure 3G-3H).

Prognostic impact of coexpression of MYC, BCL2, and BCL6 in DLBCL

The prognostic impact of protein expression of MYC, BCL2, and BCL6 is shown in Figure 4 and Supplementary Figure S2. BCL6 expression in DLBCL

did not correlate with poorer patient survival, either in the whole group or in COO subtypes (Figure 4C, 4F, and 4I). In contrast, *MYC+* or *BCL2+* expression in DLBCL correlated with significantly poorer survival for the overall patient cohort (*P* < 0.0001, Figure 4A-4B) and for patients with GCB (*MYC+*: *P* < 0.0001, Figure 4D; *BCL2+*: *P* = 0.004, Figure 4E) and ABC subtypes of DLBCL (*MYC+*: *P* = 0.032, Figure 4G; *BCL2+*: *P* < 0.0001, Figure 4H).

Our results further showed that *MYC+/BCL2+* (*P* < 0.0001, Figure 5A, Supplementary Figure S3A), *MYC+/BCL6+* (OS: *P* = 0.0001, Figure 5C; PFS: *P* = 0.0002, Supplementary Figure S3C), and *BCL2+/BCL6+* (OS: *P* = 0.014, Figure 5E; PFS: *P* = 0.033, Supplementary Figure S3E) coexpression correlated with significantly poorer survival in the overall cohort. The inferior survival of patients *MYC+/BCL2+* and *MYC+/BCL6+* DLBCL compared with all other DLBCL patients was significant for both the GCB and ABC subtypes, whereas *BCL2+/BCL6+* only correlated with poorer OS for patients with ABC-DLBCL (Supplementary Figure S4). *MYC* expression showed dependence and synergy only with *BCL2* expression (Figure 5B, Supplementary Figure S3B); *BCL6* expression had no additive adverse impact in patients with *MYC+*, *BCL2+* or *MYC+/BCL2+* DLBCL (Figure 5D, 5F-5G, Supplementary Figure S3D,

Table 3: GEP signatures of MYC/BCL2 double-hit lymphoma, isolated MYC⁺BCL2⁺ (i.e., BCL6⁻DPL) and MYC⁺BCL2⁻BCL6⁺ (i.e., BCL2⁻DPL)

A. MYC ⁺ /BCL2 ⁺ + double-hit vs others (false discovery rate < .01)		
Functional categories	Upregulated genes	Downregulated genes
Signaling	<i>BMP3, SWAP70, CELSR1</i>	<i>PLA2G7, DOCK10, RNF149</i>
Cell proliferation and growth	<i>STRBP, MUC4</i>	
Transcriptional regulation	<i>ZNF280D, DIP2C</i>	<i>PRDM1</i>
Metabolism	<i>RIMKLB, UGT8, PLA2G12A, SLC25A27, CYP39A1, C7orf10, ENPP3, HPGD</i>	
Cell death		<i>TMEM49, CFLAR, CARD16 /CASP1</i>
Immune response	<i>NCR3LG1</i>	
Cell adhesion, extracellular matrix, migration	<i>MYO3B</i>	<i>SLAMF7, SIGLEC10/12, TPM4, SRGN</i>
microRNA		<i>MIR21</i>
Others	<i>TPD52, C4orf34, FLJ41455, XKR6, FAM53B, TMEM156, CEACAM7</i>	<i>FLCN</i>
B. MYC ⁺ BCL2 ⁺ BCL6 ⁻ vs MYC ⁺ BCL2 ⁻ BCL6 ⁺ (false discovery rate < .05)		
	Upregulated in MYC ⁺ BCL2 ⁻ BCL6 ⁻	Upregulated in MYC ⁺ BCL2 ⁺ BCL6 ⁺
BCR signaling, receptors, antigen, modulators, transducers	<i>CCR7, CYSLTR1, PRSS21</i>	<i>STAP1, SORL1, RFTN1, GNA13, SWAP70, ANKRD13A, PIK3CG</i>
Proliferation, cell cycle, , gene expression DNA replication	<i>SUB1, ZBTB32, LIMD1, C16orf53</i>	<i>BCL6, MYBL1, NEK6, BRWD1, SFRS15, HMGNI, TMPO</i>
Apoptosis	<i>BCL2, CASP10</i>	<i>DNAJC10</i>
DNA repair		<i>MSH6</i>
Metabolism	<i>ALDH4A1</i>	<i>BPNT1</i>
Cell shape, cytoskeleton, microtubes, migration	<i>BBIP10</i>	<i>MARCKSL1, VNN2, OSBPL3, ACTR2</i>
Unknown function	<i>LOC100289094</i>	<i>C17orf99, KIAA0746, ZNF608</i>

Abbreviations: DH, double-hit; DP, double-positive.

S3F-S3G). Patients with MYC⁺/BCL2⁺ DLBCL but not MYC⁺/BCL6⁺ DLBCL had poorer survival (Figure 5G, Supplementary Figure S3G). The poor prognosis of patients with MYC⁺/BCL6⁺ DLBCL was attributable to the poorer survival of MYC⁺/BCL2⁺ DLBCL patients.

We further evaluated the survival of patients with isolated MYC⁺/BCL2⁻/BCL6⁻ (i.e., BCL6⁻ DPL) versus isolated MYC⁺/BCL6⁺/BCL2⁻ (i.e., BCL2⁻ DPL) DLBCL. Patients with isolated MYC⁺/BCL6⁺ DLBCL had favorable survival compared with isolated MYC⁺/BCL2⁺ DLBCL patients (OS: *P* = 0.004, Figure 5H; PFS: *P* < 0.0001, Supplementary Figure S3H).

Gene expression signatures of concurrent MYC, BCL2, and BCL6 rearrangements and isolated MYC⁺BCL2⁺ and MYC⁺BCL6⁺ coexpression

To better understand the molecular mechanisms of the effects on prognosis, we compared the GEP of patients with concurrent MYC/BCL2 or MYC/BCL6 rearrangements with the remaining patients. Only MYC/BCL2 rearranged DLBCL showed a distinctive GEP signature, whereas concurrent MYC/BCL6 rearranged DLBCL did not show DEGs compared with MYC rearranged DLBCL or the remaining DLBCL patients. The GEP signature of concurrent MYC/BCL2 rearranged DLBCL included 24 upregulated genes and 14 downregulated genes at the false discovery rate threshold of 0.01 (Table. 3A, Figure 5A). These genes were involved in signaling (upregulation of *BMP3*, *SWAP70*, and *CELSR1*, and downregulation of *PLA2G7* and *DOCK10*), cell proliferation (upregulated *STRBP* and *MUC4*), metabolism (eight upregulated genes), apoptosis (*TMEM49*, *CFLAR*, *CARD16* and

CASP1), transcription factors and genes related to cell adhesion, extracellular matrix, and migration (*MYO3B*, *SLAMF7*, *SILEC10/12*, *TPM4*, and *SRGN*).

Comparison of the GEP of MYC⁺ BCL6⁺ versus MYC⁺BCL2⁺ DLBCL did not show significant DEGs. We further compared the GEP of isolated MYC⁺BCL2⁻BCL6⁺ DLBCL versus isolated MYC⁺BCL2⁺BCL6⁻ DLBCL, which resulted in 36 DEGs at the false discovery rate threshold of 0.05 (Table 3B, Figure 6B). Upregulated genes in MYC⁺BCL2⁻BCL6⁺ compared with MYC⁺BCL2⁺BCL6⁻ included *BCL6*, a proliferation signature (*MYBL1*, *NEK6*, *BRWD1* [bromodomain and WD repeat domain containing 1], *SFRS15*, *HMGNI*, and *TMPO*), *MSH6* involved in DNA repair, *DNAJC10* which promotes apoptotic in response to endoplasmic reticulum stress, various signaling genes including *PIK3CG* (PI3K catalytic subunit gamma), *STAP1* (B-cell receptor signaling) *SORL1*, *RFTN1*, *GNA13*, *SWAP70*, and *ANKRD13A*, and genes involved in actin cytoskeleton regulation and migration (*MARCKSL1*, *VNN2*, *OSBPL3*, *ACTR2* and *etc.*). Comparably, upregulated genes in MYC⁺BCL2⁺BCL6⁻ included signatures of apoptosis (antiapoptotic *BCL2*, and paradoxically proapoptotic, *CASP10*), glutamine metabolism (*ALDH4A1*), indicating that abnormal dysregulation of apoptotic and proliferation pathways are critical in patients with MYC⁺BCL6⁺BCL2⁻. In contrast, such signaling and molecular defects were not seen in patients with MYC⁺BCL6⁻BCL2⁺. The observations provide molecular basis for the difference of outcome and survival between these two groups of DLBCL patients.

DISCUSSION

In the literature B-cell lymphomas with concurrent *MYC/BCL2* or *MYC/BCL6* rearrangements are grouped together as double-hit B-cell lymphomas [1, 2]. Patients with *MYC/BCL2* DHL have responded poorly to all traditional chemotherapy regimens and have extremely poor outcomes [1, 10, 11]. However, most of what we

know about double-hit B-cell lymphoma is derived from studies of the most common form, *MYC/BCL2* DHL. In contrast, very little has been published on DLBCL patients with concurrent *MYC/BCL6* rearrangements. The findings in this study for a group of DLBCL patients treated with R-CHOP suggest that patients with concurrent *MYC/BCL6* rearrangements do not have a poorer prognosis and that grouping these tumors with other forms of DHL could lead to inappropriate therapy.

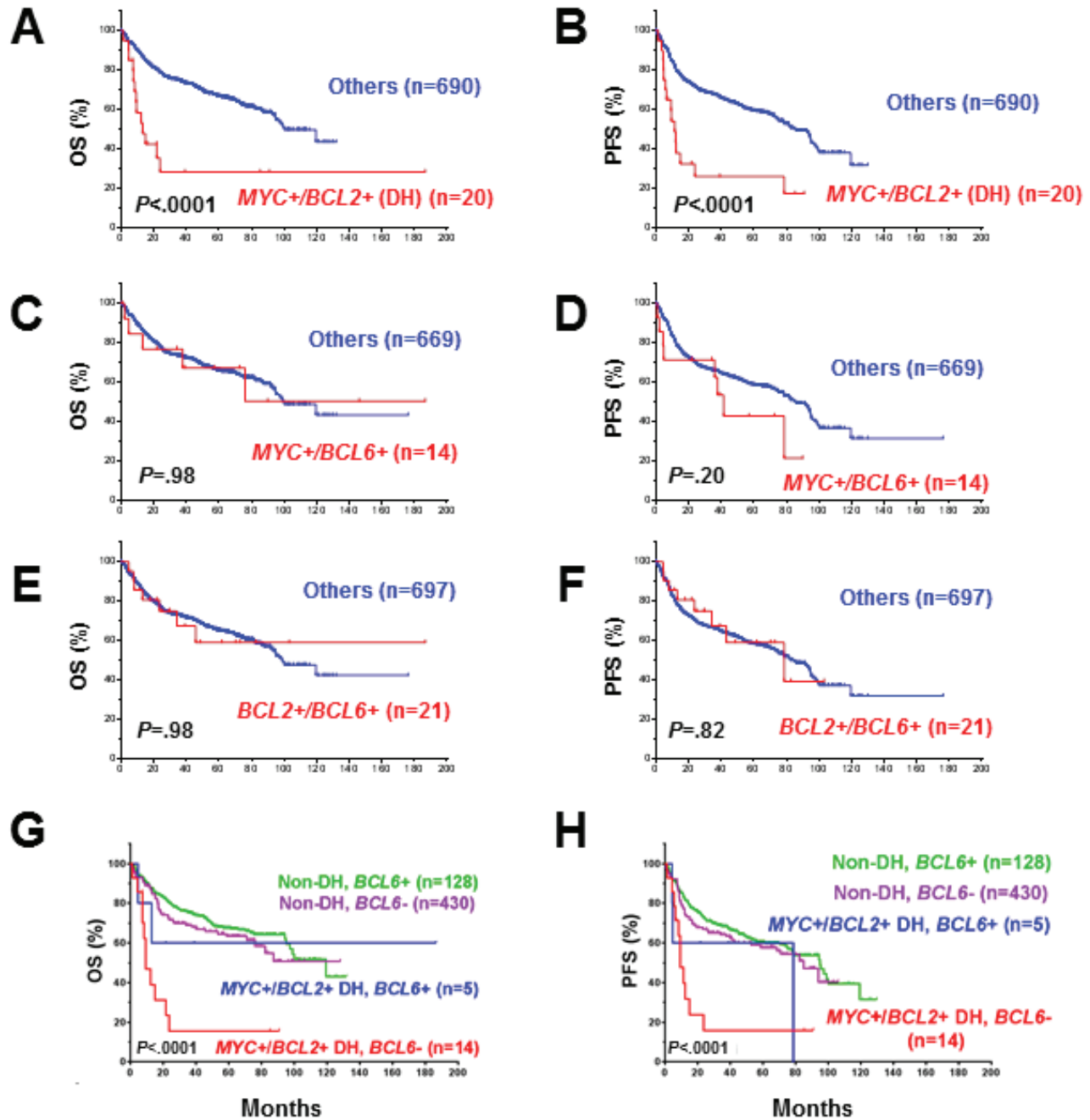


Figure 3: A.-B. Concurrent *MYC/BCL2* rearrangements correlated with significant poorer overall survival. C.-D. Concurrent *MYC+/BCL6+* rearrangements did not correlate with poorer overall survival. E.-F. Concurrent *BCL2+/BCL6+* rearrangements did not correlate with poorer overall survival. G.-H. *BCL6* attenuated the adverse prognostic impact of *MYC+/BCL2+* double-hit lymphoma.

In this large cohort of 898 cases of *de novo* DLBCL, rearrangements of *MYC*, *BCL2*, and *BCL6* were found in 11.8%, 13.6% and 23.1% of cases, respectively. *BCL6* rearrangements were more frequently observed in the ABC subtype whereas *MYC* and *BCL2* rearrangements were more frequently observed in the GCB subtype. Concurrent *MYC/BCL6* and *MYC/BCL2* rearrangements were observed in 2.0% and 2.8% of DLBCL cases, respectively. *MYC/BCL2/BCL6* triple-hit was observed in 38% of *MYC/BCL6* and 26% of *MYC/BCL2* double-hit cases. The frequencies of *MYC*, *BCL2*, and *BCL6* rearrangement and concurrent *MYC/BCL2* rearrangements are similar to those reported in DLBCL in earlier studies [10, 11, 31-34].

In this study, DLBCL patients with concurrent *MYC/BCL6* rearrangements did not have a poorer prognosis. The notion that concurrent *MYC/BCL6* rearrangements in DLBCL is not an indication of aggressive lymphoma was further substantiated by the lack of a distinctive GEP signature for *MYC/BCL6* rearranged DLBCL although *BCL6* was overexpressed in almost all *MYC/BCL6* rearranged patients except one case with an IHC of 30%. These phenomena might be explained by a previous finding that up to 43% of *BCL6* translocations involved non-*IG* loci showing complex gene expression patterns [35], limited case numbers, and 36% of *MYC/BCL6* cases showing low *MYC* expression levels (Figure 2G-2H). In contrast, *MYC/BCL2* rearranged DLBCL correlated

with significantly poorer survival, and was associated with a distinctive GEP signature suggesting increased proliferation, growth and metabolism and decreased apoptosis pathway (our results showed downregulation of both pro- and anti-apoptotic genes).

In the MD Anderson Cancer Center experience with 52 DHL patients tested for *BCL6*, 24 patients with *BCL6* gene abnormality (translocation or amplification, $n = 15$ and $n = 9$ respectively. Among them, 14 patients had *MYC/BCL2/BCL6* triple-hit) showed slightly better survival than other patients with DHL (hazard ratio: 0.59, 95% confidence interval: 0.21-1.69, $P = 0.33$) [36]. Ueda *et al.* presented a case report consistent with our results, in which a person having DLBCL with concurrent *MYC*, *BCL2*, and *BCL6* rearrangements achieved complete remission after chemoradiotherapy for two years [37]. A recent study reported the largest DHL series including 41 cases with *BCL6* rearrangement, in which patients' OS was not significantly affected by whether the DHL was *MYC/BCL2* or *MYC/BCL6* ($P = 0.537$). However, 25 (58.5%) of the 41 *MYC/BCL6* patients also had *BCL2* rearrangement (triple-hit) [38]. In contrast, in another study, *MYC/BCL6* ($n = 13$) showed significantly worse survival than *MYC/BCL2* DHL ($n = 20$) after exclusion of triple-hit lymphoma [39]. These *MYC/BCL6* DHL showed a trend toward higher *MYC* mRNA expression and a distinct gene expression profile compared to *MYC/BCL2* DHL. In a smaller study reported by Pillai *et al.* B-cell

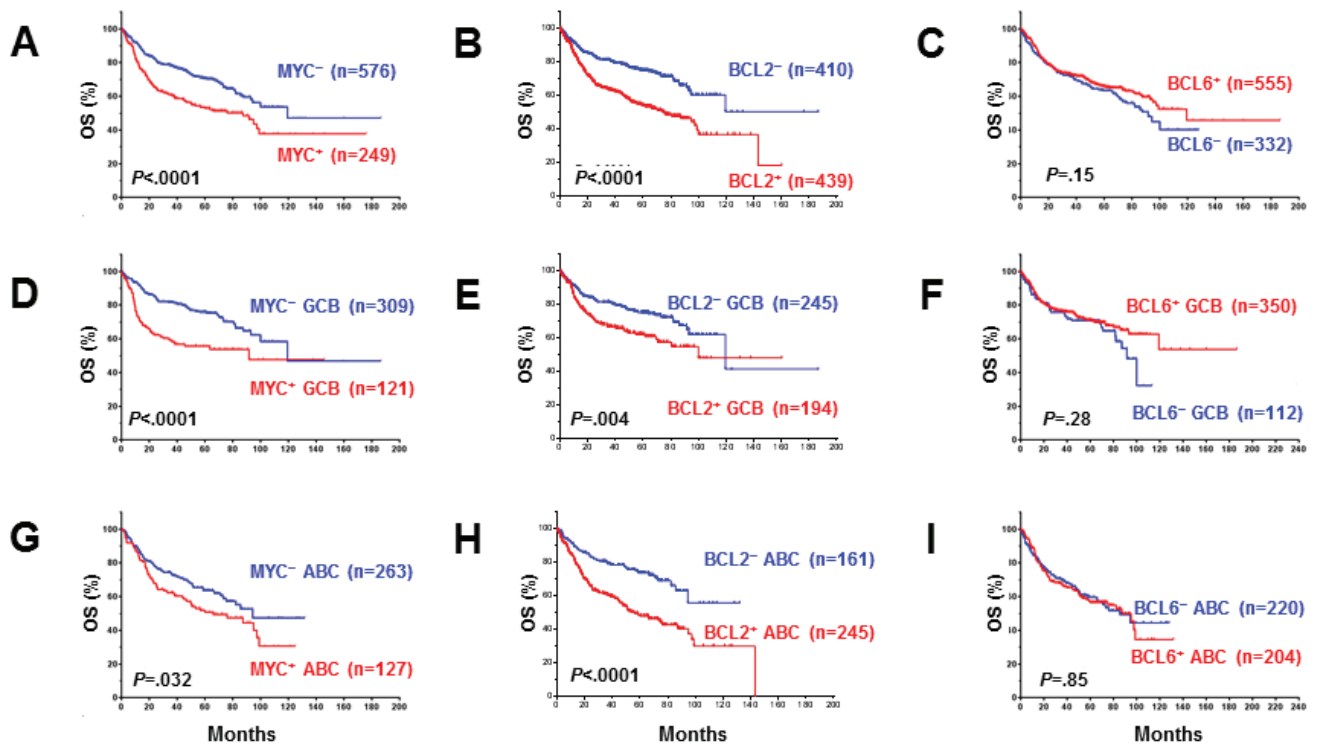


Figure 4: Univariate analysis for DLBCL patients with MYC, BCL2 and BCL6 protein expression in the overall-, GCB, and ABC-DLBCL. A.-B., D.-E., G.-H. MYC and BCL2 protein expression correlated with significantly poorer overall survival in overall, GCB- and ABC-DLBCL. C., F., I. BCL6 overexpression did not correlate with poor survival.

lymphoma with concurrent *MYC/BCL6* rearrangements was associated with aggressive clinical course and poor survival [40]. A possible explanation for the discrepancy is patient selection. In the study by Pillai *et al*, patients with BL, BL-like lymphoma, and primary effusion lymphoma were also included. Moreover, the median age of their patients was 83 years and only one of six patients with adequate information received chemotherapy in

combination with rituximab [40]. In this study, all patients met morphologic and immunophenotypic criteria for DLBCL with a median patient age of 64 years, which is comparable to that of patients with DLBCL without concurrent *MYC/BCL6* rearrangements, and all patients were treated with standard R-CHOP therapy.

Recently, the concept of *MYC/BCL2* rearranged DHL has been extended to *MYC* and *BCL2* protein

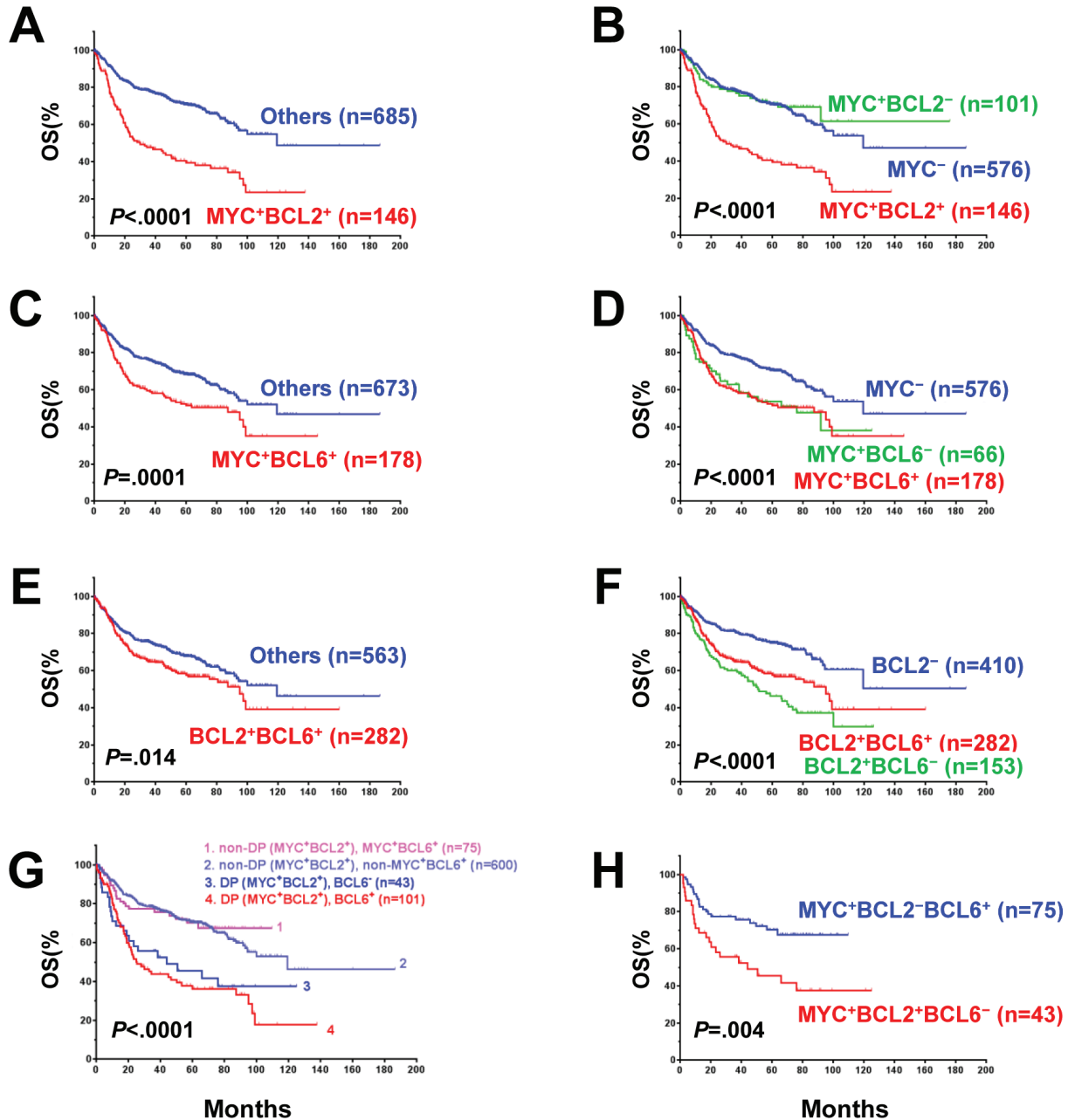


Figure 5: A., C., E. Patients with DLBCL and *MYC/BCL2*, *BCL6/MYC* or *BCL2/BCL6* co-expression had significantly poorer overall survival in the DLBCL cohort. B. *BCL2* overexpression had a synergetic effect with *MYC* overexpression and the adverse prognostic impact of *MYC* depended on *BCL2* overexpression. D. *BCL6* expression had no synergetic effect with *MYC* expression. F. *BCL6* expression appeared to attenuate the adverse prognostic impact of *BCL2* overexpression. G. The poorer overall survival of *MYC+BCL6+* patients was due to the poor survival of *MYC+BCL2+* patients. H. Isolated *MYC+BCL6+* versus *MYC+BCL2+* double-positive DLBCL had significantly better patient survival.

coexpression [11, 22, 23]. These studies showed that patients with DLBCL with MYC/BCL2 double-positive (by immunohistochemistry) also have a dismal prognosis, regardless of the status of MYC or BCL2 rearrangement

[11, 22, 23]. In this study, DLBCL patients with MYC/BCL6 coexpression showed a significantly poorer survival than DLBCL patients without MYC/BCL6 coexpression. However, this prognostic effect was significant only in

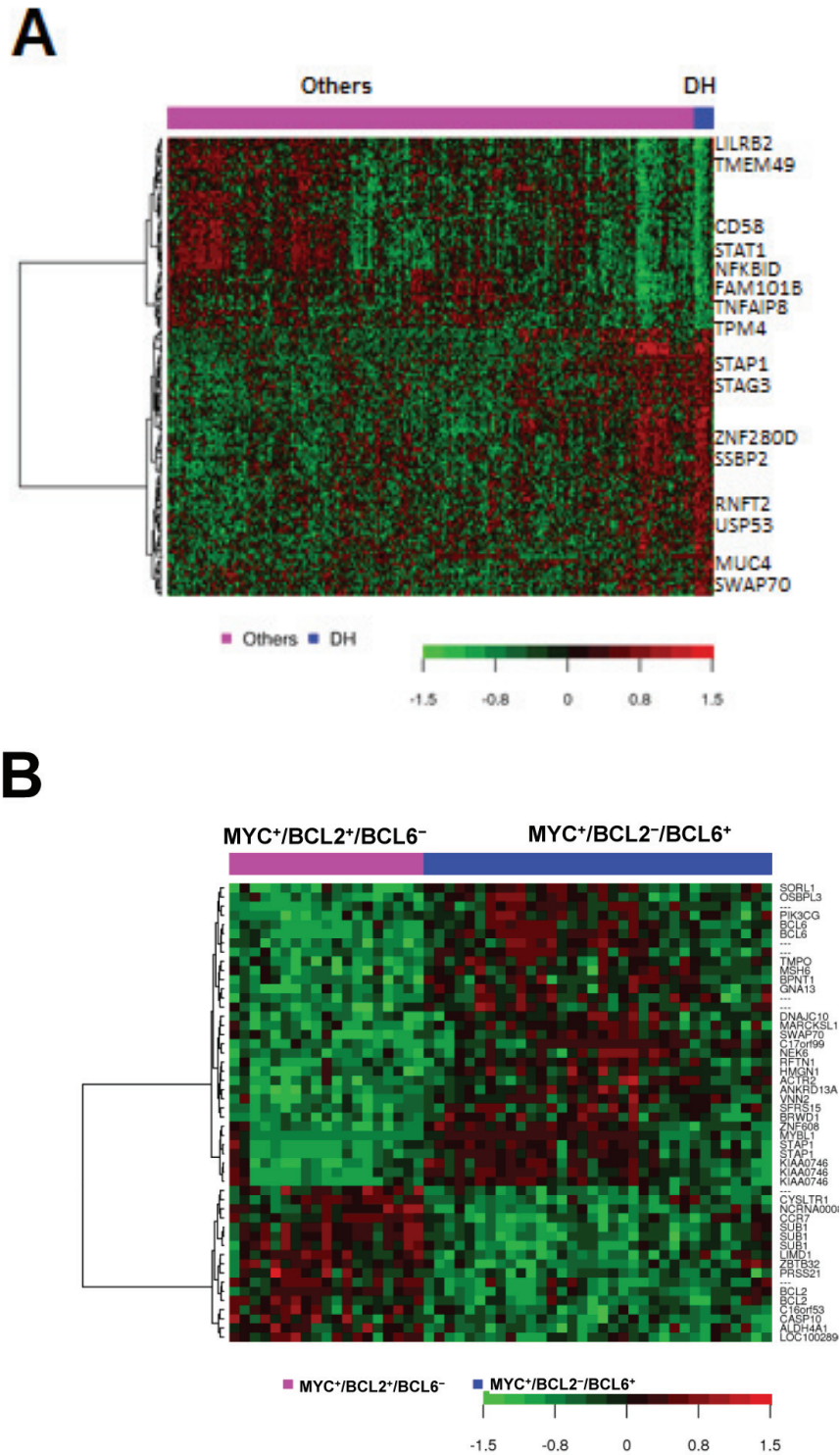


Figure 6: Gene expression signature for MYC/BCL2 double-hit DLBCL (A) and comparison of MYC⁺BCL2⁺ BCL6⁻ versus MYC⁺BCL2⁺BCL6⁺ translocation in DLBCL (B).

the presence of DLBCL with MYC/BCL2 coexpression. The isolated MYC⁺BCL2⁻BCL6⁺ (from all MYC⁺BCL6⁺) subgroup had significantly better patient survival compared with the MYC⁺BCL2⁺BCL6⁻ (from all MYC⁺BCL2⁺) subgroup. Previous studies also suggested that BCL6 expression is associated with favorable survival in patients with DLBCL [24, 30, 41, 42].

There is a biologic basis that may explain the lack of significantly adverse prognostic impact of recurrent MYC/BCL6 rearrangements and MYC/BCL6 coexpression. BCL6 represses *CCND2* and *MYC* [27-29, 43]; we have recently shown that MYC expression levels significantly impact the prognosis of MYC rearranged DLBCL [44], and our multivariate analysis suggested BCL6 expression correlated with favorable survival ($P = 0.048$ for OS and $P = 0.016$ for PFS, Table 1), therefore the adverse prognostic impact of MYC might have been diminished by high BCL6 expression in these cases. In addition to the potential role of BCL6, the GEP signature of MYC⁺BCL2⁻BCL6⁺ DLBCL suggested DNA repair and proapoptosis, in contrast with the upregulation of antiapoptotic *BCL2* in MYC⁺BCL2⁺BCL6⁻ DLBCL. Interestingly, *MYBL1* and *LIMD1* were also significantly upregulated in our MYC⁺BCL2⁻BCL6⁺ (associated with GCB) and MYC⁺BCL2⁺BCL6⁻ (associated with ABC) DLBCL subgroup respectively, which is in consistent with the correlations between a novel two-gene expression index, "LIMD1-MYBL1 Index", and GCB/ABC subtypes and clinical outcome [45, 46].

In summary, DLBCL patients with concurrent MYC/BCL6 rearrangements are not necessarily associated with an inferior prognosis when treated with R-CHOP therapy, unlike DLBCL patients with concurrent MYC/BCL2 rearrangements, probably due to different pathogenesis and MYC expression levels. In addition, DLBCL patients with MYC/BCL6 coexpression did have an inferior prognosis, but only in the presence of MYC/BCL2 coexpression, and therefore MYC/BCL6 coexpression seems to be of less prognostic importance [46]. These data support the notion that DLBCL with concurrent MYC/BCL6 rearrangements and DLBCL with MYC/BCL2 DHL are not equivalent prognostically. These results suggest that the concept of double-hit lymphoma needs to be refined. The grouping of cases of DLBCL with concurrent MYC/BCL6 rearrangements with cases of DLBCL with MYC/BCL2 rearrangements may lead to over treatment of MYC/BCL6 rearranged DLBCL patients.

PATIENTS, MATERIALS AND METHODS

Patients

A cohort of 898 patients with *de novo* DLBCL treated with standard rituximab, cyclophosphamide,

doxorubicin, vincristine and prednisolone (R-CHOP) therapy was collected as part of The International DLBCL Rituximab-CHOP Consortium Program Study [33,46]. All patients were diagnosed according to the World Health Organization (WHO) classification system and received treatment between 1998 and 2010. Cases were excluded if patients had a history of low-grade B-cell lymphoma; human immunodeficiency virus infection; or primary mediastinal, cutaneous B cell lymphoma, or central nervous system DLBCL. This study was approved by the Institutional Review Boards of each participating center, and the comprehensive collaborative study was approved by the Institutional Review Board at The University of Texas MD Anderson Cancer Center.

Tissue microarray, immunohistochemistry and fluorescence in situ hybridization

Construction of tissue microarrays, IHC staining procedures on tissue microarray sections, and scoring criteria for MYC, BCL2 and BCL6 have been described previously [23, 44, 47]. MYC (clone Y69; Epitomics, Burlingame, CA) and BCL6 (clone LN22; Leica Microsystems, Buffalo Grove, IL) expression showed a distinct nuclear pattern and BCL2 (clone 124; DAKO, Denmark) expression exhibited a cytoplasmic pattern. Cutoffs for MYC, BCL2 and BCL6 overexpression are determined as $\geq 70\%$, $\geq 70\%$, and $> 50\%$ respectively based on survival analysis as described previously, and cutoffs and positivity rates reported by other study groups.

Interphase fluorescence *in situ* hybridization (FISH) analysis was performed using formalin-fixed, paraffin-embedded tissue sections and BCL2 and BCL6 dual-color, break-apart probes (Vysis), IGH/MYC/CEP8 tricolor dual-fusion probes (Vysis) and a locus specific MYC dual-color break-apart probe (Vysis) as described previously [47].

Gene expression profiling and COO classification

Gene expression profiling (GEP) was performed on formalin-fixed, paraffin-embedded tissue samples using Affymetrix GeneChip HG-U133 Plus Version 2.0 (Affymetrix, Santa Clara, CA) as described in an earlier study [47]. The CEL files are deposited in the National Center for Biotechnology Information Gene Expression Omnibus repository (GSE#31312).⁶¹ The microarray data were quantified and normalized by the frozen robust multiarray analysis (RMA) algorithm [48]. Differential expression gene (DEG) analysis was performed using multiple *t*-tests [31, 47]. Cell-of-origin classification into either GCB or ABC subtypes was achieved by using either GEP ($n = 497$) or IHC methods ($n = 401$) according to the Visco-Young (first selection) and Choi (the second selection) algorithms [49-53].

Statistical analysis

Clinical and laboratory features of DLBCL patients at the time of presentation according to different subgroups were compared using the chi-squared test and the Spearman rank correlation test. Overall survival (OS) was calculated from the date of diagnosis to the date of last follow-up or death. Progression-free survival (PFS) was calculated from the date of diagnosis to the time of progression or death. Kaplan-Meier survival curves were used to estimate OS and PFS rates, and the log-rank (Mantel-Cox) test was used to assess differences in survival between groups. Multivariate analysis for survival was performed with IBM statistics SPSS 19 software using the Cox proportional hazards regression model (Chicago, SPSS Inc.). All differences with $P \leq 0.05$ were considered to be statistically significant.

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Authors' Contributions

Conception and design: SH, ZYXM, KHY; Research performance: QY, ZYXM, AT, KHY; Provision of study materials, key reagents and technology: QY, ZYXM, LD, AT, XW, GCM, CV, LZ, SMM, , KD, AC, AO, YZ, GB, KLR, EDH, WWLC, JHK, JH, MP, AJMF, BMP, MBM, MAP, JNW, S.H., LJM, KHY; Collection and assembly of data under approved IRB and MTA: ZYXM, AT, CV, SMM, , KD, AC, AO, YZ, GB, KLR, EDH, WWLC, JHK, JH, MP, AJMF, BMP, MBM, MAP, JNW, KHY; Data

analysis and interpretation: QY, ZYXM, LD, XW, SH, KHY; Manuscript writing: YQ, ZYXM, LD, XW, LJM, SH, KHY; Final approval of manuscript: All authors.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

EDITORIAL NOTE

This paper has been accepted based in part on peer-review conducted by another journal and the authors' response and revisions as well as expedited peer-review in *Oncotarget*.

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