Clinicopathological and genetic features of limited-stage diffuse large B-cell lymphoma with late relapse: targeted sequencing analysis of gene alterations in the initial and late relapsed tumors

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ONLINE SUPPLEMENTARY MATERIALS

SUPPLEMENTARY METHODS

■Patients

This study evaluated 334 consecutive patients with Ann Arbor stage I/II diffuse large B-cell lymphoma (DLBCL) who were treated using CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisolone) with or without rituximab between 1997 and 2012 at the National Cancer Center Hospital (NCCH). Patients who had an initial diagnosis of transformed indolent B-cell lymphoma were excluded. All patients underwent staging at initial diagnosis via routine computed tomography (CT) and histological evaluation of bone marrow (BM). Of the patients who underwent positron emission tomography (PET)-CT, some were excluded if their PET-CT results caused upstaging to Ann Arbor stage III/IV.

Late relapse (LR) was defined as the first relapse of any B-cell lymphoma (including indolent B-cell lymphoma) occurring at >5 years after the initial diagnosis in patients who achieved complete response to the initial therapy. The response to the initial therapy was generally assessed using the International Working Group response criteria.[1] The revised response criteria[2] were used for patients with available PET-CT data to evaluate the treatment response. Survival curves were generated, and clinical characteristics were examined for 16 patients who experienced late relapse. The immunohistochemical and genetic analysis also included 3 patients with limited-stage DLBCL who were initially treated at another hospital and were referred to the NCCH after late relapse. All three patients (#5, #7, and #12) had sufficient clinical information and tumor specimens from initial diagnosis.

■Statistical analysis

The Kaplan-Meier method was used to evaluate survival outcomes. Progression-free survival was determined as

the time from the initial diagnosis to disease progression or death from any cause.

■Immunohistochemical and fluorescence in situ hybridization (FISH) analysis

Tumor specimens were subjected to immunostaining for MYC, BCL2, CD10, BCL6, and MUM1 and double staining for PAX5 and programmed death-ligand 1 (PD-L1). The cell of origin was determined according to algorithm developed by Hans et al.[3], and the cut-off values are listed in Supplementary Table S3. FISH was performed using formalin-fixed paraffin-embedded (FFPE) sections and probes mapping to 9p24.1 and centromere-9 for specimens with PD-L1 tumor expression.[4] *MYC* rearrangements were evaluated in available FFPE samples using the Vysis LSI MYC dual-color break-apart rearrangement probe (5J9101; Abbott Molecular, Abbott Park, IL).

■Genetic analyses

DNA extraction

For each patient, genomic DNA (gDNA) was extracted from (i) the initial tumor, (ii) relapse tumor, and (iii) bone marrow (BM) as a control in cases with pathologically confirmed non-tumor invasion. The gDNA was mainly extracted from formalin-fixed paraffin-embedded (FFPE) sections using a Genomic DNA Extraction Kit (Qiagen, Hilden, Germany). However, control gDNA samples for patients #2, #4, #5, #6, #8, and #12, and the recurrent tumor samples for patient #4, were extracted from fresh frozen specimens using a DNeasy Blood & Tissue Kit (Qiagen). Seven of the 19 patients (#13–19) were excluded from further genetic analyses because of a lack of paired tumor specimens for gDNA extraction, or poor quality of the extracted gDNA. Patient #10, whose gDNA samples at initial diagnosis and LR were successfully extracted, was included in genetic analyses, despite no control gDNA being available from a BM specimen.

Immunoglobulin heavy chain (IgH)-clonality analysis

The gDNA samples from the initial and relapsed tumor specimens were analyzed as previously described[5] using

the BIOMED-2 multiplex PCR assay (*Invivo*scribe Technologies, San Diego, CA, USA), which included three reactions targeting IgH (tube C: FR3-J, tube D: $D_{1\text{-}6}$-J, and tube E, D_7-J). As the gDNA samples extracted from the FFPE specimens were predicted to be fragmented, we did not perform PCR assays using a master-mix of tube A and B, whose valid product size is >250 base pairs[5]. All reactions were performed in duplicate using positive controls (clonal), negative controls (polyclonal), and blank controls (H_2O). Gene scanning was performed with an ABI-Prism 3100 Sequencer (ThermoFisher Scientific, Massachusetts, USA) and Gene Scan Analysis Software (version 3.1, ThermoFisher Scientific). If at least one clear peak with an identical base-pair length was detected in the duplicate reactions, the peak was judged to originate from a clonal IgH rearrangement. When reproducible peaks were not obtained from the duplicate reactions, the sample's IgH rearrangement status was judged as unevaluable. If any clonal peaks with identical base-pair lengths were detected in the paired tumor samples using the same master-mix tube, they were judged as clonally related. If clonal peaks of apparently different base-pair lengths were detected, the paired tumors were judged as clonally unrelated. The results were judged as "not determined" in all other cases.

Targeted sequencing and mutation call

The ratio of PCR-amplifiable DNA to total dsDNA, as a value indicating the DNA quality (named as Q-value, cut-off value: 0.1)[6], was evaluated for gDNA samples of the 12 patients, although they were analyzed for targeted sequencing regardless of the Q-value. The gDNA samples extracted from the FFPE specimens were pre-treated using uracil DNA glycosylase (ThermoFisher Scientific). Then, two independent multiplex PCRs were performed to amplify 2,709 regions, covering all exons in 64 lymphoma-related genes designed by the Ion AmpliSeq Custom Panel (Thermo Fisher Scientific) (Table S4), using the Ion AmpliSeq Library Kit 2.0 (ThermoFisher Scientific). The 64 genes were selected because mutations in these genes were reported to be frequently found in lymphomas, including DLBCL, at the time when the panel was designed. The amplicon size was

157 base pairs (bp) on average (range: 124-174 bp). The two PCR-amplified products were mixed in a 1:1 volume ratio and labeled with unique barcodes using the Ion Xpress Barcode Adaptors 1-96 Kit (Thermo Fisher Scientific). Emulsion PCR was performed using an Ion Chef instrument (Thermo Fisher Scientific), and amplicon resequencing was performed using an Ion Proton sequencer (Thermo Fisher Scientific) as previously described.[7] Primer sequences were removed from the obtained sequences, and the trimmed sequences were aligned to the human reference genome hg19 using Torrent Suite (Thermo Fisher Scientific). Mutation calls were made using the CLC Genomics Workbench (CLC bio, Aarhus, Denmark) according to the criteria described in Table S5. Each identified mutation was screened via manual inspection of the alignment data.

Mutation calls in a patient without control gDNA

Patient #10 did not have available control gDNA, and shared/non-shared mutations in the paired tumor samples were identified by direct comparison. Special care was taken to avoid selecting single nucleotide polymorphisms as functional shared mutations, and there were no shared mutations between the paired tumor samples of patient #10.

Evaluation for shared and non-shared mutation

After excluding one patient (#9) due to the poor specimen sequence quality obtained at initial diagnosis, genetic mutations between the samples obtained at initial diagnosis and LR in each patient were compared. If any mutation of the same basal changes was detected in the same basal position between the pair, the mutation was defined as a shared mutation. If any mutation was detected in either of the pair, the mutation was defined as a non-shared mutation. The target lesions should be sufficiently covered, especially for evaluating non-shared mutations in order not to misinterpret the shared mutation as the non-shared mutation. Therefore, samples with coverage uniformity of < 70% were excluded from non-shared mutation analysis.[8] All samples with Q-value below 0.1 had coverage uniformity of <70%. Mutations that could not be categorized as either shared or non-shared were defined as “undetermined."

Table S1. Baseline characteristics of patients with limited-stage DLBCL

	Overall n = 334	Late relapsed DLBCL n = 16
Median age, years (range)	61 (20-85)	63 (32-77)
Gender, male : female, n	177 : 157	9 : 7
Ann arbor stage, n (%)		
I	180 (54)	11 (69)
II	154 (46)	5 (31)
LDH > ULN, n (%)	107 (32)	6 (38)
Extranodal disase ≥1, n (%)	184 (55)	9 (56)
IPI score, n (%)		
0 or 1	259 (78)	14 (88)
≥2	75 (22)	2 (12)
Initial treatment, n (%)		
CHOP (3~4 cycles) with RT	147 (44)	9 (56)
CHOP (6~8 cycles) with / without RT	172 (52)	7 (44)
Rituximab administration, n (%)	205 (61)	6 (38)

DLBCL, diffuse large B-cell lymphoma; LDH, lactate dehydrogenase; ULN, upper limit of normal; IPI, International Prognostic Index, CHOP, (cyclophosphamide,doxorubicin, vincristine and prednisolone); RT, radiation therapy

Table S2. Detailed clinical charasteristics and outcomes of 19 patients who developed late relapsed DLBCL

		At initial diagnosis							At late relapse					
Patient	Gender	Age	Lymph node involvement	Involved extranodal sites	Stage	IPI	Initial therapy	Interval, year	Histological diagnosis	Lymph node involvement	Involved extranodal sites	Stage	IPI	Outcome
#1	F	42	-	Paranasal cavity	1	0	3xCHOP, RT	6	DLBCL	+	Paranasal cavity	4	1	Alive
#2	M	70	+	Gingiva	2	1	8xR+3xCHOP, RT	8	DLBCL	+	Lower limb, mandible	4	3	Died of DLBCL
#3	M	61	-	Paranasal cavity	1	1	8xR+6xCHOP, IT, RT	9	DLBCL	+	Liver, bone marrow	4	5	Alive
#4	M	43	-	Testis	1	0	8xR-CHOP, IT, RT	12	DLBCL	+	Liver, testis, stomach, bone	4	4	Died of DLBCL
#5	M	38	+	Testis	2	0	6xCHOP	18	DLBCL	-	Central nervous system	1	1	Alive
#6¶	M	32	-	Testis	1	0	8xCHOP, IT	13	DLBCL	-	Testis (another side)	1	0	Lost follw up
#7	M	58	-	Gingiva	1	0	7xR+3xCHOP, RT	7	DLBCL	+	Testis, skin, adrenal gland	4	4	Alive
#8	M	60	+	Pleura	2	1	8xCHOP, RT	13	DLBCL	-	Bone, diaphragm	4	3	Alive
#9	F	65	+	Stomach	2	1	3xCHOP, RT	9	DLBCL	+	Intestin	4	3	Alive
#10	F	77	+	-	1	2	6xR-CHOP, RT	7	DLBCL	-	Subcutaneous (multiple)	4	3	Died of DLBCL
#11	M	72	+	-	1	2	2xR+6xCHOP, RT	7	DLBCL	-	Colon, skin, diaphragm	4	5	Died of DLBCL
#12	F	59	+	-	1	0	3xCHOP, RT	14	MALT	-	Subcutaneous (solitary)	1	2	Alive
#13	F	68	-	Nasal cavity	1	1	4xCHOP, RT	7	DLBCL	+	Stomach	4	2	Lost follw up
#14	F	70	-	Gingiva	1	1	3xCHOP, RT	5	DLBCL	-	Subcutaneous (solitary)	1	1	Lost follw up
#15	F	73	+	-	2	1	3xR-CHOP, RT	5	DLBCL	-	Subcutaneous (solitary) Nasal cavity	4	4	Lost follw up
#16	M	37	+	-	1	1	8xCHOP, RT	10	DLBCL	+	Bone marrow	4	2	Died of DLBCL
#17	M	66	+	-	I	1	3xCHOP, RT	5	DLBCL	-	Cecum	1	1	Lost follw up
#18	M	59	+	-	1	0	3xCHOP, RT	16	DLBCL+FL	+	-	3	3	Alive
#19	F	41	+	-	2	0	8xCHOP, RT	6	FL grade3a	+	-	2	1	Died of AE

DLBCL, diffuse large B-cell lymphoma; Stage, Ann Arbor clinical stage; IPI, international prognostic index; F, female; M, male; CHOP, (cyclophosphamide, doxorubicin, vincristine, prednisolone); R, rituximab; RT, radiation therapy; IT, intrathecal therapy; ¶ Radiation therapy on contralateral uninvolved testis was not performed as an initial therapy.

Table S3. Antibodies and their cut-off values

Antibody	Clone	Product by	Cut-off value (%)
MYC	Y69	Abcam, Cambridge, United Kingdom	40[9]
BCL2	124	Dako, Glostrup, Denmark	50[9]
CD10	56C6	Leica biosystems, Nussloch, Eisfeld, Germany	20[10]
BCL6	PG.B6p	Agilent, Santa Clara, US	20[10]
MUM1	MUM1p	Agilent, Santa Clara, US	20[10]
PAX5	24/Pax-5	Becton, Dickinson, New Jersey, US	-
PD-L1	E1L3N	Cell Signaling Technology, Massachusetts, US	30[11]

Table S4. Genes included into the custom panel for target sequencing

ARID1A	CARD11	FBXO11	KIT	NOTCH2	PTEN	TNFRSF14
ARID2	CD58	FLT3	KMT2D	NRAS	RHOA	TP53
ASXL1	CD79A	FOXO1	KRAS	P2RY8	S1PR2	TRAF2
ATM	CD79B	GNA13	LYN	PBRM1	SMARCA2	TRAF3
B2M	CDKN2A	ID3	MALT1	PIK3CA	SMARCA4	
BCL10	CIITA	IDH1	MAP3K14	PIK3CD	SMARCB1	
BIRC3	CREBBP	IDH2	MEF2B	PIK3R1	SOCS1	
BLIMP1	DNMT3A	IKBKB	MLL3	PIM1	SYK	
BRAF	EP300	IRAK1	MYD88	PLCG1	TET2	
BTK	EZH2	KDM6A	NOTCH1	PLCG2	TNFAIP3	

Table S5. The criteria for calling significant mutational variants

Mutation type	For sample with tumor content of > 50%	For sample with tumor content of ≤ 50%
SNV, MNV, replacement	The frequency of the sample from which is subtracted the frequency of the control ≥10%	The frequency of the sample from which is subtracted the frequency of the control ≥ 5%
Insertion, deletion	The frequency of the sample from which is subtracted the frequency of the control ≥20%	The frequency of the sample from which is subtracted the frequency of the control ≥ 10%
All variants	Quality score ≥20 Both forward and reverse reads ≥10 Coverage count ≥100 Exonic non-synonymous Frequency ≤ 5% in control	

SNV, single nucleotide variation; MNV, multiple nucleotide variation

Table S6. The pathological characteristics of 19 patients who developed late relapsed DLBCL

Patient	Timing	Histology	CD10	BCL6	MUM1	COO	PD-L1 on tumor cell	BCL2	MYC	*MYC*-R*
#1	Initial	DLBCL	-	+	+	non-GCB	-	+	-	-
	Relapse	DLBCL	-	-	+	non-GCB	-	+	+	-
#2	Initial	DLBCL	-	-	-	non-GCB	-	-	-	-
	Relapse	DLBCL	-	+	+	non-GCB	-	+	+	-
#3	Initial	DLBCL	-	NA	+	non-GCB	-	NA	NA	NA
	Relapse	DLBCL	-	+	+	non-GCB	-	NA	NA	NA
#4	Initial	DLBCL	-	-	+	non-GCB	-	-	-	-
	Relapse	DLBCL	-	+	+	non-GCB	-	-	+	+
#5	Initial	DLBCL	-	+	+	non-GCB	-	+	-	-
	Relapse	DLBCL	-	+	+	non-GCB	-	+	-	ND
#6	Initial	DLBCL	-	-	+	non-GCB	-	+	+	-
	Relapse	DLBCL	-	-	-	non-GCB	-	+	-	-
#7	Initial	DLBCL	-	-	+	non-GCB	-	+	+	-
	Relapse	DLBCL	-	+	+	non-GCB	-	+	+	-
#8	Initial	DLBCL	-	+	-	GCB	-	+	+	ND
	Relapse	DLBCL	+	+	+	GCB	+	+	-	-
#9	Initial	DLBCL	-	-	-	non-GCB	-	-	-	-
	Relapse	DLBCL	+	+	-	GCB	-	-	+	ND
#10	Initial	DLBCL	-	+	+	non-GCB	-	NA	NA	NA
	Relapse	DLBCL	-	-	+	non-GCB	-	NA	NA	NA
#11	Initial	DLBCL	-	-	+	non-GCB	-	+	+	-
	Relapse	DLBCL	-	+	+	non-GCB	-	-	-	ND
#12	Initial	DLBCL	-	-	+	non-GCB	-	-	NA	NA
	Relapse	MALT	NA	NA	NA	NA	-	+	NA	NA
#13	Initial	DLBCL	-	-	+	non-GCB	-	+	+	ND
	Relapse	DLBCL	-	+	-	GCB	-	NA	-	NA
#14	Initial	DLBCL	+	+	+	GCB	-	-	NA	NA
	Relapse	DLBCL	-	-	-	non-GCB	-	+	NA	NA
#15	Initial	DLBCL	-	-	+	non-GCB	-	+	+	-
	Relapse	DLBCL	-	-	+	non-GCB	-	+	-	-
#16	Initial	DLBCL	-	NA	NA	NA	-	NA	NA	NA
	Relapse	DLBCL	-	-	+	non-GCB	+	+	NA	NA
#17	Initial	DLBCL	-	-	-	non-GCB	-	-	-	-
	Relapse	DLBCL	+	+	-	GCB	-	-	-	NA
#18	Initial	DLBCL	-	+	-	GCB	-	-	-	-
	Relapse	DLBCL+FL	+	+	-	GCB	-	-	-	-
#19	Initial	DLBCL	-	+	-	GCB	-	+	NA	NA
	Relapse	FL grade3a	+	NA	NA	NA	-	+	NA	NA

DLBCL, diffuse large B-cell lymphoma; COO, cell of origin determined by Hans algorithm; PD-L1, programmed death-ligand 1; GCB, germinal center B-cell-like; NA, not assessed; ND, not determined; MALT, mucosa associated lymphoid tissue lymphoma; FL, follicular lymphoma

*FISH analysis for *MYC* break-apart rearrangements

Table S7. The summary of the results of IgH-clonality analysis

Patient	Timing	Histology	Amplicon size of clonal peak detected in duplicates			Clonal relationship
			FR3-JH (tube C) valid range,100-170	DH-JH (tube D) valid range,110-290, 390-420	DH-JH (tube E) valid range,100-130	
#1	Initial	DLBCL	-	**148**	-	Related
	Relapse	DLBCL	-	**148**	113	
#2	Initial	DLBCL	-	-	**115**	Related
	Relapse	DLBCL	-	-	**115**	
#3	Initial	DLBCL	-	128, **239**	-	Related
	Relapse	DLBCL	-	**239**	-	
#4	Initial	DLBCL	-	**174**	-	Related
	Relapse	DLBCL	-	**174**	-	
#5	Initial	DLBCL	**117**	-	-	Related
	Relapse	DLBCL	**117**	-	-	
#6	Initial	DLBCL	**111**	-	-	Related
	Relapse	DLBCL	**111**	-	-	
#7	Initial	DLBCL	-	-	-	Not determined
	Relapse	DLBCL	-	-	-	
#8	Initial	DLBCL	-	-	106	Unreralted
	Relapse	DLBCL	-	204	116	
#9	Initial	DLBCL	-	-	-	Not determined
	Relapse	DLBCL	-	143	-	
#10	Initial	DLBCL	136	-	-	Unreralted
	Relapse	DLBCL	117	132	-	
#11	Initial	DLBCL	-	-	-	Not determined
	Relapse	DLBCL	111	129	-	
#12	Initial	DLBCL	**110**	-	-	Related
	Relapse	MALT	**110**	-	-	

IgH, immunoglobulin heavy chain; DLBCL, diffuse large B-cell lymphoma; MALT, mucosa associated lymphoid tissue lymphoma

Table S8. The overview of the data associated with target sequencing

Patient	Tumor parcentage in the FFPE sample (%)	Q-value[6]	Mean coverage	coverage uniformity (%)	Number of variants called by our criteria*	Evaluation for gene mutation
#1	80	0.32	1207	91	2	Shared/non-shared
	80	1.57	1564	92	4	
#2	70	0.22	1205	88	3	Shared/non-shared
	70	0.58	1524	92	6	
#3	80	0.34	837	87	8	Shared/non-shared
	20	0.9	1242	92	2	
#4	80	0.29	1269	88	7	Shared/non-shared
	70	0.67	1448	87	11	
#5	80	0.009	682	**63**	12	Shared
	10	0.33	1252	82	4	
#6	70	0.17	1198	88	6	Shared/non-shared
	50	0.42	1293	92	8	
#7	80	0.21	1057	89	3	Shared
	90	0.4	1950	**46**	2	
#8	70	0.034	834	**38**	10	Shared
	50	0.53	1828	91	8	
#9	80	0.13	**62**	NA	2	Excluded for the mutational
	70	0.84	1417	92	13	
#10	70	0.39	1310	92	0	Shared/non-shared
	50	2.47	1377	93	2	
#11	80	0.42	821	85	6	Shared
	80	0.043	302	**40**	68	
#12	80	0.014	557	**39**	48	Shared
	60	0.36	1308	88	0	

*Before screened via manual inspection of the alignment data

Q-value, The ratio of PCR-amplifiable DNA to total dsDNA as a value indicating the DNA quality

Table S9. Mutations detected in the paired tumor samples of 12 patients who developed late relapsed DLBCL

Paitnes who were eligible for shared and non-shared mutation analyses

Patient	Shared or non-shared	Timimg	Gene	Chromosome	Region	Mutation type	Reference	Allele	Variant count	Coverage	Variant frequency	Average quality	Coding region change	Amino acid change
#1	Shared	Initial	MYD88	chr3	38182641	SNV	T	C	642	2023	31.7	25.2	c.794T>C	p.L265P
	Non-shared		KMT2D	chr12	49438647	SNV	G	A	228	843	27	25.6	c.4843C>T	p.Arg1615*
	Shared	Relapse	MYD88	chr3	38182641	SNV	T	C	1097	2548	43	25.1	c.794T>C	p.L265P
	Non-shared		PIM1	chr6	37138600	SNV	G	A	863	1136	75.9	24.9	c.134G>A	p.Gly45Asp
	Non-shared		PIM1	chr6	37139033	SNV	C	T	603	1376	43.8	25.5	c.373C>T	p.Pro125Ser
	Non-shared		PIM1	chr6	37139145..37139146	MNV	GG	AA	524	2189	23.9	25.3	c.485_486delGGinsAA	p.Gly162Glu
#2	Shared	Initial	TNFRSF14	chr1	2494306	SNV	G	A	196	862	22.7	23.5	c.697G>A	p.Asp233Asn
	Shared		CD79B	chr17	62006798	SNV	T	G	293	1062	27.5	24.9	c.587A>C	p.Tyr196Ser
	Shared	Relapse	TNFRSF14	chr1	2494306	SNV	G	A	357	987	36.1	24.6	c.697G>A	p.Asp233Asn
	Shared		CD79B	chr17	62006798	SNV	T	G	524	1277	41	24.9	c.587A>C	p.Y196S
	Non-shared		NOTCH2	chr1	120458147	SNV	G	A	929	2272	40.8	27.4	c.7198C>T	p.Arg2400*
	Non-shared		TNFAIP3	chr6	138198325^138198326	Insertion	-	T	627	1015	61.7	24	c.918_919insT	p.Leu307fs
	Non-shared		B2M	chr15	45007814	SNV	C	G	1154	1547	74.5	26.6	c.261C>G	p.Tyr87*
#3	Shared	Initial	MYD88	chr3	38182641	SNV	T	C	211	1046	20.1	24.4	c.794T>C	p.L265P
	Shared		CD79B	chr17	62006799	SNV	A	G	196	609	32.1	23.1	c.586T>C	p.Y196H
	Non-shared		PIM1	chr6	37139021	SNV	G	C	80	537	14.8	25.7	c.361G>C	p.Glu121Gln
	Non-shared		PIM1	chr6	37139039	SNV	C	T	82	537	15.2	25.2	c.379C>T	p.Q127*
	Non-shared		PIM1	chr6	37139204	SNV	C	T	593	3534	16.7	25.1	c.544C>T	p.Leu182Phe
	Shared	Relapse	MYD88	chr3	38182641	SNV	T	C	69	1300	5.3	25.9	c.794T>C	p.L265P
	Shared		CD79B	chr17	62006799	SNV	A	G	64	1061	6	23.1	c.586T>C	p.Y196H
#4	Shared	Initial	MYD88	chr3	38182641	SNV	T	C	783	1732	45.2	25.7	c.794T>C	p.L265P
	Shared		CD79B	chr17	62006799	SNV	A	T	482	1191	40.4	24.6	c.586T>A	p.Y196N
	Non-shared		CD58	chr1	117078610	SNV	A	T	159	497	31.9	25.7	c.605T>A	p.Leu202*
	Non-shared		P2RY8	chrX	1585264	SNV	A	C	691	1046	66	24.2	c.188T>G	p.Ile63Ser
	Shared	Relapse	MYD88	chr3	38182641	SNV	T	C	930	2472	37.6	25.5	c.794T>C	p.L265P
	Shared		CD79B	chr17	62006799	SNV	A	T	717	1891	37.9	24.3	c.586T>A	p.Y196N
	Non-shared		CD58	chr1	117087019..117087022	Deletion	TAGA	-	343	457	75	26.8	c.275_278delTCTA	p.Ile92fs
	Non-shared		PIM1	chr6	37138642	SNV	C	G	650	2966	21.9	25.9	c.176C>G	p.Ser59Cys
	Non-shared		PIM1	chr6	37138804	SNV	G	C	734	1167	62.8	23.8	c.237G>C	p.E79D
	Non-shared		PIM1	chr6	37138962	SNV	C	T	278	1290	21.5	24.5	c.302C>T	p.Ser101Phe
	Non-shared		PIM1	chr6	37139072	SNV	G	A	809	1881	43	23.7	c.412G>A	p.Ala138Thr
	Non-shared		PIM1	chr6	37139111	SNV	G	A	783	1547	50.6	26.8	c.451G>A	p.Val151Met
	Non-shared		PIM1	chr6	37139150	SNV	C	G	193	1543	12.5	24	c.490C>G	p.Leu164Val
	Non-shared		PIM1	chr6	37139210	SNV	C	G	2049	5212	39.3	25.8	c.550C>G	p.Leu184Val
	Non-shared		CD79B	chr17	62007715	SNV	G	A	927	2527	36.6	26.8	c.149C>T	p.Pro50Leu
#6	Shared	Initial	MYD88	chr3	38182641	SNV	T	C	496	1456	34	25.4	c.794T>C	p.L265P
	Shared		PIM1	chr6	37138609	SNV	G	T	163	357	45.6	25.8	c.143G>T	p.Gly48Val
	Shared		PIM1	chr6	37139167	SNV	G	C	1292	2847	45.3	26.7	c.507G>C	p.Lys169Asn
	Shared		PRDM1	chr6	106536324	SNV	G	C	1250	2208	56.6	27.4	c.183G>C	p.E61fs
	Shared		CD79B	chr17	62006680	SNV	A	G	639	1642	38.9	23.5	c.596T>C	p.Leu199Pro
	Shared	Relapse	MYD88	chr3	38182641	SNV	T	C	97	1863	5.2	25.3	c.794T>C	p.L265P
	Shared		PIM1	chr6	37138609	SNV	G	T	58	888	6.5	26	c.143G>T	p.Gly48Val
	Shared		PIM1	chr6	37139167	SNV	G	C	341	4326	7.8	26.1	c.507G>C	p.Lys169Asn
	Shared		PRDM1	chr6	106536324	SNV	G	C	151	2298	6.5	26.9	c.183G>C	p.E61fs
	Shared		CD79B	chr17	62006680	SNV	A	G	241	2262	10.6	24.3	c.596T>C	p.Leu199Pro
	Non-shared		PIM1	chr6	37139083	SNV	G	T	173	1940	8.9	27.3	c.423G>T	p.Glu141Asp
	Non-shared		CIITA	chr16	11000605	SNV	T	C	70	1220	5.7	22.5	c.1256T>C	p.Leu419Pro
	Non-shared		CIITA	chr16	11001691	SNV	C	A	85	1423	5.9	26.6	c.2342C>A	p.Ser781*
#10		Initial	No mutation was identified.								0			
	Non-shared	Relapse	MYD88	chr3	38182641	SNV	T	C	634	1959	32.3	25.4	c.794T>C	p.L265P
	Non-shared		KMT2D	chr12	49427282	SNV	G	A	1028	3097	33.1	26.3	c.11206C>T	p.Gln3736*

Patients who were eligible for only shared mutation analysis

Patient	Shared or Undetermined ¶	Timimg	Gene	Chromosome	Region	Mutation type	Reference	Allele	Variant count	Coverage	Variant frequency	Average quality	Coding region change	Amino acid change
#5	Shared	Initial*	CD79B	chr17	62006680	SNV	A	G	526	2077	25.3	24.3	c.596T>C	p.Leu199Pro
	Shared		CD79B	chr17	62006680	SNV	A	G	416	2602	15.9	23.8	c.596T>C	p.Leu199Pro
	Undetermined	Relapse	MYD88	chr3	38182641	SNV	T	C	151	1861	8.1	25.1	c.794T>C	p.L265P
	Undetermined		KMT2D	chr12	49440205	SNV	C	T	131	1645	7.9	27.1	c.4421G>A	p.Cys1474Tyr
	Undetermined		SOCS1	chr16	11348738	SNV	G	C	179	2112	8.4	25.9	c.598C>G	p.Leu200Val
#7	Shared	Initial	CD79B	chr17	62006799	SNV	A	G	266	1068	24.9	23.5	c.586T>C	p.Y196H
	Shared		CD79B	chr17	62009555	SNV	C	G	333	1138	29.2	27.7	c.67G>C	p.Ala23Pro
	Undetermined		MYD88	chr3	38182641	SNV	T	C	371	1543	24	25.3	c.794T>C	p.L265P
	Shared	Relapse*	CD79B	chr17	62006799	SNV	A	G	1390	3211	43.2	23.4	c.586T>C	p.Y196H
	Shared		CD79B	chr17	62009555	SNV	C	G	2077	4757	43.6	27.6	c.67G>C	p.Ala23Pro
#8	Shared	Initial*	PIM1	chr6	37138946	SNV	G	C	415	2201	18.8	27.4	c.286G>C	p.Val96Leu
	Shared		KMT2D	chr12	49447389	SNV	C	A	1443	5593	25.8	26.6	c.709G>T	p.Glu237*
	Shared		CREBBP	chr16	3781324..3781326	Deletion	AGG	-	1656	6944	23.8	21.9	c.5039_5041delCCT	p.S1680delS
	Shared		MEF2B	chr19	19261544	SNV	T	C	51	164	31	24.3	c.1A>G	p.Met1Val
	Shared	Relapse	PIM1	chr6	37138946	SNV	G	C	1077	2270	47.4	27.5	c.286G>C	p.Val96Leu
	Shared		KMT2D	chr12	49447389	SNV	C	A	415	1322	31.3	26.4	c.709G>T	p.Glu237*
	Shared		CREBBP	chr16	3781324..3781326	Deletion	AGG	-	318	1976	16	22.3	c.5039_5041delCCT	p.S1680delS
	Shared		MEF2B	chr19	19261544	SNV	T	C	152	928	16.3	23.3	c.1A>G	p.Met1Val
	Undetermined		EZH2	chr7	148508727	SNV	T	A	417	2543	16.3	24.9	c.1937A>T	p.Y646F
	Undetermined		TNFRSF14	chr1	2491314	SNV	T	A	1073	2242	47.8	26.3	c.357T>A	p.Cys119*
	Undetermined		SOCS1	chr16	11349034	SNV	A	G	279	1726	16.1	22.8	c.302T>C	p.Phe101Ser
	Undetermined		GNA13	chr17	63014374	SNV	T	A	147	1002	14.6	27.6	c.558A>T	p.Glu186Asp
#9		Initial	The sample was excluded from the mutational analysis because of extremely poor sequence quality.											
	Undetermined	Relapse	NOTCH2	chr1	120484314	SNV	G	A	457	917	49.8	26.9	c.2816C>T	p.Pro939Leu
	Undetermined		CARD11	chr7	2977603	SNV	A	T	902	2415	37.3	24.2	c.1081T>C	p.Y361H
	Undetermined		BRAF	chr7	140453153	SNV	A	T	230	1126	20.4	24.3	c.1782T>A	p.D594E
	Undetermined		FOXO1	chr13	41134274	SNV	T	A	615	3288	18.7	26.2	c.1354A>T	p.Met452Leu
	Undetermined		FOXO1	chr13	41134355	SNV	T	C	376	1978	19	24.9	c.1273A>G	p.Thr425Ala
	Undetermined		TRAF3	chr14	103369593	SNV	G	A	1030	2991	34.4	24.8	c.962G>A	p.Arg321Gln
	Undetermined		SOCS1	chr16	11348807	SNV	G	C	195	1133	17.2	23.2	c.529C>G	p.Leu177Val
	Undetermined		SOCS1	chr16	11348852	SNV	G	C	204	1032	19.7	21.2	:c.484C>G	p.Leu162Val
	Undetermined		S1PR2	chr19	10335268	SNV	C	G	279	1212	23	26.5	c.314G>C	p.Trp105Ser
	Undetermined		BTK	chrX	100611068	SNV	T	A	464	3070	15.1	26.7	c.1538A>T	p.Glu513Val
#11	Shared	Initial	MYD88	chr3	38182641	SNV	T	C	806	1093	73.7	25.7	c.794T>C	p.L265P
	Shared		CD79B	chr17	62006662	SNV	G	A	569	1469	38.7	26.8	c.614C>T	p.Ala205Val
	Shared		CD79B	chr17	62006795	SNV	T	C	270	695	38.8	27.1	c.590A>G	p.Glu197Gly
	Shared		CD79B	chr17	62006798	SNV	T	C	251	694	36.1	22.1	c.587A>G	p.Y196C
	Undetermined		FOXO1	chr13	41134261	SNV	T	A	651	1785	36.4	27.3	c.1367A>T	p.Asn456Ile
	Shared	Relapse*	MYD88	chr3	38182641	SNV	T	C	329	344	95.5	25.4	c.794T>C	p.L265P
	Shared		CD79B	chr17	62006662	SNV	G	A	226	675	33	26.9	c.614C>T	p.Ala205Val
	Shared		CD79B	chr17	62006795	SNV	T	C	136	424	32	26.7	c.590A>G	p.Glu197Gly
	Shared		CD79B	chr17	62006798	SNV	T	C	187	424	44.1	21.9	c.587A>G	p.Y196C
#12		Initial*												
		Relapse	No mutation was identified.											

DLBCL; diffuse large B-cell lymphoma, SNV; single nucleotide variation, MNV; multiple nucleotide variation

Undetermined¶: Mutations cannot be determined as shared or non-shared mutations because the coverage uniformity of the other pair sample was <70%.

* Non-shared muations were not evaluated because coverage uniformity of the sample was <70%.

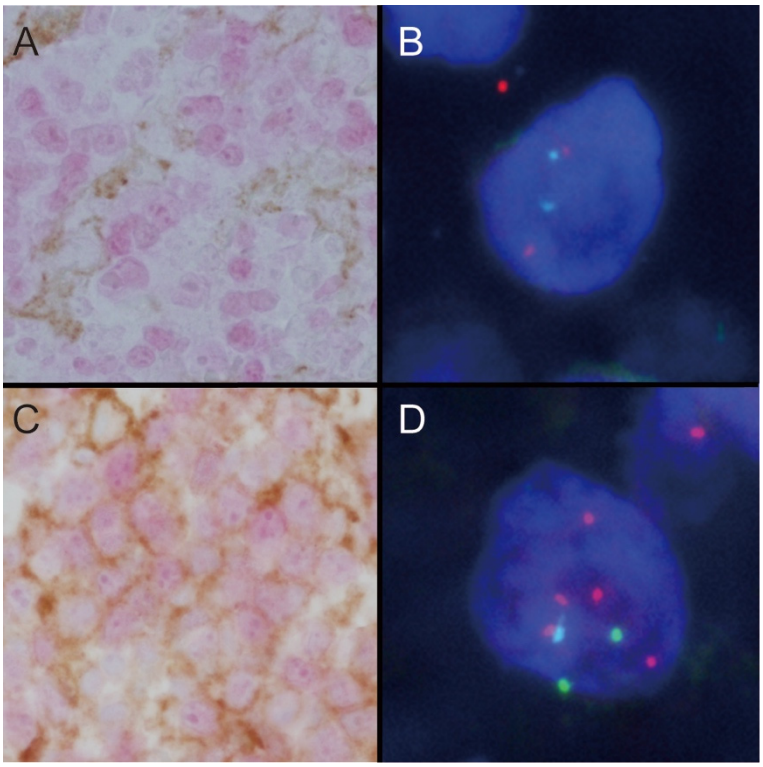

Figure S1. Double staining for PAX5 and PD-L1, with FISH analysis of *PD-L1*, which were performed using tumor specimens from patient #8

(A) The tumor specimen from the initial diagnosis was positive for PAX5 (pale red) but negative for PD-L1, although some of the non-tumor cells (negative for PAX5) were positive for PD-L1 (brown). (B) FISH analysis using an FFPE specimen from the initial diagnosis; red signals indicate *PD-L1* gene probes, and green signals indicate centromere-9 gene probes, which failed to reveal gain of the *PD-L1* gene. (C) The specimen from the late relapse was positive for PAX5 and PD-L1. (D) The FISH analysis using an FFPE specimen from the late relapse revealed five red signals and three green signals, which suggested a gain of function of the *PD-L1* gene.

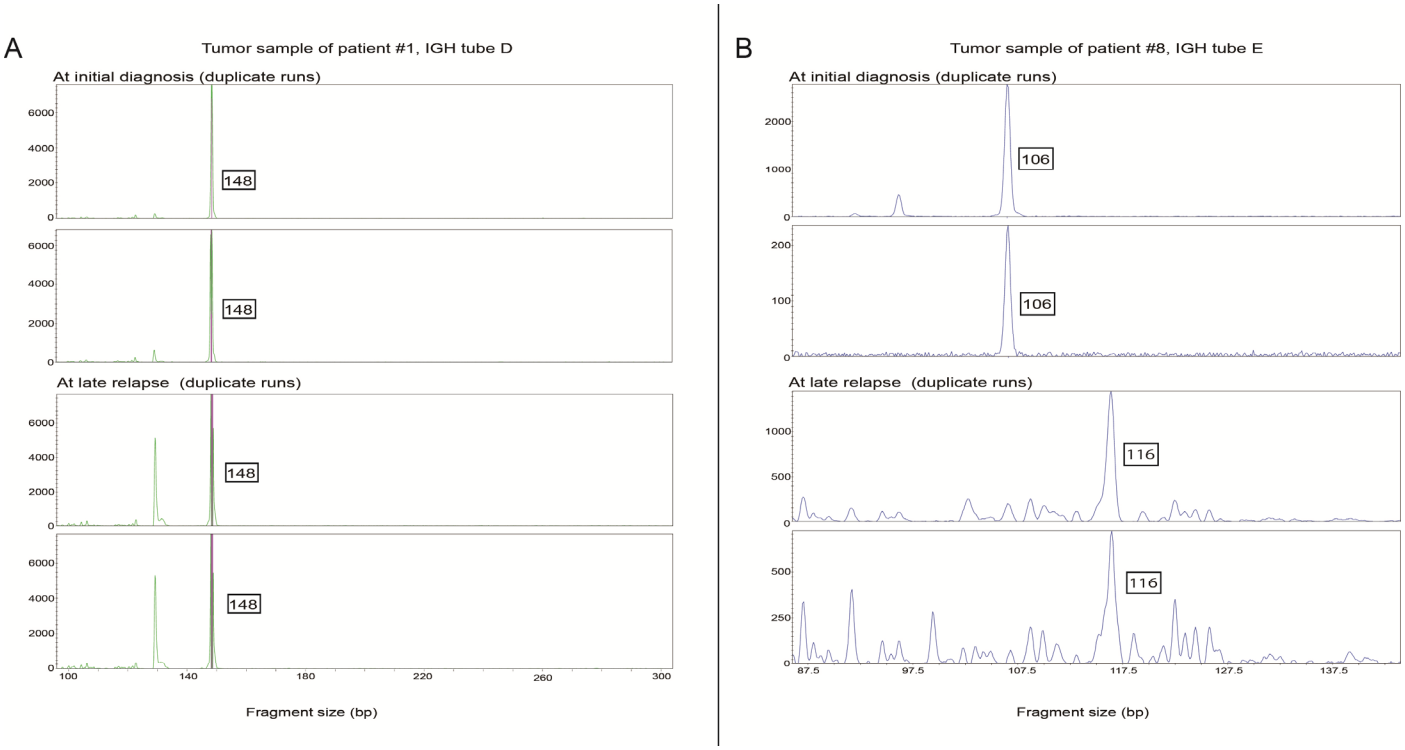

Figure S2. Representative results from the IgH-clonality analysis.

(A) The results for samples from patient #1, which were obtained using a master mix tube D. The clonal peak of 148 bp was identified in the initial and relapsed tumor samples, which indicated that the pair were clonally related.

(B) The results for samples from patient #8, which were obtained using a master mix tube E. The different clonal peaks in the paired samples (106 bp in the initial tumor sample and 116 bp in the relapsed tumor sample) indicated that the pair were clonally unrelated.

■REFERENCES

1. Cheson BD, Horning SJ, Coiffier B et al. Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas. NCI Sponsored International Working Group. J Clin Oncol. 1999;17(4):1244.
2. Cheson BD, Pfistner B, Juweid ME et al. Revised response criteria for malignant lymphoma. J Clin Oncol. 2007;25(5):579-586.
3. Hans CP, Weisenburger DD, Greiner TC et al. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood. 2004;103(1):275-282.
4. Tanaka Y, Maeshima AM, Nomoto J et al. Expression pattern of PD-L1 and PD-L2 in classical Hodgkin lymphoma, primary mediastinal large B-cell lymphoma, and gray zone lymphoma. Eur J Haematol. 2018;100(5):511-517.
5. van Dongen JJ, Langerak AW, Bruggemann M et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia. 2003;17(12):2257-2317.
6. Tanabe Y, Ichikawa H, Kohno T et al. Comprehensive screening of target molecules by next-generation sequencing in patients with malignant solid tumors: guiding entry into phase I clinical trials. Mol Cancer. 2016;15(1):73.
7. Kuboki Y, Yamashita S, Niwa T et al. Comprehensive analyses using next-generation sequencing and immunohistochemistry enable precise treatment in advanced gastric cancer. Ann Oncol. 2016;27(1):127-133.
8. Juskevicius D, Lorber T, Gsponer J et al. Distinct genetic evolution patterns of relapsing diffuse large B-cell lymphoma revealed by genome-wide copy number aberration and targeted sequencing analysis. Leukemia. 2016;30(12):2385-2395.
9. Johnson NA, Slack GW, Savage KJ et al. Concurrent expression of MYC and BCL2 in diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. J Clin Oncol. 2012;30(28):3452-3459.
10. Maeshima AM, Taniguchi H, Fukuhara S et al. Bcl-2, Bcl-6, and the International Prognostic Index are prognostic indicators in patients with diffuse large B-cell lymphoma treated with rituximab-containing chemotherapy. Cancer Sci. 2012;103(10):1898-1904.
11. Kiyasu J, Miyoshi H, Hirata A et al. Expression of programmed cell death ligand 1 is associated with poor overall survival in patients with diffuse large B-cell lymphoma. Blood. 2015;126(19):2193-2201.