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Clinicopathological and genomic analysis of double-hit follicular lymphoma: comparison with high-grade B-cell lymphoma with *MYC* and *BCL2* and/or *BCL6* rearrangements

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Most high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements are aggressive B-cell lymphomas. Occasional double-hit follicular lymphomas have been described but the clinicopathological features of these tumors are not well known. To clarify the characteristics of double-hit follicular lymphomas, we analyzed 10 cases of double-hit follicular lymphomas and 15 cases of high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements for clinicopathological and genome-wide copy-number alterations and copy-neutral loss-of-heterozygosity profiles. For double-hit follicular lymphomas, the median age was 67.5 years (range: 48–82 years). The female/male ratio was 2.3. Eight patients presented with advanced clinical stage. The median follow-up time was 20 months (range: 1–132 months). At the end of the follow-up, 8 patients were alive, 2 patients were dead including 1 patient with diffuse large B-cell lymphoma transformation. Rearrangements of *MYC*/*BCL2*, *MYC*/*BCL6*, and *MYC*/*BCL2*/*BCL6* were seen in 8, 1, and 1 cases, respectively. The partner of *MYC* was IGH in 6 cases. There were no cases of histological grade 1, 4 cases of grade 2, 5 cases of grade 3a, and 1 case of grade 3b. Two cases of grade 3a exhibited immunoblast-like morphology. Immunohistochemistry demonstrated 9 cases with $\geq$50% MYC-positive cells. There was significant difference in MYC intensity ($P = 0.00004$) and MIB-1 positivity ($P = 0.001$) between double-hit follicular lymphomas and high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements. The genome profile of double-hit follicular lymphomas was comparable with conventional follicular lymphomas (GSE67385, $n = 198$) with characteristic gains of 2p25.3-p11.1, 7p22.3-q36.3, 12q11-q24.33, and loss of 18q21.32-q23 ($P < 0.05$). In comparison with high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements, double-hit follicular lymphomas had fewer copy-number alterations and minimal common region of gain at 2p16.1 (70%), locus also significant against conventional follicular lymphomas ($P = 0.0001$). In summary, double-hit follicular lymphomas tended to be high-grade histology, high MYC protein expression, high *MYC*/IGH fusion, and minimal common region of gain at 2p16.1. Double-hit follicular lymphomas seemed to be a different disease from high-grade B-cell lymphomas

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with *MYC* and *BCL2* and/or *BCL6* rearrangements and have an indolent clinical behavior similar to follicular lymphomas without *MYC* rearrangement.
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Chromosomal translocation frequently occurs in B-cell lymphomas and well-known translocations are at the chromosomal loci of 18q21 involving *BCL2*, 3q27/*BCL6*, and 8q24/*MYC*.[1] In high-grade B-cell lymphoma, gene translocation of *MYC* is seen not only in Burkitt lymphoma, but also in about 10% of diffuse large B-cell lymphoma, and that of *BCL2* and *BCL6* is found in 30% and 30–40% of diffuse large B-cell lymphoma, respectively.[1,2] Although the combination of these gene translocations is a rare phenomenon, Kanungo *et al*[3] first reported that high-grade B-cell lymphomas with concurrent t(14;18) and 8q24/*MYC* translocations demonstrate a poor prognosis. Furthermore, high-grade B-cell lymphomas that have both *MYC* and *BCL2* translocations have been referred to as double-hit lymphoma. Double-hit lymphoma was included in 'B-cell lymphoma unclassifiable, intermediate between diffuse large B-cell lymphoma, and Burkitt lymphoma' in 2008 World Health Organization classification of lymphoid neoplasms.[1] Diagnostic criteria of B-cell lymphoma unclassifiable, intermediate between diffuse large B-cell lymphoma, and Burkitt lymphoma; however, was ambiguous in histological, immunohistochemical, and genetic characteristics. Finally, in the revised 2016 World Health Organization classification of lymphoid neoplasms, double-hit lymphoma was classified into the new category of 'High-grade B-cell lymphoma, with *MYC* and *BCL2* and/or *BCL6* rearrangements.[4] High-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements is resistant to conventional chemotherapy and shows poorer prognosis than standard diffuse large B-cell lymphoma.[2,5] High-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements frequently exhibits involvement of extra-nodal organs, the central nervous system and abnormal elevation of serum lactate dehydrogenase, and median survival duration is less than 1 year.[6] Moreover, triple-hit lymphoma with *MYC*, *BCL2*, and *BCL6* translocations were also reported.[7] Clinical factor variables of triple-hit lymphoma were similar with high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements without *BCL6* translocation, but the median survival duration of these patients was shorter than that of patients with high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements without *BCL6*.[7]

Follicular lymphoma accounts for 15–30% of all lymphoid neoplasms and is the most common histological subtype of indolent non-Hodgkin Lymphoma.[1] The natural history of follicular lymphoma is characterized by recurrent relapses and progressively shorter remissions with a median survival of 10 years.[8] Follicular lymphoma usually carry *BCL2* rearrangement and this rearrangement is found in 80–90% of cases. Secondary *MYC* rearrangement in follicular lymphoma frequently demonstrates histological transformation to diffuse large B-cell lymphoma with Burkitt-like appearance.[9] Follicular lymphoma having both *MYC* and *BCL2* translocations without high-grade transformation (double-hit follicular lymphoma) is extremely rare and two reports have been published so far.[10,11] Yoshida *et al*[10] indicated that double-hit follicular lymphoma may exhibit an indolent clinical course despite *MYC* and *BCL2* translocations, and Miao *et al*[11] suggested that double-hit follicular lymphoma, regardless of low-grade morphology, tends to have an aggressive clinical stage. In the latter series of double-hit follicular lymphoma (7 cases), the male/female ratio was 1:2.5, ranging from 34 to 64 years with a median age of 47 years. Bone-marrow involvement was observed in 43%. Grade 3 morphology was found in 86% and immunohistochemical staining of MYC protein positivity ranged from <5 to 30%.[11] The genetic features of double-hit follicular lymphoma, however, have not been reported.

To clarify the characteristics of double-hit follicular lymphoma, we analyzed and compared the clinicopathological, immunohistochemical, and genomic characteristics of 10 cases of double-hit follicular lymphoma and 15 cases of high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements. We found that double-hit follicular lymphoma tended to be high-grade (grade 3) with high MYC positivity, and the frequency of *MYC*/IGH fusion was higher than that of high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements. OncoScan's estimated percentage of altered double-hit follicular lymphoma genome was much less than that of high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements.

Materials and methods

Case Selection

We retrieved a total of 25 patients of B-cell lymphoma with rearrangements of *MYC* and *BCL2* and/or *BCL6* from Tokai University, Okayama University, St Marianna University and Kanagawa cancer center of Japan. The cases were 10 double-hit follicular lymphomas and 15 high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6*

rearrangements cases, histologically. Rearrangements of *MYC*, *BCL2*, and *BCL6* were examined by fluorescence *in situ* hybridization in all cases and routine chromosome analysis (karyotype, G-banding) in 8 cases (32%). All clinical and laboratory data for each case, along with follow-up data, were obtained from the medical records at each institution. The Institutional Review Board's approval for this study had previously been obtained (Tokai University, School of Medicine, 15I-21).

Histology and Immunohistochemistry

Formalin-fixed paraffin-embedded sections for all cases were collected for histology, immunohistochemistry, and molecular studies. Immunohistochemistry was performed using mouse monoclonal antibodies against cytoplasmic CD3 (cCD3, non-glycosylated epsilon chain of the human CD3 molecule) (clone LN10, Novocastra (NV), Leica Microsystems K.K., Tokyo, Japan), CD5 (4C7, NV), CD10 (56C6, NV), CD20 (L26, Nichirei Biosciences, Tokyo, Japan), BCL2 (BCL2/100/D5, NV), BCL6 (LN22, NV), MUM1 (IRF4 antibody (MUM1p), GeneTex, CA, USA), MIB-1 (MM1, NV), and a rabbit monoclonal antibody against MYC (Y69, Abcam K. K., Tokyo, Japan) as primary antibodies and the Leica BOND-MAX fully automatic immunohistochemistry system with the BOND Polymer Refine detection kit (DS9800) according to the manufacturer's instructions for signal detection. BOND Epitope Retrieval Solution 2 (AR9640) was used for 20 min for CD3, CD5, CD10, CD20, BCL6, MUM1, MIB-1, and MYC, and 30 min for CD10 and BCL2. A marker was considered positive if more than 30% of the tumor cells expressed the antigen for CD3, CD5, CD10, CD20, BCL2, BCL6, and MUM1 markers. MIB-1 and MYC were assessed semi-quantitatively (increments of 10%, ie, 10%, 20%, 30%). The markers were evaluated by three authors at the same time (Masashi Miyaoka, Shinichiro Hiraiwa and Naoya Nakamura). Final composition of figures was created using GIMP 2.8 software.

Fluorescence *In Situ* Hybridization

Fluorescence *in situ* hybridization was performed on 3 μm tissue sections using the *BCL2*, *BCL6*, and *MYC* break apart (split signal) DNA probes (Y5407, Y5408, and Y5410, respectively, Dako K.K., Tokyo, Japan) and *MYC*/IGH fusion probe (LSI IGH/MYC/CEP 8 Tri-Color Dual Fusion Probe, Vysis, Abbott Molecular, IL, USA). Fluorescence *in situ* hybridization was performed as previously described.[12,13] The slides were evaluated using a fluorescence microscope (Olympus BX51, Olympus K.K., Tokyo, Japan), DP73 camera and cellSens microscope imaging software (Olympus). Signals were counted in at least 100 cells and positivity was considered if more than 10% of the tumor cells exhibited a break apart signal for *BCL2*, *BCL6*, or *MYC*, and fusion signal for the *MYC*/IGH probe.

Genome-Wide Copy Number and Copy-Neutral Loss-of-Heterozygosity OncoScan Microarray Analysis

The OncoScan copy-number analysis was performed as previously described.[13] In summary, the genomic DNA was extracted from formalin-fixed paraffin-embedded using the QIAamp DNA Micro Kit (#56304, Qiagen K.K., Tokyo, Japan). DNA quality was checked using primers for housekeeping genes as previously described in the EuroClonality/BIOMED-2 guidelines[14] and the 22 samples (all double-hit follicular lymphoma cases and 12 cases of high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements) had genomic fragments equal to or larger than 300 bp. Genomic dsDNA was quantified by Qubit assay (Invitrogen, Thermo Fisher Scientific, MA, USA) and processed according to the OncoScan copy-number variation formalin-fixed paraffin-embedded assay kit (#902695, Affymetrix K.K., Tokyo, Japan). The assay, visualization and data analysis were performed under the Standard Analysis Setup (this was not a matched normal analysis) using the Affymetrix GeneChip System 3000, Affymetrix OncoScan Console 1.1 and Nexus Express Software for OncoScan 3.1. All cases but case 6 of high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements were analyzed using the OSCHP-TuScan algorithm. Case 6 (high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements) required the OSCHP-SNP-FASST2 algorithm due to Diploid Recentering. All alterations were confirmed by visual inspection, minimal common regions were identified and regions were mapped according to Human GRCh37.p13 reference. Sexual chromosomes were excluded from the final analysis. The comparison with conventional follicular lymphoma made use of the Gene Expression Omnibus (GEO) dataset GSE67385, genome variation profiling by SNP array (Affymetrix Mapping 205 K Nsp SNP Array), which comprises 198 follicular lymphomas and 79 transformed follicular lymphomas as described by Bouska A *et al*.[15]

Statistical Analysis

Comparison between groups was made using IBM SPSS Statistics Version 24 software: crosstabulations with χ^2 test (including Fisher exact test); non-parametric tests, two or more independent samples and automatically comparison distribution across groups; and survival, Kaplan–Meier analysis.

Table 1 Clinical features of double-hit follicular lymphoma and high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements

Entity	*Case*	*Age*	*Gender*	*Serum lactate dehydrogenase*	*Stage*	*PS*	*BM invasion*	*Follicular Lymphoma International Prognostic Index* / *International Prognostic Index*	*Diffuse large B-cell lymphoma transformation*	*Therapy*	*Response*	*OS*	*Outcome*
Double-hit follicular lymphoma	1	57	F	N	4	1	–	Poor risk	–	R-CHOP	Complete response	132	Alive
	2	82	F	N	1	0	–	Intermediate risk	–	R-CHOP	Complete response	90	Death
	3	75	F	+	3	0	–	Poor risk	–	R-COP	Complete response	83	Alive
	4	70	F	N	3	NA	NA	Poor risk	–	R-EPOCH	Complete response	11	Alive
	5	62	F	+	4	0	+	Poor risk	–	R-CHOP	Complete response	27	Alive
	6	50	M	+	3	1	–	Poor risk	+	R-CVP	Progressive disease	12	Death
	7	77	F	N	4	NA	+	Poor risk	–	DA-EPOCH-R	Complete response	12	Alive
	8	81	M	N	2	0	–	NA	–	R-DHAP	Complete response	28	Alive
	9	65	F	N	3	0	–	NA	–	RB	Complete response	7	Alive
	10	48	M	N	3	0	–	NA	–	R-CHOP	NA	1	Alive
High-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements	1	69	F	+	4	0	–	High risk	NA	R-CHOP	Partial response	69	Death
	2	69	M	+	4	1	+	High risk	NA	THP-COP	Progressive disease	2	Death
	3	63	F	+	4	2	+	High risk	NA	Hyper-CVAD	Progressive disease	4	Death
	4	77	M	+	3	2	NA	High risk	NA	R-CHOP	Progressive disease	5	Death
	5	49	F	N	2	0	–	Low risk	NA	R-CHOP	Complete response	91	Alive
	6	67	M	+	4	4	–	High risk	NA	R-COP	Complete response	13	Death
	7	66	M	+	2	0	–	Low-intermediate risk	NA	R-CHOP	Complete response	73	Alive
	8	61	F	+	4	0	–	High risk	NA	R-CHP	Complete response	56	Alive
	9	57	M	N	4	0	+	Low risk	NA	R-CHOP → DA-R-EPOCH	Complete response	33	Alive
	10	55	F	+	4	0	–	Low-intermediate risk	NA	NA	NA	24	Alive
	11	39	M	N	4	0	+	Low risk	NA	R-CHOP		66	Alive

Table 1 (Continued)

Entity	*Case*	*Age*	*Gender*	*Serum lactate dehydrogenase*	*Stage*	*PS*	*BM invasion*	*Follicular Lymphoma International Prognostic Index*	*Diffuse large B-cell lymphoma transformation*	*Therapy*	*Response*	*OS*	*Outcome*
								International Prognostic Index					
	12	66	M	+	3	2	–	High risk	NA	Hyper-CVAD	Complete response/ unconfirmed	38	Alive
	13	34	F	+	3	1	–	NA	NA	High-dose R-THP-COP	Complete response	78	Alive
	14	62	F	+	2	2	–	NA	NA	R-THP-COP +Rx	NA	8	Death
	15	63	F	NA	NA	NA	NA	NA	NA	R-CODOX-M/ IVAC	Progressive disease	1	Death
P-value		NS	NS	0.035	NS	NS	NS	NS	NA	NA	NS	NS	

+, High or positive; BM, bone marrow; F, female; M, male; N, within normal range; NA, not available and/or not applicable; NS, not significant; OS, overall survival.

Results

Clinical Characteristics

Double-hit follicular lymphoma (10 patients). The median age of patients was 67.5 with a range from 48 to 82 years (Table 1). The female/male ratio was 2.3. Three patients (30%) had abnormal elevation of serum lactate dehydrogenase (institutional upper limit of normal range as cut off value), 8 patients (80%) had advanced stage (Ann Arbor stage III/IV), and 2 patients (20%) had bone-marrow involvement. The Eastern Cooperative Oncology Group performance status was asymptomatic or symptomatic but completely ambulatory (0 or 1) in all 8 patients with available clinical information. The Follicular Lymphoma International Prognostic Index reported by Solal-Céligny *et al*[16] in 2004 was as follows; 6 of 7 patients (86%) were poor risk and 1 patient (14%) was intermediate risk. Eight of ten patients (80%) were treated with the standard regimen for follicular lymphoma; ie, R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) or R-CHOP-like regimens. The other two patients were treated with more intensive regimens; R-EPOCH (rituximab, etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicin) or dose adjusted-EPOCH-R chemotherapy. According to standard criteria of response to therapy reported by Cheson *et al*[17] in 1999, the response to chemotherapy was complete response in 8 patients, progressive disease in 1 patient with transformation to diffuse large B-cell lymphoma (Case 6), and not available in 1 patient. The median follow-up time was 20 months with a range from 1 to 132 months. At the end of the follow-up period, two patients were dead including case 6 with diffuse large B-cell lymphoma transformation. In case 6, diffuse large B-cell lymphoma transformation occurred 11 months after the double-hit follicular lymphoma diagnosis.

High-grade B-cell lymphomas with MYC and BCL2 and/or BCL6 rearrangements (15 patients). The median age was 63 years with a range from 34 to 77 years (Table 1). The female/male ratio was 1.1. Eleven of 14 patients had abnormal elevation of serum lactate dehydrogenase, 11 of 14 patients had advanced stage and 4 of 13 patients had involvement of the bone marrow. Eastern Cooperative Oncology Group performance status (PS) was as follows; PS 0, 7 patients, PS 1, 2 patients, PS 2, 4 patients, PS 3, no patient, PS 4, 1 patient, PS not available 1 patient. International Prognostic Index was as follows; low risk, 3 patients, low-intermediate risk, 2 patients, high-intermediate risk, 0 patient, high risk, 7 patients, not available, 3 patients. Seven patients were treated with R-CHOP or R-CHOP-like regimens. The other regimens were as follows: THP-COP (cyclophosphamide, tetrahydropyranyl adriamycin, vincristine, and prednisolone) for 1 patient, R-THP-COP (rituximab, tetrahydropyranyl adriamycin,

cyclophosphamide, vincristine, and prednisolone) and radiation for 1 patient, high-dose R-THP-COP for 1 patient, R-CODOX-M/IVAC (rituximab, cyclophosphamide, vincristine, doxorubicin, and high-dose methotrexate, alternating with ifosfamide, etoposide, and cytarabine) for 1 patient and Hyper-CVAD (hyper-fractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone) for 2 patients and DA-R-EPOCH after R-CHOP for 1 patient. Complete response or complete response/unconfirmed was achieved in 7 patients. Partial response was observed in 1 patient and progressive disease was observed in 4 patients. The median follow-up time was 33 months with a range from 1 to 91 months. At the end of the follow-up period, 7 patients had died.

In comparison with high-grade B-cell lymphomas with *MYC* and *BCL*2 and/or *BCL*6 rearrangements, double-hit follicular lymphoma was characterized by lower levels of serum lactate dehydrogenase (abnormal elevation of serum lactate dehydrogenase was seen 30% in double-hit follicular lymphoma, 79% in high-grade B-cell lymphomas with *MYC* and *BCL*2 and/or *BCL*6 rearrangements ($P=0.035$)). No differences were found regarding the overall survival ($P=0.196$ by log rank, $P=0.118$ by Breslow) (Figure 1).

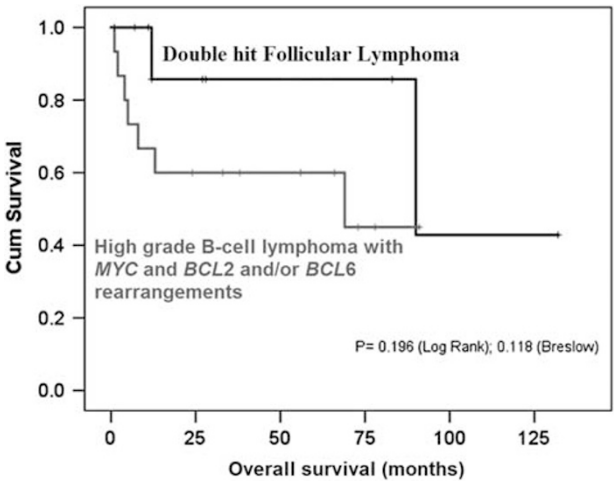

Figure 1 Overall survival curve of double-hit follicular lymphoma and high-grade B-cell lymphomas with *MYC* and *BCL*2 and/or *BCL*6 rearrangements. There is not significant difference in overall survival between double-hit follicular lymphoma and high-grade B-cell lymphomas with *MYC* and *BCL*2 and/or *BCL*6 rearrangements in this study ($P=0.196$ by log rank, $P=0.118$ by Breslow).

Histology and Immunohistochemistry

Histology. *Double-hit follicular lymphoma (Table 2; Figure 2)*: Histological grade was classified into grade 1, 0 cases; grade 2, 4 cases (40%); grade 3a, 5 cases (50%), and grade 3b, 1 case (10%). Two of the 5 cases of grade 3a (40%) exhibited immunoblast-like morphology.

High-grade B-cell lymphomas with MYC *and* BCL2 *and/or* BCL6 *rearrangements (Table 2; Figure 2)*: All cases showed diffuse proliferation of medium to large lymphoma cells. Thirteen cases showed diffuse large B-cell lymphoma morphology and two cases showed Burkitt-like morphology.

Immunohistochemistry. *Double-hit follicular lymphoma (Table 2; Figure 2)*: The results showed CD3, 0/10 (0%); CD5, 0/10 (0%); CD10, 9/10 (90%); CD20, 10/10 (100%); BCL2, 8/10 (80%); BCL6, 10/10 (100%); MUM1, 3/8 (37.5%). Average of MIB-1 positivity was 39%. Positivity of MYC ranged as follows: 7 cases had more than 70%, 2 more than 50%, and only one was almost negative with only 1% positive cells. Nine cases (90%) had $\geq 50\%$ MYC-positive cells. For aspect of MYC-positive intensity, we defined weak positive as intensity of positive cells seen in normal tonsil, 2 cases had only weakly positive cells (+), 8 cases had strongly and weakly stained cells, which were intermingled (++), no case had only strongly positive cells (+++) (Table 2).

High-grade B-cell lymphomas with MYC and BCL2 and/or BCL6 rearrangements (Table 2; Figure 2): The immunohistochemistry expression of the different markers was as follows: CD3, 0/15 (0%); CD5, 1/15 (6.7%); CD10, 13/15 (87%); CD20, 14/15 (93%); BCL2, 13/15 (87%); BCL6, 13/14 (93%); MUM1, 3/15 (20%). The cell-of-origin subtype was germinal center B-cell-like in 13/15 (87%) based on the Hans classifier. Average of MIB-1 positivity was 75.3%. Positivity of MYC ranged from 50 to 100% and 14 cases (93%) had $\geq 70\%$ MYC-positive cells. For aspect of MYC-positive intensity, all cases had only strongly positive cells (+++).

Between double-hit follicular lymphoma and high-grade B-cell lymphomas with *MYC* and *BCL*2 and/or *BCL*6 rearrangements, there was statistically significant difference in MYC intensity ($P=0.000004$) and MIB-1 positivity ($P=0.001$), ie, double-hit follicular lymphoma was characterized by lower MYC immunohistochemistry intensity (+/++ *vs* +++) and lower proliferation index as expressed by MIB-1% (39 *vs* 75.3%).

Gene translocation by fluorescence in situ hybridization and karyotype. *Double-hit follicular lymphoma (Table 2; Figure 2)*: The combination patterns of gene translocation in double-hit follicular lymphoma were as follows; 8 cases with *MYC* and *BCL*2, 1 case with *MYC* and *BCL*6, and 1 case with *MYC, BCL*2, and *BCL*6 genes (triple-hit follicular lymphoma). Translocation of *MYC*/IGH was found in 6 of 10 cases (60%).

High-grade B-cell lymphomas with MYC *and* BCL2 *and/or* BCL6 *rearrangements (Table 2; Figure 2)*: All 15 cases of high-grade B-cell lymphomas with *MYC* and *BCL*2 and/or *BCL*6 rearrangements had both translocation of *MYC* and *BCL*2, and 4 cases have translocation of *MYC*, *BCL*2, and *BCL*6 (triple-hit lymphoma). *MYC*/IGH was found in 6 of 14 cases (43%).

Table 2 Histological, immunohistochemical and cytogenetics of double-hit follicular lymphoma and high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements

Entity	*Case*	*Follicular lymphoma grade—diffuse large B-cell lymphomma subtype*	*Morphology*	*Immunohistochemistry*										*Fluorescence in situ hybridization*				*Karyotype (G-banding)*
				MYC %	*MYC int. (i)*	*MIB-1 %*	*CD3*	*CD5*	*CD10*	*CD20*	*BCL2*	*BCL6*	*MUM1*	MYC *split*	MYC/IGH *fusion*	BCL*2 split*	BCL*6 split*	
Double-hit follicular lymphoma	1	2	Centrocyte/centroblast	1	+	10	–	–	+	+	+	+	–	+	–	+	–	NT
	2	3a	Centrocyte/centroblast	70	++	70	–	–	+	+	–	+	+/–	+	–	–	+	NT
	3	2	Centrocyte/centroblast	50	+	20	–	–	–	+	+	+	+/–	+	+	+	–	NT
	4	2	Centrocyte/centroblast	90	++	30	–	–	+	+	+	+	–	+	+	+	–	47, XX, +7, t(8;14)(q24;q32), t(14;18)(q32;q21)[2/6]/46,XX[4/6]
	5	3b	Centrocyte/centroblast	70	++	30	–	–	+	+	+	+/–	–	+	+	+	–	47, XX, add(1)(p36.1), add(2)(p11.2), add(3)(q27), t(8;14)(q24;q32), +marl [15/20]/47, XX,+X [4/20]/46, XX[1/20]
	6	3a	Immunoblast-like	60	++	10	–	–	+	+	+	+/–	–	+	+	+	–	NT
	7	3a	Centrocyte/centroblast	70	++	30	–	–	+	+	+	+	+/–	+	+	+	–	NT
	8	3a	Immunoblast-like	90	++	50	–	–	+	+	+	+	NT	+	+	+	–	46, X, +X, -Y[1/20]/47, idem, add(1)(p36.1), t(2;18)(p12;q21), +12, add(14)(q32), der(18)t(2;18)[19/20]
	9	3a	Centrocyte/centroblast	90	++	70	–	–	+	+	+	+	NT	+	–	+	+	47, XX, add(6)(p21), +7, del(13)(q?), t

Table 2 (Continued)

Entity	*Case*	*Follicular lymphoma grade—diffuse large B-cell lymphomma subtype*	*Morphology*	*Immunohistochemistry*										*Fluorescence* in situ *hybridization*				*Karyotype (G-banding)*
				MYC %	*MYC int. (i)*	*MIB-1 %*	*CD3*	*CD5*	*CD10*	*CD20*	*BCL2*	*BCL6*	*MUM1*	MYC *split*	MYC/IGH *fusion*	BCL*2* *split*	BCL*6* *split*	
	10	2	Centrocyte/centroblast	80	++	70	–	–	+	+	–	+	–	+	–	+	–	(14;18)(q32;q21)[16/20]/46, XX [4/20] 46.Y, add(X)(q22), add(2)(q11.2), add(4)(q21), t(14;18)(q32;q21)[2/11], 46, XY[9/11]
High-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements	1	Non-GCB	Diffuse large B-cell lymphoma	70	+++	50	–	–	–	+	+	–	–	+	–	+	–	NT
	2	GCB	Diffuse large B-cell lymphoma	90	+++	90	–	–	+	+	+	+	–	+	No signal	+	NT	NT
	3	GCB	Diffuse large B-cell lymphoma	90	+++	60	–	–	+	+	+	+	–	+	+	+	+	NT
	4	GCB	Diffuse large B-cell lymphoma	80	+++	70	–	–	+	+	+	+	+	+	–	+	–	NT
	5	GCB	Diffuse large B-cell lymphoma	70	+++	80	–	–	+	+	–	+	–	+	–	+	–	NT
	6	Non-GCB	Diffuse large B-cell lymphoma	90	+++	70	–	–	–	+	+	+	+	+	–	+	NT	NT
	7	GCB	Diffuse large B-cell lymphoma	80	+++	80	–	–	+	+	–	+	–	+	–	+	–	NT
	8	GCB	Diffuse large B-cell lymphoma	80	+++	70	–	–	+	+	+	+	–	+	–	+	+	52, XX, t(3;3)(q27;q29), +7X2, +8X2, t(14;18)(q32;

Table 2 (Continued)

Entity	*Case*	*Follicular lymphoma grade—diffuse large B-cell lymphomma subtype*	*Morphology*	*Immunohistochemistry*										*Fluorescence in situ hybridization*				*Karyotype (G-banding)*
				MYC %	*MYC int. (i)*	*MIB-1 %*	*CD3*	*CD5*	*CD10*	*CD20*	*BCL2*	*BCL6*	*MUM1*	MYC *split*	MYC/IGH *fusion*	BCL*2* *split*	BCL*6* *split*	
	9	GCB	Diffuse large B-cell lymphoma	80	+++	80	–	–	+	+	+	+	–	+	+	+	+	q21), +19, +mar[20/20] 46, -X,Y?, +add(1)(?p21), t(8;14)(q24;q32), t(14;18)(q32;q21)[15/20]/46,-X,Y?, +del(1)(p21), t(8;14)(q24;q32), t(14;18)(q32;q21)[5/20]
	10	GCB	Diffuse large B-cell lymphoma	90	+++	80	–	–	+	+	+	+	–	+	+	+	+	NT
	11	GCB	Diffuse large B-cell lymphoma	50	+++	30	–	–	+	+	+	+	–	NT	+	+	–	46, XY, add(1)(q21), t(8;14;18)(q24;q32;q21)[16/20], 47, sl, +2 [3/20], 46, XY[1/20]
	12	GCB	Burkitt-like	70	+++	90	–	–	+	+	+	+	+	+	–	+	–	NT
	13	GCB	Diffuse large B-cell lymphoma	100	+++	100	–	–	+	+	+	+	–	+	+	+	–	NT
	14	GCB	Diffuse large B-cell lymphoma	90	+++	90	–	+	+	+	+	NT	–	+	+	+	–	NT
	15	GCB	Burkitt-like	90	+++	90	–	–	+	–	+	+	–	+	–	+	–	NT
P-value		NA	NA	NS	$P=0.000004$	$P=0.001$	NS	NS	NS	NS	NS	NS	NS	NT	NT	NT	NT	NT

+, Positive for immunohistochemistry (>30% of positive cells/all lymphoma cells); –, negative; GCB, germinal center B-cell-like (Hans classifier); Intens, protein expression intensity by immunohistochemistry (+, weak; ++ intermediate (ie, weak and strong intermingled stained cells); +++, strong); non-GCB, non germinal center-cell-like (Hans classifier); NT, not tested; NS, not significant.

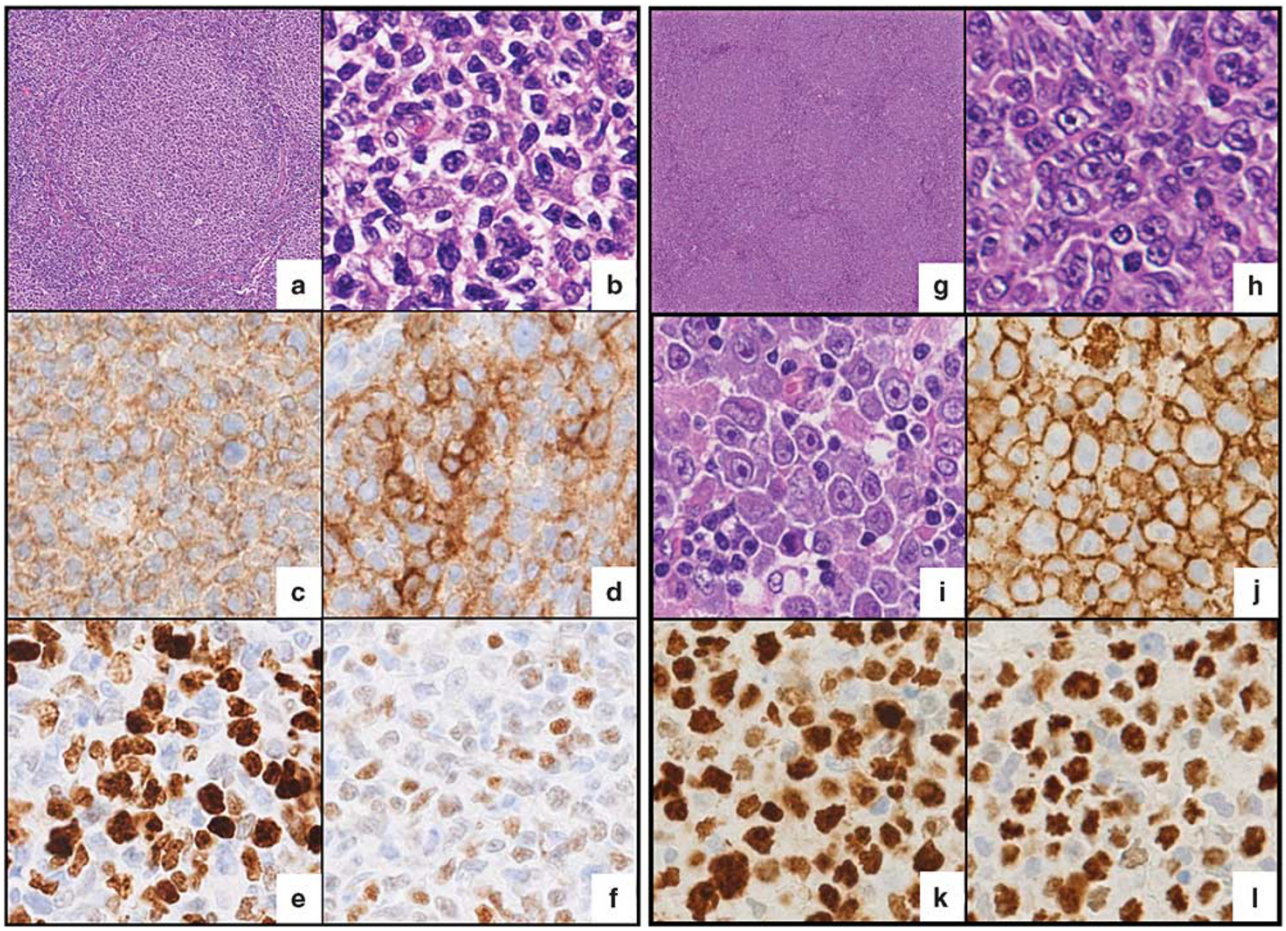

Figure 2 Histology, immunohistochemistry, and fluorescence *in situ* hybridization of double-hit follicular lymphoma and high-grade B-cell lymphoma with *MYC* and *BCL2* and/or *BCL6* rearrangements. (**a–f**) Double-hit follicular lymphoma (case 10). (**a**) Low power; neoplastic lymphocytes make nodule. (**b**) High power; neoplastic lymphocytes mainly show centrocyte morphology, sometimes show centroblast morphology (grade 2). (**c**) Neoplastic lymphocytes are positive for CD20. (**d**) Neoplastic lymphocytes are positive for CD10. (**e**) MIB-1 positivity is 70% in germinal center. (**f**) Neoplastic lymphocytes show 80% MYC-positive cells with strong and weak staining. (**g**, **h**) Double-hit follicular lymphoma (case 8). (**g**) Low power; neoplastic lymphocytes make many nodules. (**h**) High power; neoplastic lymphocytes have large nucleus with one central large nucleolus (immunoblast-like morphology, grade 3a). (**i–l**) High-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements (case 9). (**i**) Medium to large lymphoma cells show diffuse proliferation pattern. (**j**) Neoplastic lymphocytes are positive for CD20. (**k**) MIB-1 positivity is 80%. (**l**) This case shows 80% MYC-positive cells with strong staining. (**m–p**) Fluorescence *in situ* hybridization of double-hit follicular lymphoma (case 7). (**m**) *MYC* split fluorescence *in situ* hybridization; split signal is observed in more than 10% neoplastic cells. (**n**) *BCL2* split fluorescence *in situ* hybridization; split signal is observed in more than 10% neoplastic cells. (**o**) *BCL6* split fluorescence *in situ* hybridization; split signal is not observed. (**p**) *MYC*/IGH fusion fluorescence *in situ* hybridization; red signal shows *MYC*, green signal shows IGH, and blue signal shows centromere. Split signal (arrow head) and fusion signal (arrow) are seen. Fusion signal is observed in more than 10% neoplastic cells. (**q–s**) Fluorescence *in situ* hybridization of high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements (case 10). (**q**) *MYC* split fluorescence *in situ* hybridization; split signal is observed in more than 10% neoplastic cells. (**r**) *BCL2* split fluorescence *in situ* hybridization; split signal is observed in more than 10% neoplastic cells. (**s**) *MYC*/IGH fusion fluorescence *in situ* hybridization; red signal shows *MYC*, green signal shows IGH, and blue signal shows centromere. Split signal (arrow head) and fusion signal (arrow) are seen. Fusion signal are observed in more than 10% neoplastic cells.

Karyotype data of double-hit follicular lymphoma and high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements is shown in Table 2.

Genome-wide copy number and copy-neutral loss-of-heterozygosity profiles. The genomic profiles are presented in Figure 3 with Supplementary Data 1 and 2. We succeeded in obtaining good quality results for copy-number analysis and copy-neutral loss-of-heterozygosity in 10 cases of double-hit follicular lymphoma, and 12 cases of high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements (case 1, 3–10, and 13–15). All cases exhibited multiple copy-number alterations throughout the genome.

Double-hit follicular lymphoma: OncoScan's estimated percentage of altered genome ranged from 2.8 to 19.1%, with an average of 8.5%. The genomic profile was characterized by gains of 2p24.2-2p12 (40%) with minimal common region at 2p16.1-2p15 (60–70%), 7p22.3-7q36.3 (=~40%) with minimal

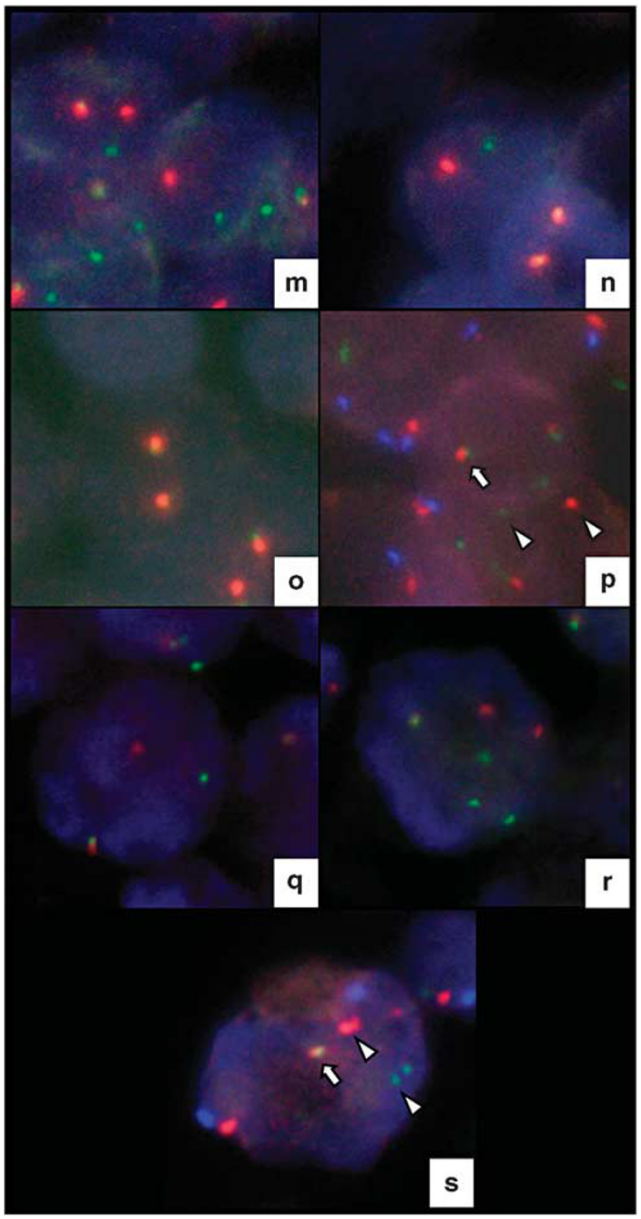

Figure 2 Continued.

common region 7p21.1 (50%), 12p11.1-12q12 (40%). Of note, 8q23.1-8q24.3 gain including *MYC* was only present in 20% of cases. The most relevant loss was 1p36.33-1p36.23 (30%, locus also identified as copy-neutral loss-of-heterozygosity); the loss of 6q16.1-6q22.31 was present only in 20% of the cases. Copy-neutral loss-of-heterozygosity was present at 1p36.33-1p36.22 (40%), 3p21.31-3p21.2 (60%), 6p21.32-6p21.31 (70%), 15q22.2-15q22.31 (50%), and 17q11.2 (50%). In comparison with conventional follicular lymphoma (follicular lymphoma, not otherwise specified, series GSE67385, $n=198$), double-hit follicular lymphoma had a similar profile. Double-hit follicular lymphoma had characteristic gains of 2p25.3-p11.1 (which includes the minimal common region of 70% gain at 2p16.1), 7p22.3-q36.3 and 12q11-q24.33, and a characteristic loss of 18q21.32-q23 ($P<0.05$).

High-grade B-cell lymphomas with MYC and BCL2 and/or BCL6 rearrangements: The altered genome ranged from 5.4 to 70.3%, with an average of 28.8%. The genome profile was more complex with multiples regions of alterations. The most frequent changes of gains were 3p12.1-3q29 (=~42%) with minimal common region at 3q29 (58%), 7p22.3-7p11.1 (=~67%) with minimal common region at 7p22.3 (75%) and 7p21.1 (75%), and 12p13.33-12q21.31 (=~67%). As in double-hit follicular lymphoma, the loss of 1p36.33-1p36.32 was also affected (33%). Other losses were 6q22.1-6q23.2 (25%) and 15q14-15q21.3 (42%). The most characteristic copy-neutral loss-of-heterozygosity was 1p32.3 (58%), 3p21.31 (58%); the 1p36.33-1p36.32 locus was affected only in =~21% of the cases. The genome profile was similar between high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements with or without *BCL6* translocation ($P>0.05$) except for chromosome 12. High-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements with *BCL6* translocation was characterized by lower frequency of 12q12-q15 copy-number gain than high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements without *BCL6* translocation (25 *vs* 100%, $P<0.05$).

Comparing double-hit follicular lymphoma with high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements, the pattern of copy-number alterations and copy-neutral loss-of-heterozygosity was different. Gains of 3p12.1-p11.1, 3q11.2-q13.33, 12q14.1-q21.31, 20p11.22-20q11.21, 20q13.2-20q13.33, loss of 15q14-15q21.3 were more prominent in high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements, but minimal common region of gain at 2p16.1 was seen in double-hit follicular lymphoma. Candidate genes in above areas were summarized in Supplementary Data 2.

Discussion

In this study, we retrospectively analyzed 10 cases of double-hit follicular lymphoma. Compared with the conventional follicular lymphoma cases, so far only 2 reports for double-hit follicular lymphoma have been reported with 2 cases and 7 cases, respectively. Double-hit follicular lymphoma in advanced stage comprised 80% of our study and 86% in the previous report.[11] These frequencies are more than two-thirds of follicular lymphoma with advanced disease (stage III or IV) at diagnosis.[1] Frequency of bone-marrow infiltration in double-hit follicular lymphoma, 43% in the previous report and 20% in our report, was not different from follicular lymphoma (40–70%).[1] Grade 3 in double-hit follicular lymphoma is a major histological grade and it has a higher incidence than with conventional follicular

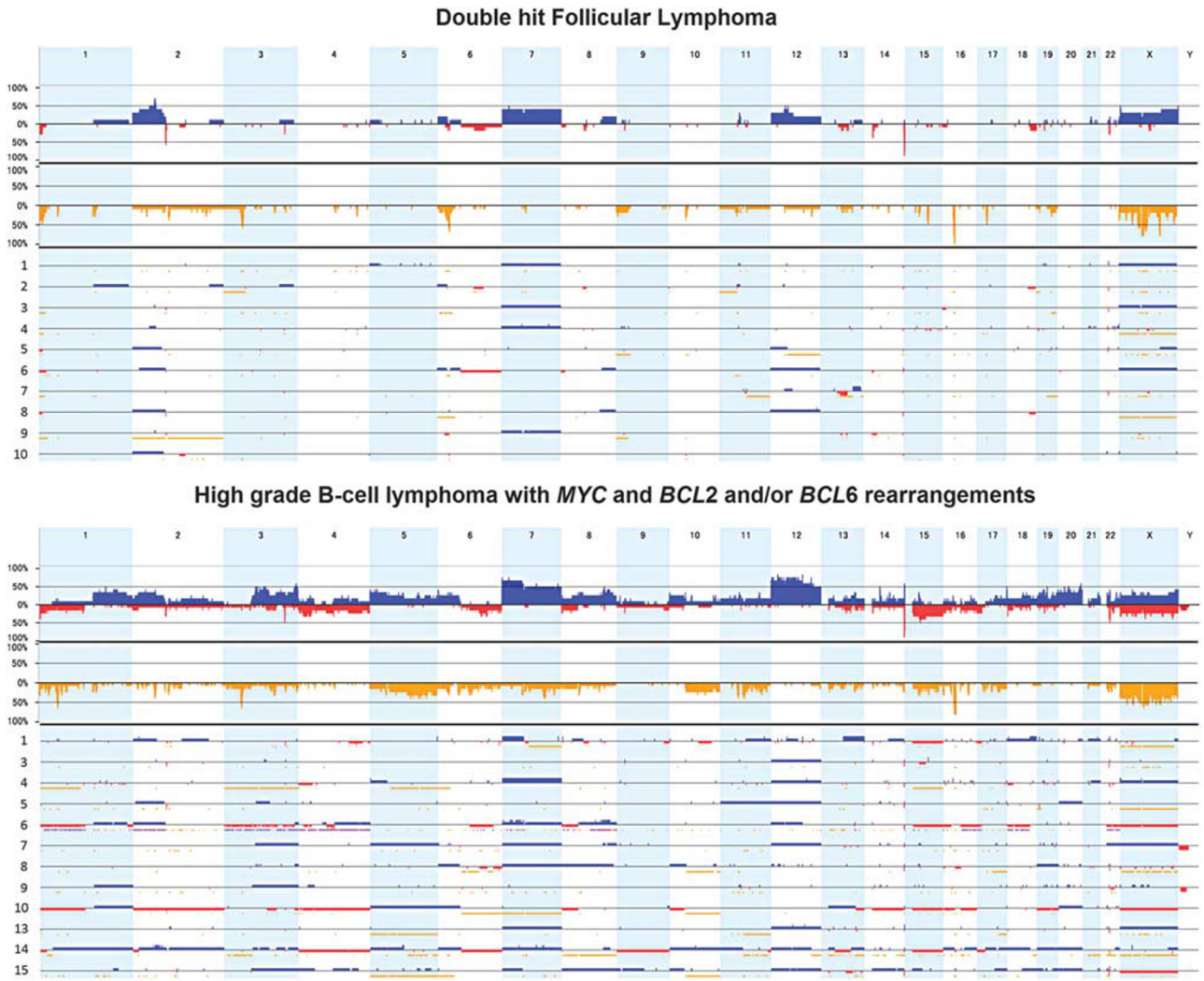

Figure 3 Copy-number alteration and copy-neutral loss-of-heterozygosity in OncoScan analysis of double-hit follicular lymphoma and high-grade B-cell lymphoma with *MYC* and *BCL2* and/or *BCL6* rearrangements. Number above in figure show chromosomal number. Blue signal, red signal, and yellow signal show copy-number gain, copy-number loss, and copy-neutral loss-of-heterozygosity, respectively.

lymphoma.[18] Transformation of follicular lymphoma into diffuse large B-cell lymphoma is one of the major causes of poor outcome and transformation into diffuse large B-cell lymphoma is reported in 27% of follicular lymphoma.[1] In our study, 1 patient (10%) showed transformation into diffuse large B-cell lymphoma and died after 12 months, 8 patients (80%) were alive in the follow-up period. Although there is not significant difference in overall survival between double-hit follicular lymphoma and high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements in this study, this is due to sample size. The standard regimen for follicular lymphoma seems to be sufficiently effective for most double-hit follicular lymphoma. Nevertheless, for both conventional follicular lymphoma and double-hit follicular lymphoma unknown which intensive chemotherapy is needed for the prevention of transformation.

The role of *MYC* in oncogenesis was first discovered in Burkitt lymphoma, in which *MYC* on chromosome 8q24 was juxtaposed to the IGH on chromosome 14q32 or, less commonly, to the IGK (2p12) or IGL (22q11) light chain genes, resulting in deregulated MYC protein expression.[19,20] *MYC* rearrangements have been identified in 7 to 11% of diffuse large B-cell lymphoma.[19,21–24] When B-cell lymphoma had $\geq$70% MYC-positive cells, the lymphoma had *MYC* translocation with a high probability.[19] Although it is reported that double-hit follicular lymphoma had $<$30% MYC-positive cells,[11] 9 cases (90%) of double-hit follicular lymphoma in our study had $\geq$50% MYC-positive cells ($\geq$70%, 7 cases). It is emphasized that strongly and weakly positive cells are intermingled with MYC-positive cells of double-hit follicular lymphoma, whereas most of the lymphoma cells in high-grade B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6*

rearrangements exhibited diffuse and strong expression of MYC ($P = 0.000004$). Moreover, 2 cases (20%) of double-hit follicular lymphoma showed immunoblast-like morphology. Immunoblastic morphology may indicate diffuse large B-cell lymphoma with *MYC* translocation.[2] Immunoblastic morphology is very unusual in follicular lymphoma and correlation with *MYC* rearrangement has been not well known yet.[25]

In our study, 27% (4/15) of high-grade B-cell lymphomas with *MYC* and *BCL*2 and/or *BCL*6 rearrangements cases were triple-hit lymphoma (case 3, 8, 9, and 10). Tomita *et al*[7] reported that 26% (7/27) of high-grade B-cell lymphomas with *MYC* and *BCL*2 and/or *BCL*6 rearrangements are triple-hit lymphoma and all triple-hit lymphoma patients died with a median survival of 4 months. In our triple-hit lymphoma patients, 1 patient was dead and 3 patients were alive in observation period, with a median survival of 28.5 months. Genomic profile of triple-hit lymphoma were similar to that of high-grade B-cell lymphomas with *MYC* and *BCL*2 and/or *BCL*6 rearrangements without *BCL*6 translocation; of note, gain at 12q12-12q15 was less prominent in triple-hit lymphoma.

The *MYC* translocation partner is an important prognostic factor in *MYC* translocation lymphoma. When IG was the *MYC* partner, the patient demonstrated a poorer prognosis than in the *MYC*/non-IG situation.[26] IGH is the *MYC* partner in 33% of high-grade B-cell lymphomas with *MYC* and *BCL*2 and/or *BCL*6 rearrangements and 83% of single hit lymphoma.[27] In our study, IGH *as* the *MYC* partner was found in 60% of double-hit follicular lymphoma, and 42.8% of high-grade B-cell lymphomas with *MYC* and *BCL*2 and/or *BCL*6 rearrangements, respectively. This result may affect the mechanism of pathogenesis, although the reason of indolent clinical course of double-hit follicular lymphoma is still not fully understood.

Array comparative genomic hybridization data of high-grade B-cell lymphomas with *MYC* and *BCL*2 and/or *BCL*6 rearrangements were already reported by a European group,[28] but to our knowledge, the genomic copy-number alterations in double-hit follicular lymphoma has not been reported. In our study, the genomic profile of double-hit follicular lymphoma was characterized by gains of 2p24.2-2p12 (40%) with minimal common region at 2p16.1-2p15 (60–70%), 7p22.3-7q36.3 (=~40%) with minimal common region 7p21.1 (50%) and 12p11.1-12q12 (40%), loss of 1p36.33-1p36.23 (30%), 6q16.1-6q22.31 (20%), copy-neutral loss-of-heterozygosity of 1p36.33-1p36.22 (40%), 3p21.31-3p21.2 (60%), 6p21.32-6p21.31 (70%), 15q22.2-15q22.31 (50%), and 17q11.2 (50%). Region at 1p36.33-1p36.22 included *TNFRSF*14. Although pattern of copy-number alterations of double-hit follicular lymphoma is similar to that of western conventional follicular lymphoma, there is significant difference in gains of 2p25.3-p11.1 (with minimal common region of 2p16.1) ($P = 0.0001$) and 12q11-q24.33 ($P = 0.0079$); and loss of 18q21.32-q23 ($P = 0.0385$) (Supplementary Data 1 and 2).[15] Therefore, it is expected that those regions with their target genes are related to the lymphoma pathogenesis. In double-hit follicular lymphoma, case 6 which is the only case showed diffuse large B-cell lymphoma transformation, has 6q loss and 8p loss. 6q large loss and 8p loss are the typical alterations acquired in B-cell lymphoma transformation to aggressive phases. The genome profile of high-grade B-cell lymphomas with *MYC* and *BCL*2 and/or *BCL*6 rearrangements was more complex with more copy-number alterations. In comparison with high-grade B-cell lymphomas with *MYC* and *BCL*2 and/or *BCL*6 rearrangements, double-hit follicular lymphoma was characterized by few copy-number alterations throughout the whole genome except an minimal common region of gain at 2p16.1 including *BCL*11*A*. Interestingly, common regions between the two entities were found at 7p21.1 and the target gene of *HDAC*9. We firstly demonstrated differences in copy-number alterations between high-grade B-cell lymphomas with *MYC* and *BCL*2 and/or *BCL*6 rearrangements and double-hit follicular lymphoma. Histological transformation of follicular lymphoma may need more genomic change other than the *MYC* gene.

In conclusion, double-hit follicular lymphoma tended to be high-grade (grade 3) with high MYC positivity. The genomic profile of double-hit follicular lymphoma is characterized by few copy-number alterations with a profile throughout whole genome and minimal common region of gain at 2p16.1. The clinical behavior of double-hit follicular lymphoma tends to be less aggressive than high-grade B-cell lymphomas with *MYC* and *BCL*2 and/or *BCL*6 rearrangements.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)