

ARTICLE



Double/triple hit lymphoma in the gastrointestinal tract: clinicopathological features, PD-L1 expression and screening strategy

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We aimed to detect the clinicopathological features and immune microenvironment of double-hit/triple-hit lymphoma in the gastrointestinal tract (GI-DHL/THL) and identify the best diagnostic strategies. A total of 114 cases, including 15 GI-DHL/THL, 42 non-GI-DHL/THL and 57 control diffuse large B-cell lymphoma (DLBCL) cases, were comparatively analyzed for their clinicopathological characteristics, the expression of the immune-regulatory checkpoint PD-L1 and immune microenvironment. We applied univariate and multivariate analyses to determine predictors of DHL/THL. GI-DHL/THL patients showed a higher prevalence of previous infection with hepatitis B virus (HBV) than those with GI-DLBCL. Morphologically, 87% of cases exhibited features of DLBCL. Regarding immunohistochemistry results, the MYC protein expression and the Ki-67 proliferation index were significantly higher in the GI-DHL/THL group than in the GI-DLBCL group. The main source of PD-L1 expression in DHL was tumor-associated macrophages, whereas some tumor cells were positive for PD-L1 in GI-DLBCL cases, as determined through multiplex immunofluorescence staining. The multivariable logistic analysis suggested that 5 variables, namely, age, Mum1, CD10, MYC, and HBV infection status, reflect the risk of DHL/THL. The GI-DHL/THL group show different clinicopathological features and immune microenvironments from DLBCL, which might suggest that different signaling pathways are involved. More work is needed to elucidate the pathogenic mechanism of GI-DHL/THL.

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INTRODUCTION

The gastrointestinal (GI) tract is the most common extranodal site involved in non-Hodgkin lymphoma (NHL)¹. Diffuse large B-cell lymphoma (DLBCL) and marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT) constitute the largest parts of GI-NHL. Similar to DLBCL in other sites, GI-DLBCL is a heterogeneous group including DLBCLs that have transformed from low-grade B cell lymphoma as well as de novo disease. The latter can be further subdivided into DLBCL, not otherwise specified (NOS), double-hit/triple-hit lymphoma (DHL/THL) and high-grade B-cell lymphoma (HGBL), NOS and other types since 2016 by the application of the gold standard test fluorescence in situ hybridization (FISH). Double-hit/triple-hit lymphoma is defined as HGBL with *MYC* and *BCL2* or/and *BCL6* rearrangements². Clinically, DHL/THL is characterized by a rapidly progressive and advanced stage of disease, high rates of central nervous system (CNS) involvement, refractoriness to conventional therapy and inferior clinical outcomes^{2,3}.

Double-hit/triple-hit lymphoma remains a rare type of lymphoma. The overall incidence varies according to reports and makes up 6–14% of DLBCL cases^{4–6}. The diagnosis and treatment of DHL/THL can still be challenging, although much progress has

been made in various aspects such as molecular genetic studies and treatment in recent years. The molecular genetic studies included whole-genome sequencing, copy-number variants, and structural variants^{7,8}. Double-hit/triple-hit lymphoma has a spectrum of morphology, for example, some appear similar to DLBCL or Burkitt lymphoma (BL), and some have blastoid or lymphoblastic morphology or gray-zone features. Most DHL/THLs are the germinal center B cell-like (GCB) phenotype as classified by the Hans algorithm, that is, CD10 and *BCL6* expression are found in most of these lymphomas (75–90%)². The differential diagnosis between DHL and DLBCL cannot be made without knowledge of the *MYC*, *BCL2*, and *BCL6* rearrangement status. A subset of DHL/THL will likely be missed regardless of the strategy pathologists use to perform FISH testing unless they order tests for every case. The availability of FISH varies significantly from country to country due to limited diagnostic laboratory resources, biopsy tissue, and expensive price^{6,9,10}. The situation may be worse in developing countries because of the high cost of FISH testing. In addition, other lymphoid and hematological neoplasm mimics, such as transformed follicular lymphoma (FL), which may have both *MYC* and *BCL2* rearrangements and lymphoblastic lymphoma, must be excluded because similar genetic abnormalities can also be

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Table 1. Clinicopathological features of 57 cases with DHL/THL and 57 cases with DLBCL.

	DHL/THL (57)	DLBCL (57)
Site		
GI	15 (26.4)	15 (26.4)
Lymph node	21 (36.8)	21 (36.8)
Non-GI nor lymph node	21 (36.8)	21 (36.8)
Hans classification: no. (%)		
GCB	37 (64.9)	22 (38.6)
Non-GCB	20 (35.1)	35 (61.4)
Follow-up		
Alive with disease (AWD)	35 (64.8)	48 (84.2)
Dead of disease (DOD)	19 (35.2)	9 (15.8)
Lost at follow-up	3	0
Median follow-up in months (range)	17 (1–43)	24 (1–65)

DHL/THL double-hit/triple-hit lymphoma, DLBCL diffuse large B-cell lymphoma, GI gastrointestinal tract, GCB germinal center B cell-like, AWD alive with disease, DOD dead of disease.

detected in these lymphomas. Regarding treatment, no standard therapeutic approach has been recommended. DA-R-EPOCH, BL regimens and R-CHOP are the most common regimens used for treating DHL^{5,9}.

Although DHL/THL is as common in the GI tract as DLBCL¹¹, no detailed description has been reported to date. Thus, the present study aimed to characterize the clinicopathological features of these lymphomas by comparatively analyzing a series of GI-DHL/THL, non-GI-DHL/THL and GI-DLBCL cases. The PD1/PD-L1 pathway is critical in immunotherapy of malignancies including certain subtypes of lymphoma because it has been shown to be an important immune checkpoint in recent years. Most lymphomas show low efficacy of immune checkpoint inhibitor monotherapy, but the underlying reasons remain unclear^{12,13}. In the present study, PD-L1 expression and the immune microenvironment were explored in an attempt to better understand their role in immunotherapy. In addition, we attempted to develop a comprehensive model combining immunohistochemistry (IHC) signatures and clinical features to predict the risk of DHL/THL in patients with DLBCL, providing a useful tool for pathologists.

MATERIALS AND METHODS

Study population

Fifty-seven cases of DHL/THL and 57 control cases of DLBCL were retrospectively collected from 3 institutions (Department of Pathology of Sun Yat-sen University Cancer Center, Zhongnan Hospital of Wuhan University, Xijing Hospital and School of Basic Medicine). Specifically, the 57 cases in the DHL/THL cohort consisted of 15 cases of GI-DHL/THL, 21 cases of lymph node DHL/THL and 21 cases of extranodal DHL/THL. Fifty-one of these cases were included in our previous publication¹¹. The inclusion criteria for control DLBCL cases were those who underwent a FISH study at diagnosis and showed no *MYC* rearrangement from March 2016 to September 2019. For comparison, 57 control cases of DLBCL were consecutive patients who met the inclusion criteria and randomly matched with DHL/THL group according to the anatomic site (GI, LN, and non-GI/LN). The 57 DLBCL cases in the control cohort consisted of 15 cases of GI-DLBCL and 42 cases of DLBCL in non-GI locations (Table 1). The present study was approved by the Research Ethics Committee of these 3 institutes. Clinical data were retrospectively searched from the electronic medical records, including age, sex, tumor site, clinical features, initial presentation, imaging data, Ann Arbor stage, serum lactate dehydrogenase (LDH) levels, hepatitis B virus (HBV) infection status and other laboratory results from the initial examination for definite diagnosis of DHL/THL¹¹.

Pathology review and ancillary testing

The tissue samples were formalin-fixed and paraffin-embedded (FFPE) specimens from either biopsies or resections. All cases underwent primary review by two hematopathologists in each institute and secondary review by Drs. Rao and Tian. Ancillary testing included IHC staining, in situ hybridization (ISH) and FISH, which were performed according to previously published methods¹¹. The antibodies used for IHC are shown in Supplementary Table S2. The tumor cell-of-origin (COO) GCB and non-GCB subtypes were distinguished for CD10, BCL6, and MUM1 expression using thresholds of $\geq 30\%$ to define positivity. Tumors were defined as double-expressor lymphoma (DEL) when *MYC* and *BCL2* protein expression was present in at least 40% and 50% of tumor cells, respectively¹⁴. All 114 cases were tested for *MYC*, *BCL2*, and *BCL6* rearrangements. The probe for *MYC* (8q24) rearrangement was a break-apart probe (01N63-020, Vysis; Abbott Laboratories, IL). The probe for *BCL6* (3q27) rearrangement was also a break-apart probe (01N23-020, Vysis; Abbott Laboratories). In addition, the *IGH/BCL2* fusion translocation t(14; 18) probe (08L60-020, Vysis; Abbott Laboratories) was used for the test of *BCL2*. FISH test was performed according to the manufacturer's instructions.

PD-L1 expression and the immune microenvironment of DHL and DLBCL

Rabbit monoclonal anti-PD-L1 antibody was obtained from Cell Signaling Technology (clone E1L3N, dilution 1:200; Cell Signaling Technology, Danvers, MA, USA). There has been no consensus on the positive threshold of PD-L1 in lymphoma. We defined score 1 as positive staining in $\leq 5\%$ of tumor cells and score 2 as positive staining in $> 5\%$ of tumor cells in the current study^{15,16}. At the same time, the intensity of immunohistochemistry was graded as negative, weak, intermediate or high intensity.

Manual multiplex immunofluorescence (mIF) staining was performed in 4- μ m sections obtained from FFPE blocks by using the Opal 7-Color IHC Kit (PerkinElmer, Waltham, MA). The stained slides were scanned with a Vectra multispectral microscope (PerkinElmer)¹⁷. Prediluted, fluorophore-conjugated antibodies against PD-L1 (same as above), CD20 (from Leica), CD4 (DAKO), CD8 (DAKO), *MYC* (DAKO) and CD68 (PG-M1, DAKO) were used. Human tonsil FFPE tissues were used with and without primary antibodies as positive and negative (autofluorescence) controls, respectively. The procedure was performed as described in a previously published reference¹⁷.

Follow-up

Overall survival was defined as the period from the day of diagnosis to death or the last follow-up. We extended the follow-up period to August 2021 for patients who were alive at the last follow-up in 2019, as described in the previous publication¹¹.

Statistical analysis

Normally distributed data are summarized as the mean (range). The median (range) is presented for non-normally distributed data. The Mann-Whitney *U* test or Student's *t* test was used to compare continuous variables. Categorical variables were expressed as frequencies and were compared using Chi-squared analysis, Fisher's exact test, Yates' continuity-corrected chi-square test or the Mann-Whitney *U* test. Overall survival was analyzed by the Kaplan-Meier method, and comparisons were performed with the log-rank test. Univariate and multivariate logistic regression analyses were performed to determine predictors of DHL/THL. We randomly divided the entire dataset into a training dataset (70% of the original samples) and a validation dataset (30% of the original samples). Missing values were imputed based on the means. All variables with *p* values less than 0.05 in univariate analysis were selected for multivariate analysis. Backward feature selection was performed with the Akaike information criterion. The variables selected by the above procedure were applied to develop the final model on the training dataset. The discriminative ability of the model was measured by area under receiver operating characteristic curve (ROC, R package pROC), and the optimal cutoff with sensitivity of 80% was calculated. In addition, the discrimination of the model was internally validated by applying the model to validation dataset without any adjustment. The results of the modeling process were also converted to a nomogram, which depicted the relative importance of each predictor and could be used to calculate the estimated value of DHL/THL risk. We used R statistical software (version 4.0.1) to complete the analysis. Statistical analysis was performed with SPSS 22.0 software to analyze the clinical data. A *p* value < 0.05 was considered statistically significant.

Table 2. Clinicopathologic characteristics of DHL/THL in GI.

Case #	Sex	Age (y)	Location	History (mo)	Timing of diagnosis	B symptoms	CNS involvement	Stage	Regimen for the first-line treatment	Survival after diagnosis (mo)	Current status	EBER	HBV infection status	Morphology	DHL/THL
1	F	70	Stomach	NA	Transformation ^a	No	No	III	R-CHOP	43	AWD	-	NHHI	D	D1
2	M	23	Colon	2	De novo	Yes	No	IVA	CHOP	37	AWD	-	NHHI	D	D2
3	F	56	Stomach	1	De novo	NA	No	IV	R-CHOP	1	DOD	NA	NA	D	D1
4	F	49	Rectum	1	De novo	No	No	IV	R-CHOP	6	DOD	-	PHI	D	D1
5	M	42	Small intestine	3	De novo	Yes	No	II	R-CHOP+HD-MTX+IT	25	AWD	-	NHHI	D	D2
6	M	57	Stomach	3	NA	NA	NA	NA	NA	2	DOD	+	NA	D	D2
7	M	81	Stomach	1	De novo	Yes	No	IV	NA	2	DOD	-	PHI	D	D2
8	F	59	Stomach	1	De novo	Yes	No	IIIE	DA-REPOCH +HD-MTX+IT	9	AWD	-	NHHI	D	THL
9	M	82	Small intestine	5	De novo	No	NA	IV	R+lenalidomide	5	AWD	+	PHI	B	D2
10	F	52	Stomach	6	De novo	Yes	NA	IV	NA	5	DOD	-	NA	D	D2
11	M	47	Stomach	1	De novo	Yes	NA	IV	NA	1	DOD	-	PHI	B	D1
12	M	46	Colon	1	De novo	No	No	IV	R-CHOP-E	LFU	LFU	-	PHI	D	D1
13	M	48	Caecum	2	NA	No	No	IVA	CHOP	16	AWD	-	NA	D	D1
14	M	62	Jejunum	6	De novo	No	No	II	NA	18	AWD	-	PHI	D	D2
15	F	20	Stomach	NA	NA	NA	NA	NA	NA	LFU	LFU	-	NA	D	D2

DHL/THL double-hit/triple-hit lymphoma, GI gastrointestinal tract, F female, M male, AWD alive with disease, DOD dead of disease, CHOP the regimen of cyclophosphamide, doxorubicin, vincristine, and prednisone, DA-R-EPOCH dose-adjusted regimen of rituximab, etoposide, prednisone, vincristine, cyclophosphamide and doxorubicin, HD-MTX high-dose methotrexate, IT intrathecal injection, LFU lost to follow-up, LN lymph node, NA not applicable, R-CHOP the regimen of rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone, R-CHOP-E the regimen of R-CHOP with the addition of etoposide, PHI previous HBV infection, NHHI no history of HBV infection, Morphology B BCLU, Morphology D DLBCL, D1 MYC/BCL2, D2 MYC/BCL6.

^aTransformation: this case was transformed from DLBCL.

Table 3. Comparison of clinicopathological features between GI-DHL/THL and other groups.

	GI-DHL/THL (15)	Non-GI-DHL/THL (42)	Lymph node-DHL/THL (21)	GI-DLBCL (15)
Age, mean (range), years	52.9 (20.0–82.0)	62 (19.0–81.0)	50.3 (19.0–62.0)	62.6 (37.0–91.0)
Sex: no. (%)				
Female	6 (40.0)	17 (40.5)	8 (38.1)	7 (46.7)
Male	9 (60.0)	25 (59.5)	13 (61.9)	8 (53.3)
History, median (range), months	2 (1.0–6.0)	1 (0.25–34)	1 (0.25–34.0)	2 (1.0–6.0)
B symptoms: no. (%)				
Yes	6 (50.0)	14 (35.0)	7 (36.8)	5 (33.3)
No	6 (50.0)	26 (65.0)	12 (63.2)	10 (66.7)
NA	3	2	2	
Stage: no. (%)				
I/ II	3 (23.1)	12(30.8)	5 (25.0)	11 (73.3)
III/ IV	10 (76.9)	27 (69.2)	15 (75.0)	4 (26.7)
NA	2	3	1	0
CNS involvement: no. (%)				
Yes	0 (0)	4 (11.4)	0 (0)	0 (0)
No	10 (100)	31 (88.6)	17 (100)	15 (100)
NA	5	7	4	
Bone marrow involvement: no. (%)				
Yes	0 (0)	3 (7.9)	2 (11.1)	0 (0)
No	8 (100)	35 (92.1)	16 (88.9)	15 (100)
NA	7	4	3	
Hans classification: no. (%)				
GCB	11 (73.3)	26 (61.9)	14 (66.7)	7 (46.7)
Non-GCB	4 (26.7)	16 (38.1)	7 (33.3)	8 (53.3)
IPI score: no. (%)				
<3	3 (30.0)	20 (55.6)	9 (52.9)	10 (66.7)
≥3	7 (70.0)	16 (44.4)	8 (47.1)	5 (33.3)
NA	5	6	4	0
DEL				
Yes	7 (46.7)	26 (61.9)	13 (61.9)	4 (26.7)
No	8 (53.3)	16 (38.1)	8 (38.1)	11 (73.3)
LDH, median (range), U/L	304.4 (159.1–1020)	270.5 (122.6–2617)	412 (122.6–1405)	204.0 (106–644)
HBV infection status				
NHHI	4 (40.0)	20 (50.0)	9 (45.0)	13 (86.7)
PHI	6 (60.0)	20 (50.0)	11 (55.0)	2 (13.3)
NA	5	2	1	0
Follow-up				
AWD	7 (53.9)	28 (68.3)	11 (55.0)	11 (73.3)
DOD	6 (46.1)	13 (31.7)	9 (45)	4 (26.7)
LFU	2	1	1	0
Median follow-up in months (range)	16 (1–43)	19 (1–40)	22.5 (1–36)	21 (9–65)
Immunophenotype				
CD10				
Positive	11 (73.3)	24 (57.1)	12 (57.1)	6 (40.0)
Negative	4 (26.7)	18 (42.9)	9 (42.9)	9 (60.0)
BCL6				
Positive	12 (80.0)	39 (92.9)	20 (95.2)	11 (73.3)
Negative	3 (20.0)	3 (7.1)	1 (4.8)	4 (26.7)
MUM1				
Positive	6 (40.0)	23 (56.1)	11 (55.0)	10 (66.7)
Negative	9 (60.0)	18 (43.9)	9 (45.0)	5 (33.3)

Table 3. continued

	GI-DHL/THL (15)	Non-GI-DHL/THL (42)	Lymph node-DHL/THL (21)	GI-DLBCL (15)
NA	0	1	1	0
BCL2				
Positive	10 (66.7)	34 (85.0)	16 (80.0)	9 (60.0)
Negative	5 (33.3)	6 (15.0)	4 (20.0)	6 (40.0)
NA	0	2	1	0
Ki-67 (%), median (range)	90 (60–98)	90 (30–95)	90 (30–95)	80 (20–90)
MYC (%), median (range)	70 (20–100)	70 (0–100)	70 (45–100)	40 (5–85)
EBER				
Positive	2 (14.3)	1 (2.6)	1 (5.6)	0 (0)
Negative	12 (85.7)	38 (97.4)	17 (94.4)	15 (100)
NA	1	3	3	
DHL type				
MYC/BCL2	6 (42.9)	11 (27.5)	8 (38.1)	-
MYC/BCL6	8 (57.1)	29 (72.5)	13 (61.9)	-

Bold font indicates $p < 0.05$.

GI-DHL/THL double-hit/triple-hit lymphoma in the gastrointestinal tract, GI-DLBCL diffuse large B-cell lymphoma in the gastrointestinal tract, DHL/THL double-hit/triple-hit lymphoma, GCB germinal center B cell-like, AWD alive with disease, DOD dead of disease, LFU lost at follow-up, PHI previous HBV infection, NHHI no history of HBV infection, NA not available.

Table 4. Expression of PD-L1 in patients with DHL/THL or DLBCL.

	DHL/THL (15)	DLBCL (57)	<i>p</i>
PD-L1 IHC intensity			0.878
Negative	4 (26.7)	4 (7.0)	
Weak	2 (13.3)	26 (45.6)	
Moderate	5 (33.3)	16 (28.1)	
Strong	4 (26.7)	11 (19.3)	
PD-L1 IHC extent			0.003
Score 1	7 (46.7)	7 (12.3)	
Score 2	8 (53.3)	50 (87.7)	

Bold font indicates $p < 0.05$.

DHL/THL double-hit/triple-hit lymphoma, DLBCL diffuse large B-cell lymphoma, Score 1 positive staining in $\leq 5\%$ of tumor cells, Score 2 positive staining in $> 5\%$ of tumor cells.

RESULTS

Clinical characteristics and pathological findings of GI-DHL/THL

The demographic and clinical information of GI-DHL/THL patients and the morphological features of their tumors are summarized in Table 2. There were 9 males and 6 females in this group. The median age of the patients was 52.9 years (ranging from 20 to 82 years). The stomach was the most common site of GI-DHL/THL (8/15, 53%), followed by the small intestine (3/15, 20%), colon (3/15, 20%) and rectum (1/15, 7%). The main manifestations at onset of disease included abdominal pain, bloating, vomiting, abdominal mass, melena, and frequent and loose stools. Six patients (40%) had a past history of previous infection with HBV. Ten of the patients (75%) presented with Ann Arbor stage III/IV. No CNS or bone marrow involvement was identified. The International Prognostic Index (IPI) scores of nearly half of the cases (7/15) were above 3 (Table 3). They were mainly treated with R-CHOP or DA-R-EPOCH as frontline immunochemotherapy.

The cohort contained 14 cases of DHL and 1 case of THL, and the prevalence of MYC/BCL6 DHL (8/15, 53.3%) was higher than that of MYC/BCL2 DHL (6/15, 40%) (Table 2). The only case of THL was located in the gastric angle of a 59-year-old female.

Morphologically, 13 cases (87%) exhibited features of DLBCL, while the other 2 had the appearance of unclassifiable B-cell lymphoma, with features intermediate between DLBCL and BL. Regarding IHC staining results (Tables 3), 73.3% of the cases (11/15) were classified as belonging to the GCB subtype, and DEL constituted half of the cases (7/13, 53.8%; MYC IHC was not available in 2 cases). The median MYC protein expression was 70%. The average Ki-67 proliferation index was 90%, ranging from 60% to 98%.

As of August 2021, six patients had died of the disease, and seven in the cohort were still alive. The other two were lost to follow-up. The survival time ranged from 1 to 43 months, the latter being the full length of the follow-up period.

Parallel comparison between GI-DHL/THL and non-GI-DHL/THL or other extranodal DHL/THL cases

There was no significant difference between GI-DHL/THL and overall non-GI DHL/THL ($n = 42$), GI-DHL/THL and exclusively nodal DHL/THL ($n = 21$), or GI-DHL/THL and other extranodal DHL/THL ($n = 21$) (not shown) in clinical characteristics, such as age, sex, B symptoms, stage, CNS or bone marrow involvement, serum LDH level, and IPI score (Table 3). As for pathological parameters, there were no prominent differences, such as morphology, COO, rates of DEL, expression of selected immunomarkers such as the MYC protein, Ki-67 index, or EBER status. MYC/BCL6 DHL constituted a larger percentage than MYC/BCL2 DHL in all 3 groups, but there was no significant difference between groups.

Comparison of clinicopathological features and immunophenotypic differences between GI-DHL/THL and GI-DLBCL cases

GI-DHL/THL patients were found to be younger than GI-DLBCL patients (Table 3). More patients (10/13, 76.9%) presented with Ann Arbor stage III/IV disease in the GI-DHL/THL group than in the GI-DLBCL group (4/15, 26.7%) ($p < 0.05$). The rate of previous HBV infections was significantly higher in the GI-DHL/THL group than in the GI-DLBCL group. There were no prominent differences in other clinical parameters, such as sex, B symptoms, CNS or bone marrow involvement, serum LDH level, and IPI score (Table 3). There was also no significant difference in morphology, COO, rate of DEL or EBER status. However, the expression of the MYC protein

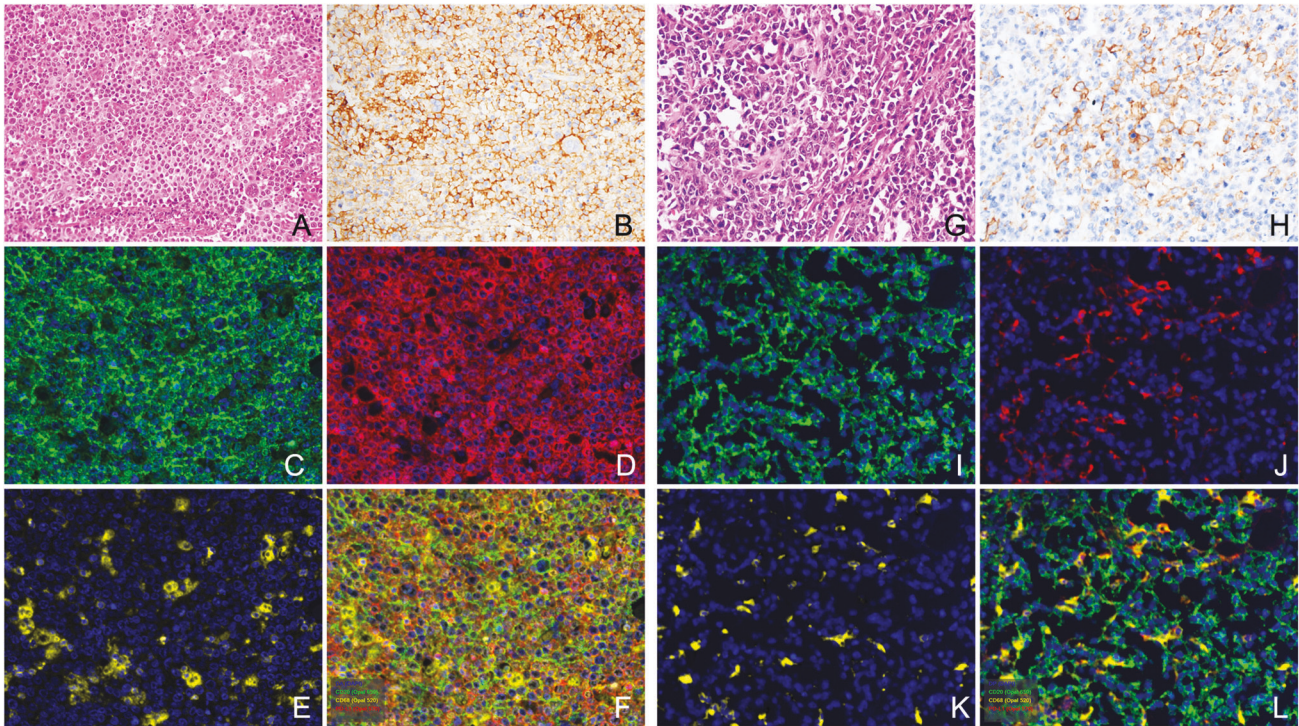


Fig. 1 Morphology, PD-L1 expression (IHC) and microenvironment of DLBCL (images A–F) and DHL (G–L) cases in the GI tract. Regarding the microenvironment, coexpression of CD20, PD-L1 and CD68 were detected by multiplex immunofluorescence. **A, G:** H&E staining; **B, H:** PD-L1 expression (IHC); **C, I:** CD20 (green); **D, J:** PD-L1 (red); **E, K:** CD68 (yellow); **F, L:** merge image for CD20, PD-L1 and CD68 (original magnification of all images $\times 400$).

and the Ki-67 proliferation index were significantly higher in the GI-DHL/THL group than in the GI-DLBCL group ($p < 0.05$). No other parameters or immunomarkers showed differential expression between these two groups.

PD-L1 and mIF results in DHL/THL and DLBCL cases

Fifteen tissue samples from DHL (representing 10 GI and 5 non-GI cases) and 57 tissue samples from DLBCL were available for IHC of PD-L1. The intensity and extent of expression differed between DHL/THL and DLBCL cases, but only the extent was significantly different between these two groups ($p < 0.05$) (Table 4). DLBCLs mainly showed a weak to moderate PD-L1 staining pattern (Table 4 and Fig. 1A, B), and 50 cases (87.7%) showed over 5% positive tumor cells, whereas DHLs demonstrated intermediate intensity but sparse staining ($< 5\%$ of tumor cells) (Table 4 and Fig. 1G, H). That is, more lymphoma cells in DLBCL cases showed PD-L1 positivity, while hardly any PD-L1-expressing tumor cells were identified in DHL cases. Consistent with the IHC results, mIF showed that coexpression of CD20 and PD-L1 was more common in DLBCL (Fig. 1C, D, F and I, J, L). Coexpression of PD-L1 and CD20 was less frequently observed in DHL, while cells positive for both PD-L1 and CD68 were observed, which demonstrated that PD-L1 was mainly expressed in macrophages in DHL (Fig. 1D–F and J–L). Seldom were cells positive for PD-L1 and CD4 or CD8 (not shown). Interestingly, one DLBCL was incidentally found to have strong and intense expression of CD4 in the mIF image (not shown).

DHL/THL screening strategy

The overall DHL/THL cohort, consisting of 57 cases, showed a younger age at the onset of disease, a higher incidence of previous HBV infection, and higher percentages of the GCB type and DEL phenotype than the cohort of 57 DLBCL cases (Table 5). In accordance with the more common GCB phenotype, higher expression of CD10 and lower expression of Mum-1 were

observed in the DHL/THL cohort. MYC and the Ki-67 proliferation index were significantly different between these two groups, that is, the DHL/THL cohort had higher MYC protein expression and faster growth. During the follow-up period, the proportion of patients died of the disease in the DHL/THL group was significantly higher than that in the DLBCL group ($p < 0.05$). There were no differences in clinical characteristics, MYC IHC, or the Ki-67 proliferation index between DHL/THL and DLBCL of the GCB type (not shown).

The final logistic model was developed from the 5 variables selected using backward feature selection. The formula was as follows: risk score = $0.03 - 0.05 \times \text{Age} - 1.65 \times \text{Mum1} + 0.20 \times \text{CD10} + 0.06 \times \text{MYC} + 1.61 \times \text{HBV infection status}$; the results are shown in Supplementary Table S1. The categorical variables in the formula were defined as follows: Mum1 IHC (0 = negative, 1 = positive); CD10 IHC (0 = negative, 1 = positive); HBV infection status (0 = no history of HBV infection, 1 = previous HBV infection). The area under the curve (AUC) was 0.89 (95% confidence interval: 0.81–0.96) in the training dataset (Fig. 2A). The optimal cutoff was 0.47, which suggested that DLBCL patients with risk scores greater than 0.47 were more likely to have DHL/THL. The sensitivity was 0.81, and the specificity was 0.77. Applying the model to the validation dataset, we obtained an AUC of 0.85 (95% confidence interval: 0.72–0.99) (Fig. 2B). With the same cutoff, the sensitivity and specificity in the validation dataset were 0.86 and 0.77, respectively. According to the nomogram (Fig. 2C), the contributions of all variables could be simply added, and the results reflected the individual risk of DHL/THL, which provides a straightforward model for pathologists to decide whether to perform FISH testing on patients with DLBCL. We have used GI-DHL/THL and GI-DLBCL to verify the model, and obtained an AUC of 0.982, which showed that the model could also be applied to GI-DHL/THL cases (Supplementary Fig. 1).

Table 5. Comparison of clinicopathological features in DHL/THL and DLBCL cases.

	DHL/THL (57)	DLBCL (57)	<i>p</i>
Age, mean (range), years	51.8 (19.0–82.0)	59.1 (23–91)	0.008
Sex: no. (%)			
Female	23 (40.4)	27 (47.4)	0.450
Male	34 (59.6)	30 (52.6)	
History, median (range), months	1.0 (0.25–34.0)	2.0 (1.0–36.0)	0.566
B symptoms: no. (%)			
Yes	20 (38.5)	12 (21.1)	0.046
No	32 (61.5)	45 (78.9)	
NA	5	0	
Stage: no. (%)			
I/ II	15 (28.9)	23 (40.3)	0.208
III/ IV	37 (71.1)	34 (59.7)	
NA	5	0	
CNS involvement: no. (%)			
Yes	4 (8.9)	0 (0)	0.075
No	41 (91.1)	57 (100)	
NA	12	0	
Bone marrow involvement: no. (%)			
Yes	3 (6.5)	0 (0)	0.172
No	43 (93.5)	57 (100)	
NA	11	0	
Hans classification: no. (%)			
GCB	37 (64.9)	22 (38.6)	0.005
Non-GCB	20 (35.1)	35 (61.4)	
IPI score: no. (%)			
<3	23 (50.0)	32 (56.1)	0.535
≥3	23 (50.0)	25 (43.9)	
NA	11	0	
DEL			
Yes	33 (57.9)	21 (36.8)	0.024
No	24 (42.1)	36 (63.2)	
LDH, median (range), U/L	272.1 (122.6–2617)	216 (106–1598)	0.149
HBV infection status			
NHHI	24 (48.0)	43 (75.4)	0.003
PHI	26 (52.0)	14 (24.6)	
NA	7	0	
Follow-up			
AWD	35 (64.8)	48 (84.2)	0.019
DOD	19 (35.2)	9 (15.8)	
LFU	3	0	
Median follow-up in months (range)	17 (1–43)	24 (1–65)	0.016
Immunophenotype			
CD10			
Positive	35 (61.4)	20 (35.1)	0.005
Negative	22 (38.6)	37 (64.9)	
BCL6			
Positive	51 (89.5)	50 (87.7)	0.768
Negative	6 (10.5)	7 (12.3)	

Table 5. continued

	DHL/THL (57)	DLBCL (57)	<i>p</i>
Mum-1			
Positive	29 (51.8)	43 (75.4)	0.009
Negative	27 (48.2)	14 (24.6)	
NA	1	0	
BCL2			
Positive	44 (80.0)	45 (78.9)	0.890
Negative	11 (20.0)	12 (21.1)	
NA	2	0	
Ki-67 (%), median (range)	90 (30–98)	80 (20–90)	0.024
MYC (%), median (range)	70 (0–100)	30 (0–85)	<0.001

Bold font indicates $p < 0.05$.

DHL/THL double-hit/triple-hit lymphoma, DLBCL diffuse large B-cell lymphoma, GCB germinal center B cell-like, AWD alive with disease, DOD dead of disease, LFU lost at follow-up, PHI previous HBV infection, NHHI no history of HBV infection, NA not available.

Progression and survival

The prognosis of patients with GI-DHL/THL was worse than that of patients with GI-DLBCL (Fig. 3A). In addition, the overall survival of the DHL/THL group was also worse than that in the DLBCL group (Fig. 3B), in line with the follow-up mentioned above. Survival analysis showed no difference between GI-DHL/THL and non-GI-DHL/THL or among DHL/THL in GI, nodal and other extranodal sites (not shown).

DISCUSSION

Thirty to forty percent of extranodal NHLs occur in the GI tract¹. Additionally, the GI tract is the most common extranodal site of DLBCL². By whole-exome sequencing, the genomic mutation profile of GI-DLBCL has been found to differ from that of other DLBCLs in that the former has higher mutation frequencies in TP53, MUC16, CCND3, HIST1H1C and ID3 but lower mutation frequencies in MYD88, CREBBP, BCL2, KMT2D, PIM1 and EZH2¹⁸. Importantly, the GI tract is also one of the most common sites of DHL/THL¹¹. Interestingly, there was no CNS or bone marrow involvement in the present GI-DHL/THL cases, which is inconsistent with previous reports^{2,3}. A larger sample size and longer follow-up will be needed to test the possible implication that GI-DHL/THL is slightly more indolent or less aggressive than DHL/THL in other sites. It was found that the prevalence of previous HBV infection was higher in the GI-DHL/THL group than in the GI-DLBCL group; thus, previous HBV infection may be a risk factor for GI-DHL/THL. This is consistent with a previous publication and the results of the final logistic analysis in the present study¹¹. No other clinical parameter showed a significant difference between GI-DHL/THL and non-GI-DHL/THL or GI-DLBCL.

Morphologically, 13 GI-DHL/THL cases (87%) exhibited features of DLBCL, while the other 2 had the appearance of unclassifiable B cell lymphoma, with features intermediate between DLBCL and BL in the current study. The differential diagnoses of GI-DHL/THL might include, but are not limited to, poorly differentiated carcinoma, neuroendocrine carcinoma and other aggressive B cell lymphomas. The most important differential diagnoses might be DLBCL-NOS, MYC single-hit DLBCL, and transformed double-hit/triple-hit follicular lymphoma. However, differential diagnosis is not challenging when the past medical history, observation of the follicular dendritic cell (FDC) meshwork, and the performance of additional FISH testing for BCL2 and BCL6 are utilized. From the

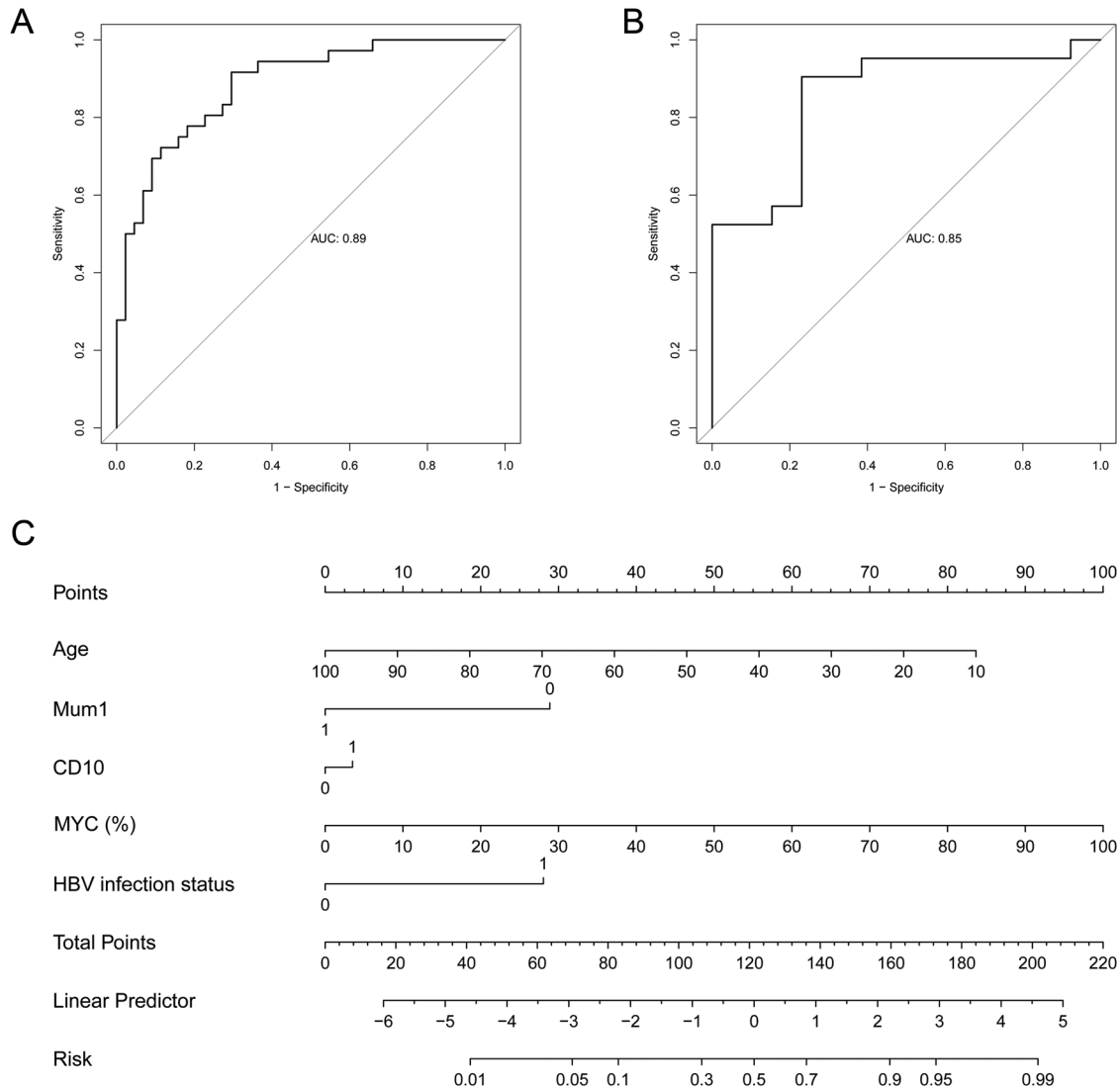


Fig. 2 **Nomogram and model performance.** **A** ROC curve analysis in the training dataset. **B** The ROC curve analysis in the validation dataset. **C** Nomogram based on IHC signatures and clinical features.

present study results, a high Ki-67 proliferation index and MYC expression are helpful to prompt testing for MYC rearrangement. In our study, MYC/BCL6 rearrangement was more common than MYC/BCL2 rearrangement in the GI tract, as in other sites.

Immune checkpoint inhibitors, such as PD1/PD-L1 inhibitors, have been reported since 2018 to be involved in various tumors, including classical Hodgkin lymphoma. The prognostic value of PD-L1 in the treatment of DLBCL remains to be verified because of conflicting results reported in the literature. In the present study, by means of IHC and mIF, we found that PD-L1 is mainly expressed by tumor cells and tumor-associated macrophages (TAMs) in DLBCL, whereas TAMs are the main source of PD-L1 expression in DHL/THL, suggesting that DHL has a different microenvironment from DLBCL. Previous findings indicated that PD-L1 is predominantly expressed in TAMs but not lymphoma B cells based on dual IHC staining¹⁹. This is slightly different from our findings. Few T cells have been found either in GI-DLBCL or DHL/THL. The result of mIF was consistent with the result of IHC but provided us with a more straightforward way to verify the spatial information and microenvironment of the tumor cells. The present study suggests that DHL/THL is a “colder” tumor than DLBCL, which is in line with the fact that DHL/THL needs stronger therapeutic regimens and has a worse prognosis than DLBCL. In

addition, TAM-associated targeted treatment may be helpful for DHL/THL in the near future because TAMs outnumber other immune cells. Because of the small sample size, we could not stratify the cases for further investigation.

Although FISH is a standard technique for testing DHL/THL, its availability has some restrictions due to financial issues, tissue quantity, and the resources of the diagnostic laboratory. In addition, there is the possibility of missing those DHL/THL tumors with cryptic mutations or only copy number alterations^{7,20}. For instance, one study identified a unique set of alternative DHL/THL cases that feature concurrent translocations and copy number gains in MYC, BCL2, and/or BCL6, but this special entity cannot readily be identified using standard FISH alone²⁰. A recommended method is to test for MYC rearrangement in all tumors with DLBCL morphology and then test for BCL2 and BCL6 rearrangements in the cases where FISH detects MYC rearrangement⁶. However, a new alternative screening strategy is still necessary. LMO2 loss may be a good predictor of the presence of MYC translocation in CD10-positive DLBCL²¹. However, we could not find a correlation between the LMO2 IHC signal and rearrangement in DHL/THL (Supplementary Table S3).

There has been considerable controversy over what subset of patients with DLBCL might have DHL/THL in the absence of FISH

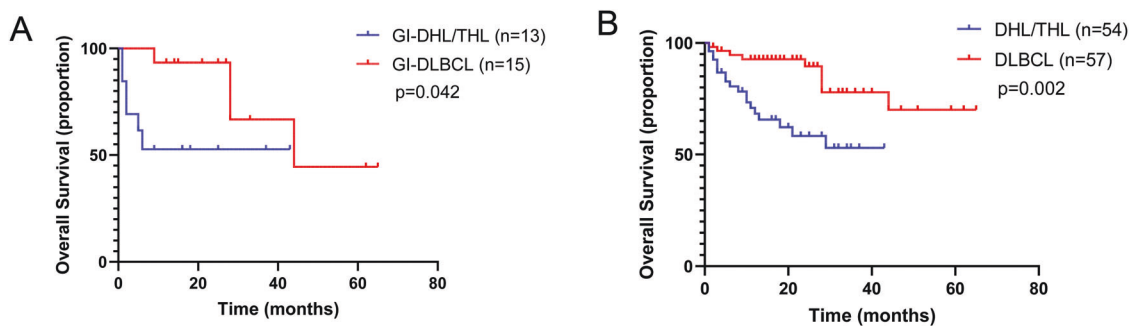


Fig. 3 Survival analysis. **A** Comparison of the prognosis of patients with GI-DHL/THL and GI-DLBCL. Two of 15 patients in GI-DHL/THL group were lost to follow-up. **B** Comparison of the prognosis of patients with overall DHL/THLs and DLBCLs. Three of 57 patients in DHL/THL group were lost to follow-up, including 2 cases from GI and 1 case from lymph nodes.

results. Screening strategies such as MYC IHC or COO plus DEL miss approximately one-quarter to one-third of DHL/THL cases^{6,8}. Therefore, comprehensive screening strategies are urgently needed. We developed a comprehensive model with two types of independent predictors (IHC signatures and clinical features) using the following variables: age, Mum1, CD10, MYC, and HBV infection status. Furthermore, a nomogram was proposed to provide pathologists with a visual tool with the candidate predictors.

In the IPI scoring system, age over 60 years old is a prognostic risk factor. In this study, the age of onset of DHL/THL was younger than that of DLBCL, which suggested that early onset might indicate more aggressive biological behavior.

It is known that CD10 and BCL6 are considered as markers of the GCB phenotype and MUM1 is the marker of the non-GCB phenotype per the Hans classification²². Consistent with a previous publication²³, we also found that a great majority of DHL/THL cases are of the GCB subtype. In the present study, the percentage of CD10 positive cases in the DHL/THL group was significantly higher than that in the DLBCL group. Meanwhile, the percentage of MUM1 positive cases in the DHL/THL group was significantly lower than that in the DLBCL group. Other studies have shown that almost all cases of *MYC/BCL2* DHL have the GCB phenotype, while *MYC/BCL6* DHL has a lower frequency of the GCB phenotype²⁴. Therefore, if only DLBCLs with the GCB type are checked by FISH testing, a subset of DHL/THL will be missed, especially *MYC/BCL6* DHL. Abdullah et al. reported that MYC IHC was a predictor for DHL/THL²⁵. MYC IHC was also found to be a risk factor in our model. Hepatitis B surface antigen (HBsAg)-seropositive patients have a significant two- to threefold increase in their risk of developing B cell NHL compared with HBsAg-seronegative patients, and the increase is especially large for DLBCL²⁶. Several lines of investigation have shown that HBV infection status is a risk factor for NHL and influences the choice of immunochemotherapy drugs^{27,28}. We found that the HBV infection rate of DHL/THL patients was significantly higher than that of DLBCL patients, which indicated that HBV might be an important risk factor affecting the tumorigenesis of DHL/THLs. Ha Nguyen et al. validated a novel gene expression assay (DLBCL90) to distinguish DHL/THL containing a *BCL2* translocation, which requires sequencing data²⁹. Interestingly, all variables in our models were easily obtained, which means that it is more practical than the DLBCL90 assay. We proposed the model based on multivariate logistic regression analysis of selected variables. The ROC of the validation dataset had an AUC of 0.85, and that of the training dataset had an AUC of 0.89, which supported the ability of the model to detect DHL/THL. We believe the model will help to identify patients with DLBCL who require FISH testing and reduce unnecessary testing. This model can also be applied to DHL/THL cases of different locations and was verified with GI-DHL/THLs in the present study.

The present study has several limitations. First, the sample size was small, and potential selection bias could not be excluded, although there have been no previous reports of GI-DHL/THL. Second, the majority of the GI-DHL/THL samples were obtained from biopsies, and we were therefore unable to perform further molecular testing such as next-generation sequencing. More work is needed to elucidate the pathogenic mechanism resulting in this special entity.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

ST and HR designed the research; JG, JX and YC performed the experiments; XX, HC and JZ assisted with the experiments; JG and YC analyzed the results and made the tables and figures; ST, ZW and HR reviewed the cases and ST wrote the paper. This manuscript was approved by all authors.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Research Ethics Committee of Zhongnan Hospital of Wuhan University, Sun Yat-sen University Cancer Center and Xijing Hospital and performed in accordance with the Declaration of Helsinki.

ADDITIONAL INFORMATION

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