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ORIGINAL ARTICLE

Clinicopathological features of HCV-positive splenic diffuse large B cell lymphoma

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Abstract

The hepatitis C virus (HCV) is a single-stranded RNA virus which is thought to be involved in the onset of B cell lymphoma. HCV-positive diffuse large B cell lymphoma (DLBCL) has been reported to clinically manifest in extranodal lesions (e.g., in the liver, spleen, and stomach). Here, we investigated HCV-positive and -negative primary splenic DLBCL (p-spDLBCL) and non-primary splenic DLBCL (ordinary DLBCL). Furthermore, to examine HCV lymphomagenesis, RNA in situ hybridization (ISH), RT-PCR (reverse-transcription polymerase chain reaction), and NS3 immunostaining of HCV viral nonstructural proteins were performed. HCV-positive p-spDLBCL patients presented fewer B symptoms (asymptomatic) and better performance status, with elevated presence of splenic macronodular lesions and more germinal center B cell (GCB) sub-group cases than HCV-negative p-spDLBCL patients. However, HCV-positive ordinary DLBCL patients were found to have more non-GCB sub-group cases than HCV-negative ordinary DLBCL patients. HCV-positive DLBCL patients showed 20.6% (7/34) NS3 positivity, 16.7% (1/6) HCV-RNA in situ positivity, and 22.2% (2/9) detection of HCV-RNA in tumor tissue by RT-PCR. Splenic samples were found to have a higher frequency of HCV detection than lymph node samples, thus suggesting that HCV may be closely related to lymphomagenesis, especially in splenic lymphoma.

Keywords Hepatitis C virus · Primary splenic DLBCL · NS3 · RNA in situ hybridization · Malignant lymphoma

Introduction

Hepatitis C virus (HCV) infection is present in 1.6 million people worldwide. HCV is a single-stranded ribonucleic acid (ssRNA) virus classified by genotype as HCV1-6, with the prevalence of each genotype varying based on geographic region [1]. HCV infection has been well-demonstrated to contribute to the development of chronic hepatitis (CH), liver cirrhosis (LC), and hepatocellular carcinoma (HCC), while malignant lymphoma, cryoglobulinemia, chronic kidney disease, lichen planus, and porphyria have also been reported to be extrahepatic lesions caused by HCV infection [2].

In epidemiological studies investigating the link between HCV infection and malignant lymphoma, an association between HCV infection and B cell lymphoma has been reported (odds ratio [OR], 10.8) [3]. In particular, the HCV infection rate among patients with diffuse large B cell lymphoma (DLBCL) has been reported as between 10.9–17.4% [4, 5],

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thus making HCV the most frequent infection in B cell lymphoma patients. Furthermore, many reports have indicated that HCV eradication therapy in HCV-positive B cell lymphoma patients resulted in lymphoma regression [6, 7]. In particular, a strong correlation was reported between viral clearance and lymphoma regression in splenic marginal zone lymphoma patients after HCV eradication therapy [6]. Thus, it is currently believed that there is a strong possibility that HCV is involved in the onset of malignant lymphoma.

HCV does not appear to integrate into host cell genomes, and no obvious oncogene is involved. Although the mechanism of B cell lymphomagenesis is not clearly understood, several hypotheses have been previously proposed. The onset of HCV-positive lymphoma is thought to be associated with HCV-induced chronic antigen stimulation and inflammation, similar to how *Helicobacter pylori* has been linked with mucosa-associated lymphoid tissue (MALT) lymphoma [8]. Additionally, it has been reported that oncogene mutation rate is markedly elevated by HCV infection of B cells [9], and that the non-structural protein NS3 may be involved in oncogenesis by inhibiting the activity of p53 [10]. Therefore, the infection of B cells with HCV is likely involved in the onset of malignant lymphoma [11, 12].

The clinical characteristics of HCV-positive DLBCL cases have been reported to include extranodal lesions of the liver, spleen, and stomach [13]. Such cases have also been reported to be more common in the elderly and presenting B symptoms more frequently, as well as showing elevated lactate dehydrogenase levels, poor International Prognostic Index (IPI), and multi-site extranodal involvement [14–17]. Many reports have indicated a higher incidence of liver damage during chemotherapy, although no difference in overall survival and progression-free survival was identified [16].

Here, we investigated the clinicopathological features of HCV-positive DLBCL, particularly focusing on primary spDLBCL (p-spDLBCL) and non-primary spDLBCL (ordinary DLBCL) of both HCV-positive and -negative cases. Furthermore, to elucidate HCV lymphomagenesis, RNA in situ hybridization (ISH), reverse-transcription polymerase chain reaction (RT-PCR), and NS3 immunostaining of HCV viral nonstructural proteins were performed.

Materials and methods

Patients and samples

We reviewed specimens obtained from patients diagnosed with DLBCL between 2005 and 2016 at the Department of Pathology, Kurume University. This study included (1) 12 cases of HCV-positive p-spDLBCL, (2) 46 cases of HCV-negative p-spDLBCL, (3) 25 cases of HCV-positive non-primary-spDLBCL (ordinary DLBCL), and (4) 120 cases of HCV-negative ordinary DLBCL. These 203 cases of DLBCL used in this study were also used in previous studies conducted by our research group [18, 19]. All cases were reviewed by hematopathologists (OK and MH) and were diagnosed according to World Health Organization (WHO) classifications [20]. P-spDLBCL was defined, as reported in previous studies [21–23] according to two clinical patterns: Type A, presenting only lymphadenopathy of the splenic hilum and lacking systemic lymphadenopathy, and Type B, presenting bone marrow, liver, or peripheral blood involvement in addition to splenic lesions but still without systemic lymphadenopathy. Clinical information was obtained by reviewing patient medical charts. The use of clinical samples and information was approved by the Research Ethics Committee of Kurume University and was performed in accordance with the Declaration of Helsinki.

Defining HCV infection

HCV infection was defined to be present in each patient if positive results were observed using second and third generation immunoassay kits (Monolisa anti-HCV Plus, Sanofi Diagnostics Pasteur, France; AxSYM HCV Version 3.0, Abbott Laboratories, Parsippany, NJ). Quantitative real-time polymerase chain reaction was used to determine HCV-RNA abundance (COBAS AmpliPrep/COBAS TaqMan HCV test; Roche Molecular Systems, Pleasanton, CA, USA). Genotype was determined by using patient serum [24]. High viral titer of HCV-RNA was defined as ≥ 5.0 log IU/ml [25].

Immunophenotyping by flow cytometry

Flow cytometric immunophenotypic analysis was performed with a flow cytometer (FACS-Calibur, Becton-Dickinson, Mountain View, CA, USA) and the BD Cell Quest software program (Becton-Dickinson). For flow cytometric immunophenotypic analysis, antibodies (clones) against CD5 (T1) (Beckman Coulter, CA) and CD10 (J5) (Beckman Coulter) were used.

Immunohistochemical analysis

Paraffin-embedded sections for each sample were immunostained. The following antibodies (clones) were used for immunohistochemical analysis: CD5 (4C7) (Leica Microsystems, Wetzler, Germany), CD10 (56C6) (Leica Microsystems), CD20 (L-26) (DAKOCytomation, Glostrup, Denmark), BCL2 (124) (DAKOCytomation), BCL6 (P1F6) (Leica Microsystems), and MUM1 (MUM1p) (DAKOCytomation). A sample was considered positive for a marker if more than ~30% of the neoplastic cells yielded positive labeling.

NS3 immunostaining

Immunohistochemical staining of NCL-HCV-NS3 (Leica Microsystems) was carried out using 2.5-mm-thick, formalin-fixed, paraffin-embedded tissue sections as previously reported [26]. The slides were deparaffinized with xylene, followed by ethanol. After rehydration with water, antigen retrieval was performed with EDTA buffer (pH 7.0) in a microwave oven at 95 °C for 20 min. Endogenous peroxidase activity was blocked via incubation in 3% hydrogen peroxide for 5 min. The slides were incubated with anti-NS3 mouse monoclonal antibodies (NS3; 1:50 dilution; MMM33, Leica Microsystems) for 30 min, following which they were incubated with an EnVision1 System horseradish peroxide-labeled anti-mouse polymer (Dakocytomation) for 30 min. NS3 visualization was performed by incubating the slides with diaminobenzidine for 5 min. The slides were then counterstained with hematoxylin, dehydrated with ethanol, and mounted under coverslips.

Fluorescence in situ hybridization analysis

Fluorescence in situ hybridization (FISH) was performed to assess *IGH-BCL2* translocation (Leica Microsystems) and *BCL6* splitting (Leica Microsystems) as previously described [27].

In situ hybridization for HCV RNA

HCV RNA in situ hybridization was performed on formalin-fixed paraffin-embedded (FFPE) specimens using a HCV1b core probe and a RNAscope® 2.0 assay kit according to the manufacturer's protocols [28].

Tissue RT-PCR in HCV-positive lymphoma

Total RNA was isolated from FFPE tissue sections using a RecoverAll Total Nucleic Acid Isolation Kit (Ambion, Foster City, CA) according to the manufacturer's instructions. RNA samples were quantified with an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). cDNA was reverse transcribed from 5 μg of total RNA using the SuperScript IV First-Strand Synthesis System and random hexamer primers (Invitrogen, Carlsbad, CA). Nested PCR was performed using the AmpliTaq Gold DNA Polymerase Master Mix (Applied Biosystems, Foster City, CA) and primers corresponding to the highly conserved genomic sequence of the HCV 5′-noncoding region. The primer sequences were as follows: outer (1st PCR) primer pair HCV-FW1 (sense: 5′-ctgtgaggaactactgtcttcacgca-3′) and HCV-FW2 (anti-sense: 5′-ttcacgcagaaagcgtctagccatg-3′), and inner (2nd PCR) primer pair HCV-RV1 (sense: 5′-aattccggtgtactcaccggttccg-3′) and HCV-RV2 (anti-sense: 5′-ggtgtactcaccggttccgcagac-3′). Twenty microliters of cDNA was subjected to first-round PCR, with the second PCR step employing 1 μl of the product from the first PCR. The first-round PCR was performed under the following conditions: initial denaturation at 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 10 min. The second-round PCR consisted of 40 cycles of 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s. The amplified products were electrophoresed using a 3% agarose gel and visualized under ultraviolet light after staining with ethidium bromide. After nested PCR, a 107 bp fragment was expected for HCV. The quality of cDNA was monitored using RT-PCR with β-actin primers (SFW sense: 5′-caagagatggccacggctgct-3′, A5RV anti-sense: 5′-tcgtggatgccacaggactcc-3′). cDNA samples yielding both a 155 bp product for β-actin mRNA in the absence of contamination and a 250 bp genomic amplification product were used for experimental amplification.

Statistical analysis

Chi-squared test was used to compare the clinicopathological features between HCV-positive p-spDLBCL and HCV-negative p-spDLBCL. Fisher's exact test was used to compare the clinicopathological features between HCV-positive ordinary DLBCL and HCV-negative ordinary DLBCL. Survival curves of progression-free survival (PFS) and overall survival (OS) were calculated using the Kaplan–Meier method. The endpoints of PFS were defined as the time of relapse due to DLBCL and death, while the endpoint of overall survival was defined as death. Statistical analyses were carried out with JMP version 11 (SAS Institute, Tokyo, Japan). A P value < 0.05 was considered to indicate statistical significance.

Results

Clinicopathological features of HCV-positive primary splenic DLBCL (12 cases)

Table 1 presents 12 cases of HCV-positive p-spDLBCL. The median age was 68.5 years (range, 59–83 years), with a ratio of 1.4 men for each woman. Initial symptoms consisted of 1 case of B symptom (8.3%), 1 case of upper abdominal discomfort (8.3%), and 10 asymptomatic cases (83.4%). Splenic macronodular lesions were observed in 11 of the 12 cases (91.7%). Regarding DLBCL cell-type of origin, 10 cases were of the germinal center B cell type (GCB type; 83.3%) and two cases were of the non-germinal center B cell type (non-GCB type; 16.7%). After FISH analysis, 0 cases (0%) of *BCL2-IGH* translocation and 5 cases (41.7%) of *BCL6* splitting were found. Nine patients were given R-CHOP therapy (composed of rituximab, doxorubicin, cyclophosphamide, vincristine, and prednisone) as initial treatment. One patient was given

Table 1 Clinicopathological features of HCV-positive primary splenic DLBCL (p-spDLBCL) 12 cases

Case	Age	Sex	Clinical type	Primary symptoms	Splenic macronodular lesions	Involvement site	IPI	Immunohistochemistry						COO	FISH analysis		Initial therapy	Therapeutic effect	Follow-up periods (months)	Disease status
								CD5	CD10	CD20	BCL2	BCL6	MUM1		*IGH/BCL2*	*BCL6* split				
1	81	M	A	B symptoms	+	SP	High int	−	−	+	−	−	+	non-GCB	−	−	R-CHOP	CR	40	CR/alive
2	66	M	A	−	+	SP	Low	−	+	+	+	−	+	GCB	−	+	R-CHOP	CR	52	CR/dead
3	69	F	A	−	+	SP	Low int	−	+	+	−	+	−	GCB	−	−	R-CHOP	CR	53	CR/alive
4	81	F	A	−	+	SP	Low int	−	−	+	+	+	−	GCB	−	−	R-CHOP	CR	37	CR/alive
5	82	M	A	Abdominal discomfort	+	SP	Low int	−	+	+	−	+	−	GCB	−	+	RTX	CR	50	CR/alive
6	60	M	A	−	+	SP	Low int	−	+	+	+	−	+	GCB	−	+	R-CHOP	CR	47	CR/alive
7	76	M	A	−	+	SP	Low int	−	−	+	−	+	−	GCB	−	−	R-CHOP	CR	18	CR/dead
8	66	F	A	−	+	SP	Low	−	−	+	+	+	−	GCB	−	+	R-CHOP	CR	94	CR/dead
9	59	M	A	−	+	SP	Low	−	−	+	+	−	+	nonGCB	−	−	R-CHOP	CR	87	CR/alive
10	60	M	A	−	+	SP	Low int	−	−	+	−	+	−	GCB	−	−	NA	NA	NA	NA
11	83	F	A	−	+	SP	Low int	−	+	+	+	+	+	GCB	−	+	No therapy	NA	4	CR/alive
12	68	F	B	−	−	SP,BM	High int	+	+	+	+	+	+	GCB	−	−	R-CHOP	PD	29	PD/dead

M, male; *F*, female; *SP*; spleen; *BM*, bone marrow; *IPI*, International Prognostic Index; *COO*, cell of origin; *RTX*, rituximab; *CR*, complete remission; *NA*, not available; *PD*, progressive disease; *GCB*, germinal center B cell; *R-CHOP*, rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone

Table 2 Comparison of HCV-positive primary splenic DLBCL with HCV-negative primary splenic DLBCL (p-spDLBCL)

		HCV-positive p-spDLBCL ($N = 12$)	HCV-negative p-spDLBCL ($N = 46$)	P value
Clinical information				
Age median[range](years)		68.5 [59–83]	66.5 [27–84]	
Age > 60 year	Yes	91.7% (11/12)	73.9% (34/46)	0.26*
	No	8.3% (1/12)	26.1% (12/46)	
Sex: male	Yes	58.3% (7/12)	58.7% (27/46)	1.00
	No	41.7% (5/12)	41.3% (19/46)	
Hepatomegaly	Yes	0.0% (0/12)	15.2% (7/46)	0.33*
	No	100.0% (12/12)	84.8% (39/46)	
Splenomegaly	Yes	100.0% (12/12)	97.8% (45/46)	1.00*
	No	0.0% (0/12)	2.2% (1/46)	
Bone marrow involvement	Yes	8.3% (1/12)	29.5% (13/44)	0.26*
	No	91.7% (11/12)	70.5% (31/44)	
Peripheral blood involvement	Yes	0.0% (0/12)	4.3% (2/46)	1.00*
	No	100.0% (12/12)	95.7% (44/46)	
Extranodal involvement > 1	Yes	0.0% (0/12)	19.6% (9/46)	0.18*
	No	100.0% (12/12)	80.4% (37/46)	
B symptoms	Yes	8.3% (1/12)	51.1% (23/45)	0.009*
	No	91.7% (11/12)	48.9% (22/45)	
Performance status > 1	Yes	8.3% (1/12)	48.9% (22/45)	0.02*
	No	91.7% (11/12)	51.1% (23/45)	
Ann Arbor stage III/IV	Yes	8.3% (1/12)	32.6% (15/46)	0.15*
	No	91.7% (11/12)	67.4% (31/46)	
Elevated LDH level	Yes	66.7% (8/12)	94.4% (34/46)	0.72*
	No	33.3% (4/12)	5.6% (12/46)	
IPI, high int, or high	Yes	16.7% (2/12)	40.9% (18/44)	0.18*
	No	83.3% (10/12)	59.1% (26/44)	
Splenic macronodular lesions	Yes	91.7% (11/12)	50.0% (23/46)	0.01*
	No	8.3% (1/12)	50.0% (23/46)	
Type of infiltration				
Type A		91.7% (11/12)	67.4% (31/46)	0.15*
Type B		8.3% (1/12)	32.6% (15/46)	
Immunohistochemistry				
CD5 expression	Positivity	8.3% (1/12)	35.6% (16/45)	0.09*
	Negativity	91.7% (11/12)	64.4% (29/45)	
CD10 expression	Positivity	50.0% (6/12)	17.8% (8/45)	0.05
	Negativity	50.0% (6/12)	82.2% (37/45)	
CD20 expression	Positivity	100.0% (12/12)	100% (45/45)	1.00
	Negativity	0.0% (0/12)	0.0% (0/45)	
BCL2 expression	Positivity	50.0% (6/12)	55.6% (25/45)	0.76
	Negativity	50.0% (6/12)	44.4% (20/45)	
BCL6 expression	Positivity	75.0% (9/12)	48.9% (22/45)	0.19*
	Negativity	25.0% (3/12)	51.1% (23/45)	
MUM1 expression	Positivity	41.7% (5/12)	55.6% (25/45)	0.52
	Negativity	58.3% (7/12)	44.4% (20/45)	
Cell of origin				
GCB type		83.3% (10/12)	28.8% (13/45)	0.002*
Non-GCB type		16.7% (2/12)	71.2% (32/45)	
Follow up (months)		46.9 months (4.0–94.3 months)	36.8 months (0.8–133.5 months)	0.46

LDH, lactate dehydrogenase; *IPI*, International Prognostic Index; *GCB*, germinal center B cell

*Fisher's exact test

rituximab monotherapy. Regarding treatment outcomes, there were 9 cases of CR and 1 case of PD. The median follow-up period was 47.0 months (4–94 months). Seven patients survived and 4 patients died. The causes of death were 1 case of malignant lymphoma, 1 case of sudden death, 1 case of hepatocellular carcinoma, and 1 case of secondary myelodysplastic syndrome. HCV-eradication therapy was not performed at the time of DLBCL diagnosis in HCV-positive DLBCL, except for one case (ordinary DLBCL case 19).

Comparison of clinicopathological features from HCV-positive and -negative primary splenic DLBCL patients

Table 2 shows the clinicopathological findings from HCV-positive and -negative p-spDLBCL patients. HCV-positive p-spDLBCL patients had fewer B symptoms ($P = 0.009$), fewer cases of PS2-4 ($P = 0.02$), more splenic macronodular lesions ($P = 0.01$), and more GCB type cases ($P = 0.002$)

compared with HCV-negative p-spDLBCL patients. There were no differences in OS ($P = 0.71$) or PFS ($P = 0.30$) between HCV-positive and -negative p-spDLBCL cases (Fig. 1).

Comparison of clinicopathological features from HCV-positive and -negative non- primary splenic DLBCL (ordinary DLBCL) patients.

Table 3 shows the clinicopathological findings from HCV-positive and -negative ordinary DLBCL patients. HCV-positive ordinary DLBCL patients presented lower CD10 expression ($P = 0.01$) and more non-GCB type cases ($P = 0.04$) compared with HCV-negative ordinary DLBCL patients. There were no differences in OS ($P = 0.24$) or PFS ($P = 0.21$) between HCV-positive and -negative ordinary DLBCL cases (Fig. 2).

Genotype and viral load of HCV-positive DLBCL patients

The HCV genotype was examined for 21 cases (Table S1). Of the 21, 8 were HCV-positive p-spDLBCL and 13 were HCV-positive ordinary DLBCL cases. Of the 8 HCV-positive p-spDLBCL cases, 87.5% (7/8) presented Genotype 1b and 12.5% (1/8) presented Genotype 2a. Of the 13 HCV-positive ordinary DLBCL cases, 92.3% (12/13) ($P = 1.00$ vs. HCV-positive p-spDLBCL) presented Genotype 1b and 7.7% (1/13) ($P = 1.00$ vs. HCV-positive p-spDLBCL) presented Genotype 2a. During their first visit, virus loads were examined for 36 cases (Table S1). Viral loads were classified as "high" in 66.7% (6/9) of HCV-positive p-spDLBCL cases and 93.3% (14/15) of HCV-positive ordinary DLBCL cases ($P = 0.13$).

Detection of HCV in lymphoma cells

Analysis of NS3 expression in HCV-positive primary splenic DLBCL and ordinary DLBCL samples

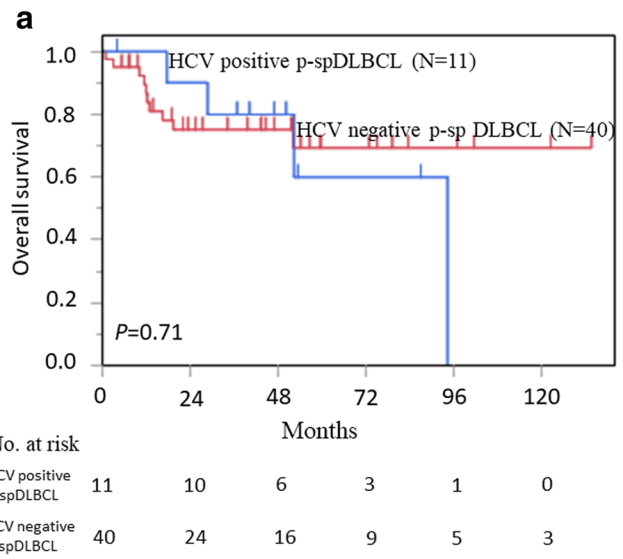
NS3 expression was examined via immunohistochemistry in 34 cases of HCV-positive p-spDLBCL and ordinary DLBCL (Table S1), with 20.6% (7/34) of cases found to be NS3-positive (Fig. 3). Of these, 4 were HCV-positive p-spDLBCL cases (40.0%, 4/10) and 3 were ordinary DLBCL cases (12.5%, 3/24) ($P = 0.16$).

HCV-RNA in situ hybridization

HCV RNA in situ hybridization results are shown in (Table 4). One tumor cell sample (16.7%) presented HCV-RNA while the other five samples (83.3%) did not (Fig. 3).

RT-PCR examination of tumor tissue from HCV-positive primary splenic DLBCL and ordinary DLBCL patients

RT-PCR was performed on samples from 9 patients with either HCV-positive p-spDLBCL or ordinary DLBCL (Table 4). These samples comprised 5 splenic tumor tissues and 4 lymph node tumor tissues, with 2 patients (22.2%) presenting HCV-RNA in tumor tissue and 7 patients not (77.8%) (Fig. 4).



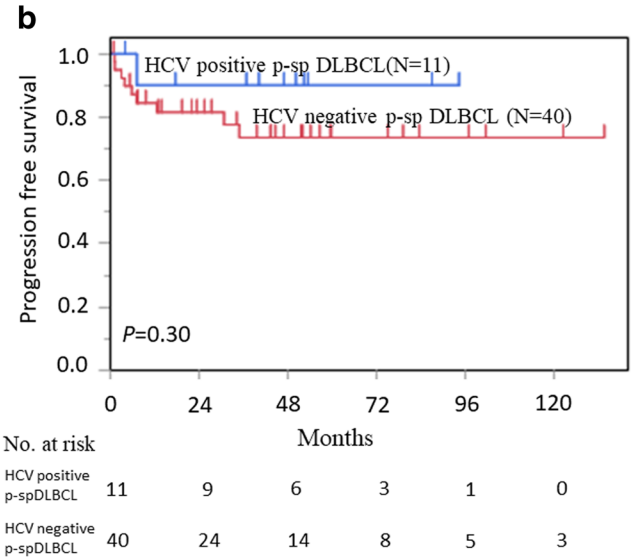


Fig. 1 Overall survival and progression-free survival in HCV-positive and -negative primary splenic DLBCL (p-spDLBCL) patients. (A) Overall survival curve. There was no difference in OS ($P = 0.71$) between HCV-positive and -negative p-spDLBCL patients. (B) Progression-free survival curve. There was no difference in PFS ($P = 0.30$) between HCV-positive and -negative p-spDLBCL patients

Table 3 Comparison of HCV-positive DLBCL without primary splenic and HCV-negative DLBCL without primary splenic (ordinary DLBCL)

		HCV-positive ordinary DLBCL ($N = 25$)	HCV-negative ordinary DLBCL ($N = 120$)	P value
Clinical information				
Age median[range](years)		73 [49–88]	70.5 [21–90]	
Age > 60 year	Yes	88.0% (22/25)	76.7% (92/120)	0.29
	No	12.0% (3/25)	23.3% (28/120)	
Sex: male	Yes	64.0% (16/25)	57.5% (69/120)	0.55
	No	36.0% (9/25)	42.5% (51/120)	
Bone marrow involvement	Yes	8.3% (2/24)	9.3% (10/107)	1.00*
	No	91.7% (22/24)	90.7% (97/107)	
Peripheral blood involvement	Yes	4.0% (1/25)	1.7% (2/120)	0.44*
	No	96.0% (24/25)	98.3% (108/120)	
Extranodal involvement > 1	Yes	20.0% (5/25)	22.5% (27/120)	0.78
	No	80.0% (20/25)	77.5% (93/120)	
B symptoms	Yes	24.0% (6/25)	19.2% (23/120)	0.59
	No	76.0% (19/25)	80.8% (97/120)	
Performance status > 1	Yes	24.0% (6/25)	33.6% (39/116)	0.34
	No	76.0% (19/25)	66.4% (77/116)	
Ann Arbor stage III/IV	Yes	64.0% (16/25)	52.6% (60/114)	0.30
	No	36.0% (9/25)	47.4% (54/114)	
Elevated LDH level	Yes	64.0% (16/25)	59.7% (71/119)	0.69
	No	36.0% (9/25)	41.3% (48/119)	
IPI, high int, or high	Yes	56.0% (14/25)	54.5% (60/110)	0.90
	No	44.0% (11/25)	45.5% (50/110)	
Infiltration site				
Liver	Yes	8.0% (2/25)	2.5% (3/120)	0.21*
	No	91.0% (23/25)	97.5% (117/120)	
Spleen	Yes	20.0% (5/25)	10.0% (12/120)	0.18
	No	80.0% (20/25)	90.0% (108/120)	
Stomach	Yes	20.0% (5/25)	10.0% (12/120)	0.18
	No	80.0% (20/25)	90.0% (108/120)	
Immunohistochemistry				
CD5 expression	Positivity	15.8% (3/19)	15.1% (18/119)	1.00*
	Negativity	84.2% (16/19)	84.9% (101/119)	
CD10 expression	Positivity	9.9% (2/22)	35.8% (43/120)	0.01*
	Negativity	90.1% (20/22)	64.2% (77/120)	
CD20 expression	Positivity	100.0% (25/25)	100% (120/120)	1.00*
	Negativity	0.0% (0/25)	0.0% (0/120)	
BCL2 expression	Positivity	45.5% (15/22)	66.7% (48/72)	0.89
	Negativity	54.5% (7/22)	33.3% (24/72)	
BCL6 expression	Positivity	59.1% (13/22)	79.2% (95/120)	0.04
	Negativity	40.9% (9/22)	20.8% (25/120)	
MUM1 expression	Positivity	59.1% (13/22)	65.0% (78/120)	0.60
	Negativity	40.9% (9/22)	35.0% (42/120)	
Cell of origin				
GCB type		22.7% (5/22)	46.7% (56/120)	0.04
Non-GCB type		77.3% (17/22)	53.3% (64/120)	
Follow up (months)		27.8 months (1.9–122.1 months)	35.9 months (0.1–90.1 months)	0.62

LDH, lactate dehydrogenase; *IPI*, International Prognostic Index; *GCB*, germinal center B cell

*Fisher's exact test

Discussion

We found that HCV-positive p-spDLBCL patients presented fewer B symptoms (asymptomatic), lower PS2-4, and more splenic macronodular lesions. In a previous report [29], 15 HCV-positive splenic DLBCL cases all presented splenic macronodular lesions, a finding consistent with our results. It is believed that HCV-positive p-spDLBCL patients presenting few B symptoms and who were relatively asymptomatic for good PS faced a high probability of coincidentally finding DLBCL in follow-up imaging for HCV-related liver disease (chronic hepatitis, cirrhosis, and hepatocellular carcinoma).

HCV-positive p-spDLBCL patients presented more GCB-type than HCV-negative p-spDLBCL patients. However, HCV-positive ordinary DLBCL patients were found to present more non-GCB type than HCV-negative ordinary DLBCL patients. In a study using transgenic mice expressing full-length HCV cDNA in B cells, DLBCL was identified in 25% of mice within 600 days of birth [30]. An array analysis of these DLBCL specimens identified abnormal expression

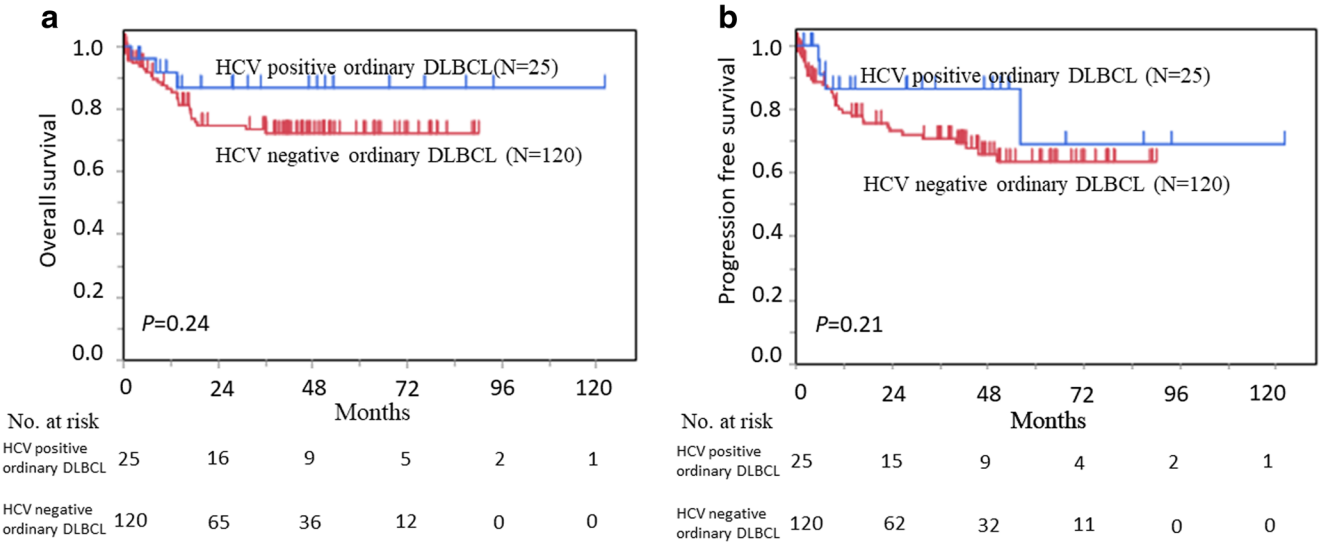


Fig. 2 Overall survival and progression-free survival in HCV-positive non-primary splenic DLBCL (ordinary DLBCL) and HCV-negative non-primary splenic DLBCL (ordinary DLBCL). (A) Overall survival curve. There was no difference in OS ($P = 0.24$) between HCV-positive and -negative ordinary DLBCL. (B) Progression-free survival curve. There was no difference in PFS ($P = 0.21$) between HCV-positive and -negative ordinary DLBCL

levels of genes involved in alternative and canonical NFκB pathways, such as *c-FOS*, *A20*, *C3*, and *LTβR*. [31] Thus, HCV-positive DLBCL may manifest as non-GCB-type DLBCL since it is believed that increased NFκB signaling strongly influences lymphomagenesis. Furthermore, clinical studies involving HCV-positive DLBCL patients have also reported more non-GCB type than GCB type [13, 17]. Here, consistent with these results, we found that 77.3% (17/22) of the HCV-positive ordinary DLBCL patients were non-GCB-type. However, 83.3% (10/12) of HCV-positive p-spDLBCL patients presented GCB-type. The reason for this is unclear. Given that the cell of origin is different, HCV-positive p-spDLBCL could present different molecular characteristics than HCV-positive ordinary DLBCL.

In our study, comparison of overall survival between HCV-positive DLBCL (p-spDLBCL and ordinary DLBCL) and -negative DLBCL did not reflect any statistical significance. However, HCV-positive DLBCL, especially HCV-positive ordinary DLBCL, had appreciable OS. In ordinary DLBCL, the clinical features did not show statistically significant difference except in the cell of origin. According to a previous report, HCV-positive DLBCL has poor prognosis compared with HCV-negative DLBCL [17, 32]. In addition, regarding the cell of origin (COO), ABC type (non-GCB type) is

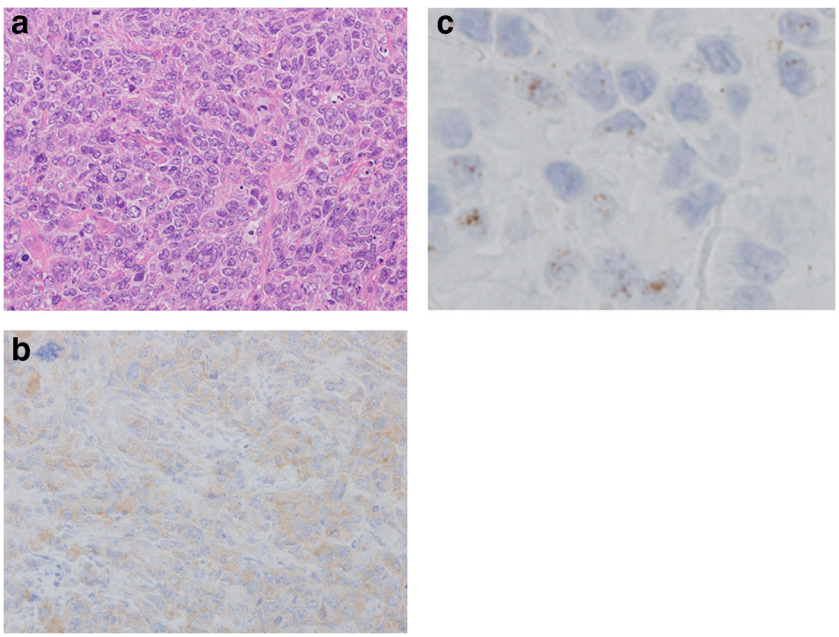


Fig. 3 Analysis of immunohistochemistry and HCV-RNA in situ hybridization (ISH) in HCV-positive DLBCL samples. (A) Representative HCV-positive DLBCL sample morphology (HE, × 200). (B) Immunohistochemistry indicating NS3-positive tumor cell cytoplasm (× 200). (C) HCV-RNA ISH. Some tumor cells were positive for HCV-RNA signal (× 400).

Table 4 HCV RT-PCR, NS3 staining, and RNA in situ hybridization in HCV-positive DLBCL

No.	Case	Peripheral blood		Tissue		NS3 immunostaining	RNA in situ hybridization
		Genotype	HCV-RNA (logIU/ml)	Sample	Tissue RT-PCR		
A	p-spDLBCL case 2	NA	3.1	Spleen	−	+	NA
B	p-spDLBCL case 4	1b	3.7	Spleen	−	−	−
C	p-spDLBCL case 8	1b	6.9	Spleen	+	+	+
D	p-spDLBCL case 10	1b	6.5	Spleen	+	+	−
E	Ordinary DLBCL case 4	1b	6.4	Spleen	−	+	−
F	Ordinary DLBCL case 6	2a	5.3	Lymph node	−	−	NA
G	Ordinary DLBCL case 10	1b	6.4	Lymph node	−	−	−
H	Ordinary DLBCL case 14	NA	7.7	Lymph node	−	−	NA
I	Ordinary DLBCL case 15	1b	6.4	Lymph node	−	−	−
Positive control		1b	6.4	Liver	+	+	+
Negative control		−	−	Spleen	−	−	ND

NA, not available; *ND*, not detectable

generally associated with poor prognosis [32]. Compared with these reports, our results were significantly different. Based on recently reported next-generation sequencing data on DLBCL, ABC type cells (non-GCB type) contain a relatively good prognosis group with characteristics of BCL6 translocation and NOTCH2 mutation [33, 34]. Although no genetic mutation experiment was conducted in this study, we hypothesized that HCV-positive ordinary DLBCL may include many cases belonging to this group.

RT-PCR, HCV RNA-ISH, and NS3 analyses were performed on HCV-positive DLBCL patient samples, with tumor cell-HCV expression being observed in some cases. In particular, NS3 was detected in 20.6% (7/34) of investigated samples, while HCV-RNA was found in only 16.7% (1/6) of samples analyzed with in situ hybridization (ISH) and HCV was only identified in 22.2% (2/9) of tumor tissues studied using RT-PCR. Here, we found that HCV was detected more often in splenic samples than lymph node samples. This may be because the spleen is likely to contain peripheral blood and serum containing circulating virus. However, previous reports have indicated that HCV core antigen was higher in the spleen than the other organs [30].

It has been reported that HCV infects normal B cells [35]. Specifically, in vitro, HCV infection has been shown to increase mutagenesis rates in various genes, including *TP53*, *BCL6*, *CTNNB1*, and *IGH*, through the induction of activation-induced cytidine deaminase (AID) and double-strand breaks [9]. It has been suggested that, even during transient HCV infection, there is a possibility of B cell genetic mutation potentially contributing to lymphomagenesis [9]. In HCV-related HCC cases, the tumor tissue has been found to have markedly lower viral replication and viral load compared with non-tumor tissue [36]. Thus, HCV infection may be important during the initial stages of B cell tumorigenesis. Additionally, it must be noted that the fact that HCV was not detected in tumor cells does not necessarily imply that HCV infection was not involved in lymphomagenesis. Alternatively, it may be that chronic antigen stimulation, rather than direct HCV infection of B cells, is involved in lymphomagenesis.

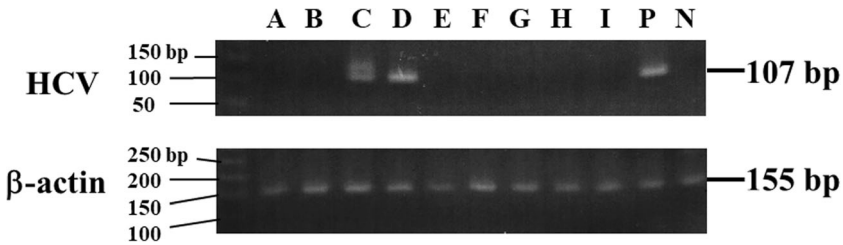


Fig. 4 RT-PCR for HCV-positive DLBCL tissue. Two cases (case C and D) expressed HCV-RNA in tumor tissue (107 bp). All cases expressed β-actin (155 bp). HCV-positive DLBCL cases (A–I), positive control (P), negative control (N)

In conclusion, we report a study in which HCV ISH, HCV RT-PCR, and NS3 immunohistochemisty were performed on HCV-positive DLBCL samples, and HCV was identified in only a few cases within the tumor tissue. HCV-positive primary splenic DLBCL was found to have unique clinicopathological features. Association of HCV infection with HCV-positive primary splenic DLBCL is more likely related to either chronic antigen stimulation or direct viral effect.

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Authors' contributions J.S. and H.M. were responsible for conception and design; T.S., K.M., T.E., T.M., K.K., K.N., and K.A. provided study materials or patients; J.S., F.A., K.Y., T.S., K.M., T.E., T.M., K.K., K.N., and K.A. performed the collection and assembly of data; J.S., H.M., T.T., and K.O. performed data analysis and interpretation; J.S. wrote the manuscript; and all authors approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

The use of clinical samples and information was approved by the Research Ethics Committee of Kurume University and was performed in accordance with the Declaration of Helsinki.

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