SHORT COMMUNICATION

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Hepatitis C virus positive diffuse large B-cell lymphomas have distinct molecular features and lack BCL2 translocations

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Background: The clinical presentation of patients with hepatitis C virus (HCV)-positive diffuse large B-cell lymphoma (DLBCL) is different from their HCV-negative counterparts, but the underlying molecular and pathological characteristics are largely under investigated. The virus has a role in lymphomagenesis, as witnessed by the curative potential of antiviral therapy in HCV-related low-grade B-cell lymphomas.

Methods: We performed a case-control study including 44 HCV-positive cases of *de novo* DLBCL, comparing them with 132 HCV-negative patients as controls (ratio 3 to 1). Cases and controls were matched for age, lactate dehydrogenase level and international prognostic index at presentation. Patients were studied by gene expression profiling for cell-of-origin determination and to perform differential expression analysis between groups, fluorescence *in-situ* hybridisation and immunohistochemistry for MYC, BCL2 and BCL6, TP53 mutations, and diagnostic specimens reviewed to exclude transformation from low-grade lymphoma.

Results: Compared to the HCV-negative controls, patients with HCV-positive *de novo* DLBCL had differential expression of genes that regulate innate immune response and modulate apoptotic pathways, have higher proliferative index, and lack *BCL2* translocations.

Conclusions: HCV-positive DLBCL have distinct molecular and pathological features compared to the HCV-negative counterparts.

Diffuse large B-cell lymphoma (DLBCL), the most frequent type of B-cell lymphoma, accounts for about 30% of all lymphoid malignancies. This entity, which encompasses distinct morphologic, molecular, and clinicopathologic subgroups (Swerdlow *et al*, 2016), has been associated with hepatitis C virus (HCV) infection (Mele *et al*, 2003; Ennishi *et al*, 2010; Merli *et al*, 2014; Visco and Finotto, 2014). The clinical presentation of patients with HCV-positive DLBCL is different from their HCV-negative counterparts, since patients are older, have more frequent spleen and/or liver involvement, elevated serum lactate dehydrogenase

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(LDH) and a high international prognostic index (IPI) (Ennishi *et al*, 2010; Merli *et al*, 2014; Visco and Finotto, 2014). However, the pathological characteristics of *de novo* DLBCL arising in patients with HCV infection are under-investigated. A substantial subset of HCV-positive DLBCL cases represent transformation from a pre-existing low-grade – especially marginal zone – lymphoma (t-DLBCL), and as such they have been postulated as being part of a multistep process that starts from type-II mixed cryoglobulinemia (MC) and the achievement of B-cell clonality (Peveling-Oberhag *et al*, 2013).

A role for the virus in lymphomagenesis is also suggested by the curative activity of antiviral therapy for patients with HCV-related low-grade B-cell lymphomas, even when non-interferon containing regimens are used (Arcaini *et al*, 2016). Such an effect implies a specific role for the virus in maintaining B-cell proliferation, although the mechanisms involved in HCV-mediated lymphomagenesis remain unknown.

In the era of DLBCL sub-classification, with cell-of-origin (COO) algorithms and fluorescence *in-situ* hybridisation (FISH) for *BCL2* and/or *MYC* rearrangements having entered clinical practice, and being included in the 2016 revision of the World Health Organization (WHO) classification, we investigated the pathological characteristics of 44 HCV-positive patients with *de novo* DLBCL with no clinical or pathological evidence of transformation from low-grade lymphoma, and compared them with HCV-negative DLBCL controls matched for clinical variables at presentation.

MATERIALS AND METHODS

Fifty-one newly diagnosed, previously untreated DLBCL in HCV-positive patients were organised from The International DLBCL Rituximab-CHOP Consortium Program Study. The database includes *de novo* DLBCL treated with standard rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone (R-CHOP) therapy, diagnosed according to the WHO classification system and treated between 1998 and 2010. A total of 44 HCV-positive cases were included in the study based on the availability of adequate biological material for FISH, COO definition based on gene expression profile (GEP) or immunohistochemistry (IHC), complete clinical data and final diagnosis after histological review. Cases with a history or pathological evidence of previous low-grade lymphoma, or arising in a setting of congenital or acquired immunodeficiency, including patients with human immunodeficiency virus infection, were excluded.

For comparative purposes, we performed a case-control study from the entire cohort of 437 DLBCL patients that were tested at lymphoma diagnosis for serum HCV-antibodies, and selected 132 HCV-negative DLBCL patients as controls (ratio 3 to 1). Controls were matched for age, LDH level, and IPI score, which are known to represent a bias for HCV-positive cases compared to the HCV-negative controls. This study was approved by the Institutional Review Boards of each participating centre, and the comprehensive collaborative study was approved by the Institutional Review Board at The University of Texas MD Anderson Cancer Center.

Gene expression profiling was performed on formalin-fixed, paraffin-embedded tissue extracts in all included patients using a GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA) with total RNAs as described previously (Visco *et al*, 2012). The CEL files were deposited in the National Center for Biotechnology Information Gene Expression Omnibus repository (GSE no. 31312). Gene expression profiling classified 104 cases as GCB/ABC subtypes (19 HCV+, 85 HCV−), and the COO of the remaining 72 cases was determined according to the Visco–Young (first selection) and Choi (second selection) algorithms (Visco *et al*, 2012). FISH was performed using *BCL2* and *BCL6* dual-colour, break-apart probe (07J75-001 from Vysis, Downers Grove, IL, USA) on paraffin-embedded tissue sections according to the Vysis protocol. FISH for the *MYC* gene was performed with a locus-specific identifier *IGH/MYC/CEP* 8 tri-colour, dual fusion probe (DFP, 05J75-001 from Vysis) and, due to shortcomings of the former in identifying alternative (non-*IGH*) *MYC* rearrangement partners, a locus-specific identifier *MYC* dual-colour, break-apart probe (BP, 05J91-001 from Vysis). Construction of the tissue microarrays, IHC staining procedures on tissue microarray sections, and scoring criteria for MYC and BCL2 have been described previously (Tzankov *et al*, 2014). Cutoffs for MYC (clone Y69; Epitomics, Burlingame, CA, USA) and BCL2 (clone 124; DAKO, Santa Clara, CA, USA) overexpression were ⩾40% and ⩾50%, respectively, as indicated in the WHO classification (Swerdlow *et al*, 2016). The MIB-1 (Ki-67) expression was quantified by estimating the percentage of positive lymphoma cells among the total number of malignant lymphoid cells. Immunoreactivity was determined without knowledge of survival, clinical data, or GEP data.

Serum antibodies against HCV were tested at the time of lymphoma diagnosis using an enzyme-linked immunosorbent assay (ELISA; HCV 3.0; Ortho Diagnostic System, Raritan, NJ, USA) and confirmed by recombinant-based immunoblot assay (Chiron RIBA; Ortho Diagnostic System) in all 176 patients. Most HCV-positive patients (39 of 44, 89%) were also tested for RNA

Table 1. Clinical and pathological characteristics of HCV-positive (HCV+) DLBCL compared to HCV-negative (HCV−) controls

Characteristics	HCV+ (*n*=44)	HCV− (*n*=132)	*P*
Age, years<60	23 (52%)	66 (50%)	0.69
Gender: male	25 (57%)	73 (55%)	0.86
Stage: III–IV	22 (50%)	78 (59%)	0.31
LDH level: elevated	32 (73%)	91 (69%)	0.53
IPI score: >2	18/39 (46%)	42/121 (35%)	0.25
GCB/ABC by GEP			
GCB	9/19 (47%)	37/85 (44%)	
ABC	9/19 (47%)	41/85 (48%)	0.89
UNCL	1/19 (6%)	7/85 (8%)	
GCB/ABC by IHC			
GCB	18/40 (45%)	56/122 (46%)	0.92
Non-GCB	22/40 (55%)	69/122 (54%)	
CD37 expression: +	13/32 (41%)	55/116 (47%)	0.54
MYC translocation: +	2/31 (6%)	5/103 (5%)	0.72
BCL2 translocation: +	0/33 (0%)	23/123 (19%)	**0.004**
BCL6 translocation: +	5/30 (17%)	24/101 (24%)	0.49
Double hit (MYC/BCL2)	0/26 (0%)	2/99 (2%)	0.46
Double hit (MYC/BCL6)	1/23 (4%)	1/81 (1%)	0.21
TP53 mutations: +	3/21 (14%)	23/84 (27%)	0.21
BCL2 protein expression: ⩾50%	17/38 (45%)	83/123 (67%)	0.01
MYC protein expression: ⩾40%	21/41 (51%)	66/125 (53%)	0.86
Double Expressors (DEL)	8/36 (22%)	45/120 (38%)	0.08
MIB-1 expression: >70%	18/27 (67%)	35/84 (42%)	**0.02**
IgA expression: 100%	3/21 (14%)	1/82 (1%)	**0.005**
IgG expression: 100%	6/21 (29%)	6/82 (7%)	**0.006**
IgM expression: 100%	9/21 (43%)	22/82 (27%)	0.15

Abbreviations: ABC = activated B-cell type; DEL = double expressors lymphoma for BCL2'and MYC proteins; GCB = germinal centre B-cell type; HCV = hepatitis C virus; Ig = immunoglobulins; IHC = immunohistochemistry; IPI = international prognostic index; LDH = lactate dehydrogenase; MIB-1 = Mindbomb E3 ubiquitin protein ligase 1 (Ki-67); UNCL = unclassifiable. Significant values are shown in bold.

sequences of HCV by reverse transcriptase polymerase chain reaction using primers for the 5′ noncoding region, and all resulted positive. Virus genotype was rarely available (5 out of 44).

The non-parametric Mann–Whitney test and Fisher exact test were used to compare quantitative and categorical variables across groups of patients, respectively. Statistical analyses were performed using Stata 12.1 software.

RESULTS

The median age of HCV-positive cases (59 years) was similar to HCV-negative controls (61 years, $P = 0.44$ by Mann–Whitney test), as were all main clinical parameters. This was a logical consequence of our match of patients by means of age, LDH, and IPI risk groups at lymphoma diagnosis (Table 1). No difference in terms of COO was found between HCV-positive and -negative patients, both by GEP and IHC. Surprisingly, *BCL2* translocation was not identified in any of the 33 HCV-positive DLBCL patients assessed, but was present in 23 of 123 HCV-negative controls (19%, $P = 0.004$). Accordingly, BCL2 protein was significantly less expressed in HCV-positive cases compared to HCV-negative controls, although double expressor cases (MYC and BCL2) were equally represented ($p = 0.08$). No significant difference was found in terms of *BCL2* gene amplification between HCV-positive cases and controls (6 and 11%, respectively, $P = 0.43$). Translocations of *MYC* and *BCL6* genes were equally represented in the HCV-positive and control groups (Table 1). Among other analysed IHC variables we found that HCV-positive cases had significantly higher MIB-1 (Ki-67) expression (median expression value 80 *vs* 70%, $P = 0.04$ by Mann–Whitney test), and did express immunoglobulins (IgA and IgG) more frequently than the HCV-negative counterparts (Table 1). No difference in overall survival and/or progression-free survival was observed between the HCV-positive cases and controls (data not shown).

By GEP, we found 23 genes that were differentially expressed between HCV-positive cases and HCV-negative controls without *BCL2* translocation (Figure 1). Among them, the *suppressor of IKBKE 1* (*SIKE-1*), which modulates the innate immunity response by suppressing virus-triggered activation pathways was upregulated, and the *C-Maf inducing protein* (*CMIP*) gene, which encodes a protein that plays a role in T-cell signalling pathway was downregulated in HCV-positive cases. *Fas apoptosis inhibitory molecule* (*FAIM*) and *cyclin-G2* (*CCNG2*) genes, that regulate B-cell signalling and differentiation by protecting against apoptosis were upregulated in HCV-positive cases.

DISCUSSION

We conclude that HCV-positive *de novo* DLBCL cases retain molecular features that differ from their HCV-negative DLBCL counterparts. The absence of *BCL2* gene translocations in this series, together with a consistently lower incidence of BCL2 protein expression, points to the existence of different mechanisms that drive oncogenesis in these patients. This is of interest because a high prevalence of t(14;18)(q32;q21)/*IGH-BCL2* associated with increased BCL2 expression has been detected in peripheral blood mononuclear cells of patients with HCV infection or MC (Zuckerman *et al*, 2001), although no study is available in DLBCL.

The data we present suggest that *BCL2*-translocation-positive cells in the blood do not appear to be precursors that are important for subsequent steps of lymphomagenesis, at least for DLBCL (Zuckerman *et al*, 2001; Mollejo *et al*, 2014). One may speculate that *BCL2* aberrations are involved in the onset of initial B-cell proliferations, such as MC, but the pathogenesis of HCV-associated DLBCL is driven by alterations of different pathways (i.e., *NOTCH*) (Peveling-Oberhag *et al*, 2013; Visco and Finotto, 2014; Arcaini *et al*, 2015). Indeed, modulation of the apoptotic

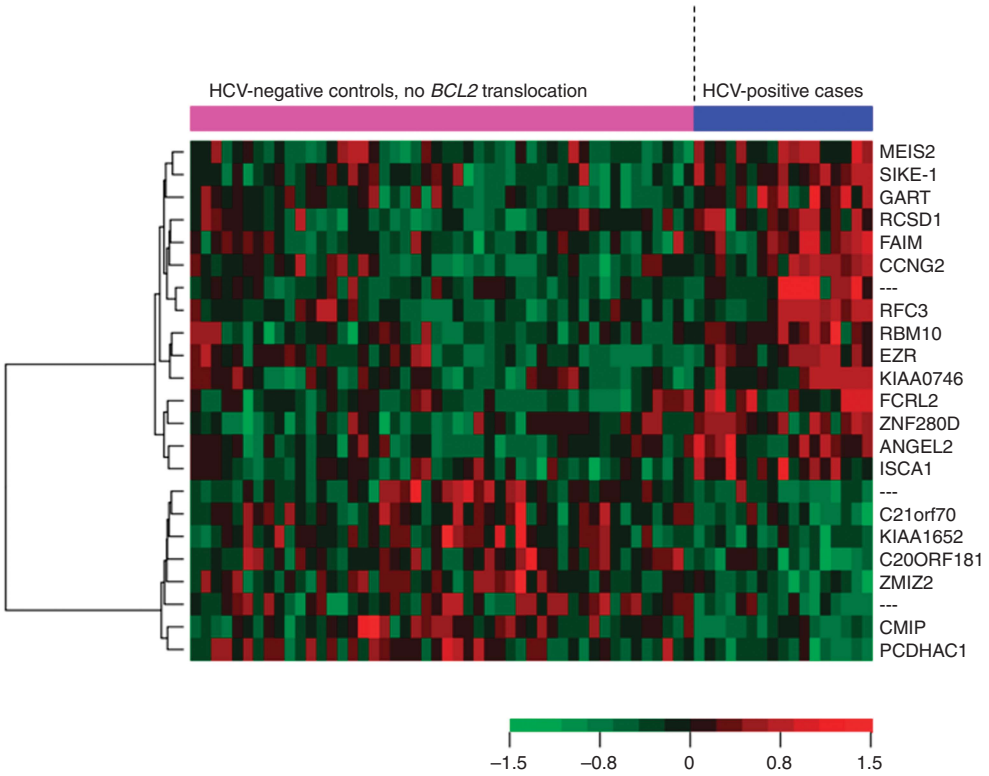

Figure 1. Heat map of hierarchical clustering of GEP on DLBCL patients, comparing HCV-positive patients (blue), with HCV-negative controls without BCL2 translocation (purple). With a false discovery rate (FDR) threshold of 0.20 there were 23 significant transcripts corresponding to the gene list on the right.

response by degradation of *BCL2* interacting domain (Bid) is one strategy used by HCV to escape immune surveillance, neutralise host defences, and establish chronic infection (Simonin *et al*, 2009). Accordingly, *FAIM* and *CCNG2* genes were upregulated in the HCV-positive cases compared to HCV-negative controls without BCL2 translocation (Figure 1). Also notably, the *CMIP*, which plays a role in T-cell signalling, was downregulated, and *SIKE-1*, which suppresses virus-triggered interferon activation, was upregulated, supporting the importance of immune regulation pathways in the pathogenesis of HCV-positive DLBCL.

The data presented differ from events in HCV-related hepatocellular carcinoma, where HCV infection is related to mutations of the *TP53* gene (Teramoto *et al*, 1994). In this study *TP53* was equally mutated in HCV-positive and -negative lymphomas. Conversely, we found a bias of HCV-positive patients in terms of higher tumour proliferation (MIB1 expression) and immunoglobulin expression by the tumour (IgG and IgA), consistent with a late, secretory, post-germinal centre COO, a feature more commonly observed in virus-related DLBCL (Gloghini *et al*, 2013). Further studies, including independent validation data sets are needed to confirm our findings and to confirm our preliminary observations.

The literature has paid relatively little attention to HCV-positive DLBCL as compared with HCV-positive low-grade lymphomas (Mele *et al*, 2003; Peveling-Oberhag *et al*, 2013; Visco and Finotto, 2014; Arcaini *et al*, 2016). A recent study has shown NOTCH pathway mutations in 25% HCV-positive DLBCL. Importantly, NOTCH pathway mutations were found to be restricted to HCV-positive DLBCL, although they were associated with the co-existence of a low-grade component in the diagnostic biopsy specimen (Arcaini *et al*, 2015). These data support the notion that a subgroup of DLBCL arising in subjects carrying HCV infection represent transformation of an underlying clinically unrecognised indolent lymphoma. Our study, which included patients without transformation from low-grade lymphoma, provides information likely helpful in further pathological exploration of these tumours.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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