EXHIBIT B



REVIEW

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From hepatitis C virus infection to B-cell lymphoma

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In addition to liver disorders, hepatitis C virus (HCV) is also associated with extrahepatic immune manifestations and B-cell non-Hodgkin lymphoma (NHL), especially marginal zone lymphoma, *de novo* or transformed diffuse large B-cell lymphoma and to a lesser extent, follicular lymphoma. Epidemiological data and clinical observations argue for an association between HCV and lymphoproliferative disorders. The causative role of HCV in NHL has been further supported by the response to antiviral therapy. Pathophysiological processes at stake leading from HCV infection to overt lymphoma still need to be further elucidated. Based on reported biological studies, several mechanisms of transformation seem however to emerge. A strong body of evidence supports the hypothesis of an indirect transformation mechanism by which sustained antigenic stimulation leads from oligoclonal to monoclonal expansion and sometimes to frank lymphoma, mostly of marginal zone subtype. By infecting lymphocytes, HCV could play a direct role in cellular transformation, particularly in *de novo* large B-cell lymphoma. Finally, HCV is associated with follicular lymphoma in a subset of patients. In this setting, it may be hypothesized that inflammatory cytokines stimulate proliferation and transformation of *IgH–BCL2* clones that are increased during chronic HCV infection. Unraveling the pathogenesis of HCV-related B-cell lymphoproliferation is of prime importance to optimize therapeutic strategies, especially with the recent development of new direct-acting antiviral drugs.

Key words: hepatitis C virus, non-Hodgkin lymphoma, oncogenesis, models of transformation

Introduction

Persistent hepatitis C virus (HCV) infection is an etiological agent of chronic hepatitis that may evolve toward cirrhosis and hepatocarcinoma. In addition, HCV is associated with extrahepatic manifestations, especially lymphoproliferative disorders, including type II 'mixed' cryoglobulinemia (MC) [1] and non-Hodgkin lymphoma (NHL).

This review aims to summarize evidence from epidemiological and clinical studies that have provided strong support for an etiological role of HCV in NHL development and maintenance. In addition, current knowledge about physiopathology of B-cell NHL associated with HCV infection will be presented.

Clinical features of HCV-positive NHL

HCV-associated lymphoma is mainly of B-cell histological subtypes. Low-grade marginal zone lymphoma (MZL) particularly those of splenic origin and diffuse large B-cell lymphoma



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(DLBCL) are the most common subtypes, followed by follicular lymphoma [2–4].

At the clinical level, HCV-positive NHL displays peculiar features when compared with their HCV-negative counterpart. They usually occur following a long period of infection (more than 15 years) [5]. HCV-related low-grade B-cell lymphoma often involves extranodal sites particularly spleen and salivary glands where the virus can replicate [6]. HCV-positive DLBCL frequently affects spleen, liver and stomach and international prognostic index (IPI) is usually high, especially due to LDH elevation [7–9].

Among HCV-associated DLBCL, two groups may be identified: one with only large B cells named *de novo* DLBCL and one associated with infiltration of small B cells, suggesting a transformation of MZL into aggressive DLBCL [7]. Interestingly, the proportion of transformed DLBCL is significantly higher in HCV-positive patients (32%), when compared with HCV-negative patients (6%) [7].

As aforementioned, HCV-positive FL are less commonly observed [10–14] and any peculiar clinical feature has been so far described in this subgroup. Interestingly, *IGH–BCL2* positive clones are not restricted to patients with HCV-positive FL and may be observed more extensively during chronic HCV infection, especially when associated with MC [15, 16] and in others sub-types of HCV-positive NHL [16–18]. This point will be discussed in more detail later in the review.

Epidemiology of HCV infection in NHL

Early-epidemiological studies suggested a significant increased risk of B-NHL in HCV-infected patients only in high prevalence areas [19, 20]. Further large studies as well as meta-analyses [2, 21-26] finally documented an overall increased risk of B-NHL in patients with chronic HCV infection when compared with HCV-negative controls (overall relative risk estimation: 2.4; 95% CI: 2.0-3.0) [26] yet higher in geographic areas with elevated prevalence of HCV [25, 26]. In addition, a recent large case-control study from North America confirmed a significantly higher prevalence of HCV infection in NHL patients (OR: 1.5; 95% CI: 1.2-1.8), especially B-NHL (OR: 1.6; 95% CI: 1.3-1.9), when compared with controls [3]. Subtype-specific analyses revealed that HCV-seropositivity was greater in MZL and DLBCL but not in follicular lymphoma cases [3, 27]. Taken together, these data indicate a greater propensity to develop NHL, especially MZL and DLBCL, in the setting of HCV infection with most prominent risk in populations with high HCV prevalence.

Antiviral therapy in HCV-related NHL

We first reported that HCV-positive patients with splenic lymphoma with villous lymphocytes (SLVL) could benefit from interferon (IFN)-based antiviral therapy alone or in combination with ribavirin [28, 29]. Further prospective studies as well as systematic meta-analyses confirmed these results in HCV-associated low-grade B-cell lymphomas, with a global response rate to antiviral therapy of 70% [10–13, 30–34]. First-line treatment of chronic HCV infection is now based on the sole use of IFN-free direct-acting antivirals (DAA), which results in complete virologic response in more than 95% of the patients [35]. In this line, DAA (mostly sofosbuvir-based regimens) efficacy has been recently evaluated in 46 patients with HCVpositive indolent B-cell NHL, consisting mostly of MZL (n=37) [36]. Sustained virologic response at week 12 was obtained in 98% of patients with chronic HCV infection. Hematological response rate (67%) was similar to those obtained after IFN-based antiviral therapy [32] and was remarkably high in MZL (73%). Estimated 1-year progression-free and overall survival were 75% and 98%, respectively. These results demonstrate that HCV-infected patients with indolent B-NHL, especially of marginal zone type, benefit from DAA-based antiviral therapy.

In clinical practice, HCV-positive DLBCL patients are usually treated as their HCV-negative counterpart with anthracyclinebased chemotherapy coupled with rituximab. However, sustained viral response after antiviral therapy has been demonstrated to be associated with a better overall survival in HCVrelated MZL [10, 13] but also in DLBCL patients [37]. In view of this, two HCV-positive DLBCL cases have been successfully treated with concurrent concurrent immunochemotherapy and DAA-based antiviral therapy [38, 39].

Antiviral therapy has been shown to induce disappearance of circulating t(14; 18)-bearing B-cell clones in HCV-positive patients, followed by the reemergence of the same t(14; 18) clone upon virologic relapse [40, 41]. Clinical studies have also reported efficacy of IFN-based (complete response, n=2) [11, 42] as well as DAA-based (partial response, n=2 [36]; complete response, n=1 [14]) antiviral therapy in few HCV-positive FL. These results are promising but need to be confirmed in a larger cohort of HCV-positive FL.

In summary, antiviral therapy efficacy in HCV-associated lymphoma supports a causative role for HCV in NHL development, at least in the MZL subgroup.

HCV infection and B-cell transformation

During the two last decades, two distinct albeit not exclusive models of infection-driven transformation have emerged. On one hand, direct lymphocyte transformation by lymphotropic transforming viruses (EBV, HHV8, or HTLV1) expressing viral oncogenes has been clearly demonstrated. On the other hand, a model based on indirect mechanism of lymphocytes transformation ultimately leading to clonal expansion has been proposed, among which *Helicobacter pylori*-associated gastric MALT lymphoma might be the best characterized [43].

Accumulating evidence suggests that several mechanisms of transformation may contribute, alone or combined, to HCV-related lymphomagenesis.

Microenvironment-driven lymphomagenesis: continuous external stimulation of lymphocytes receptors by viral antigens and cytokines

Evidences of chronic antigenic stimulation. MC is associated with HCV in more than 90% of cases [44] and is characterized by a monoclonal IgM with or without overt cryoglobulinemia-associated

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vasculitis [44, 45]. About 8%-10% of patients with MC ultimately develop lymphoproliferative disorders [46, 47] and cryoglobulinemia carriers exhibit a 35-times increased risk of NHL [48]. MC is therefore considered as a clinically benign pre-lymphomatous disease characterized by bone marrow and liver B-cell clones resembling a picture of low-grade NHL [49]. In MC patients, clonal expansion of selective VH1-69+ memory B cells [50, 51] indicates that B-cell repertoire of HCV-associated MC is highly restricted. VDJ pattern analysis of these patients has also shown that more than one clone sustain the lymphoproliferation [52]. Restricted usage of V_H1-69 and V_K3-20/15 regions has also been demonstrated in patients with HCV-associated NHL [53-55], thereby giving strong support to an antigenic selection driven process underlying lymphoma development in HCV-positive patients. VDJ pattern analysis carried out sequentially at both times of MC and overt B-cell lymphoma in one patient confirmed that lymphoma originated from one of the clones over-stimulated during MC [56]. In addition, sequencing of Ig variable regions has revealed that they are product of somatic hypermutation [54], strengthening the role of chronic antigenic stimulation in the development of HCV-associated lymphoma (Figure 1).

Role of Ig-HCV immunocomplexes. HCV-related MC IgM display rheumatoid factor (RF) activity and are in particular autoreactive against IgG anti-HCV antibodies [57] (Figure 1). It has thus been speculated that MC IgM RF might emerge from crossreaction between virus-associated epitope and IgG autoantigen. However, no cross-reactivity of the IgM RF B-cell receptor (BCR) or serum IgM RF with HCV antigens has far been demonstrated.

In addition, it has been shown that BCR from HCV-associated lymphoma patients was actually not able to bind to HCV antigens [58]. Furthermore, stereotyped BCR sequences that contribute to an highly biased repertoire in HCV-associated B-NHL, have been also detected in other HCV-negative B-cell malignancies, e.g. MALT lymphoma (some associated with RF), chronic lymphocytic leukemia, non-malignant B cells with RF activity, and nonmalignant marginal zone splenic B cells [55]. These data suggest that HCV-associated lymphoma may most likely arise from precursors with autoimmune properties rather than B cells specifically aimed at eliminating the virus.

Role of CD81-E2 binding. Several studies have supported a major role for HCV envelope glycoprotein E2 in indirect transformation. E2 binding to its receptor on B cells (i.e. CD81) [59] facilitates the assembly of the CD81/CD19/CD21 costimulatory complex [60]. Hence, E2-CD81 engagement plays a role in activating B cells by lowering their threshold of activation.

HCV E2 binding to CD81 receptor has also been shown to induce somatic hypermutation of the immunoglobulin gene locus [61] through activation-induced deaminase (AID) activation, regardless HCV replication or direct infection of B cells by HCV (Figure 1). This process has also been demonstrated in the context of malaria infection, in which chronic stimulation by Plasmodium elicits protracted AID expression in GC B-cells leading to chromosome translocations [62].

Role of cytokines and microenvironment. Upregulation of BAFF, a critical survival factor for B cells, has been demonstrated in HCV chronic infection [63]. Interestingly, BAFF levels are found



Figure 1. Model of indirect HCV-related B-cell transformation: continuous external stimulation of lymphocytes receptors by viral antigens and cytokines.

being lower in HCV patients without MC, intermediate with those with cryoglobulinemia and higher in those with NHL [64]. In addition, a specific BAFF promoter polymorphism reported to induce an increased transcriptional activity has been found with a higher frequency in HCV-infected patients with MC group than in the HCV-infected patients without MC (Figure 1). This suggests that mechanisms enhancing Ig production and B-cell survival may play a relevant role in HCV MC pathogenesis [65].

Other cytokines and growth factors, such as IL-6, IL-17, IL-10 and TGF- β , have been also reported to contribute to B-cell proliferation in HCV infection [66–68] (Figure 1).

Direct transformation of B-cell by HCV through oncogenic effects mediated by intracellular viral proteins

HCV-associated lymphoma, especially aggressive NHL, might occur without history of MC or low-grade lymphoma. Besides, they do not constantly exhibit a bias toward a specific VH usage [55]. The data presented hereafter indeed suggest that transformation may occur in some cases independently of chronic antigenic stimulation but rather by direct infection of B cells (Figure 2).

Evidences from murine models. Several murine models have shown that intracellular virus proteins might contribute to direct oncogenic transformation. For instance, combination of IFN regulatory factor-1 (irf-1) inactivation and persistent expression of HCV structural proteins, e.g. CN2, results in mice in the development of lymphoproliferative disorders after a latency comprised between 180 and 600 days [69]. In this model, decreased activation of caspases-3/7 and caspase-9 and increased levels of IL-2, IL-10 and Bcl-2, as well as increased Bcl-2 expression, were the primary events found to induce lymphoproliferation. Another transgenic mouse model expressing HCV core protein showed frequent development of follicular center cell type lymphoma (80% at >20 month of age), with HCV core mRNA detected in lymphoma tissue [70]. Mice expressing the full HCV genome in B cells develop DLBCL with a significantly higher Case 3:16-md-02741-VC Document 2934-2 Filed 03/07/19 Page 5 of 10 Annals of Oncology



Figure 2. Model of direct HCV-related B-cell transformation: oncogenic effects mediated by intracellular viral proteins.

frequency compared with their genetically identical counterpart without HCV expression [71]. A possible transforming mechanism could involve serum-soluble interleukin-2 receptor a subunit (sIL-2Ra), which was found in substantially elevated levels [72].

HCV lymphotropism. Although a growing number of studies have detected presence of HCV RNA negative strand in B cells from patients chronically infected with HCV as well as from HCV-positive MC patients [73–76], whether HCV infects B cells is still not universally accepted. However, expression of HCV-encoding protein, NS5 and NS3, in peripheral blood mononuclear cells [73–75] indicates that HCV not only replicates but also produces HCV proteins, ruling out the possibility that finding of negative strand of HCV RNA is solely due to passive absorption by circulating HCV in peripheral blood.

CD81, which is expressed in B cells has been shown to be an entry receptor for HCV [59]. Nevertheless, all HCV strains are not lymphotropic. It has been recently demonstrated that HCV tissue tropism is genetically determined by the properties of viral envelope proteins and the 5'-UTR sequences. In addition, the co-stimulatory receptor B7.2 (CD86) has been shown to mediate

HCV lymphotropism toward memory B-cells. Its binding to HCV leads to inhibition of memory B-cell function and enhances the differentiation of memory B cells into IgM-secreting plasmablasts [77].

In situ *detection of HCV proteins on NHL tissues.* Expression of NS3 protein in primary B-cells has been detected by immunohistochemistry in most cases of HCV-positive DLBCL (12/14) in contrast to HCV-positive MZL (4/14) or follicular lymphoma (1/6) [78]. These findings indicate that transformation of B cells toward DLBCL is favored by their infection by HCV and suggest a possible direct role of HCV in lymphomagenesis.

DNA damages induction. By activating error-prone polymerases and AID [79], HCV is able to cause mutations in *'immunoglobulin heavy chain'*, *BCL6*, *TP53* and *beta-catenin* genes of *in vitro* HCV-infected B-cell lines and HCV-associated peripheral blood mononuclear cells, lymphomas and hepatocellular carcinomas [79]. Expression of HCV core protein (C) and non-structural protein 3 (NS3) has also been associated with the induction of nitric oxide synthase (NOS) and reactive oxygen species (ROS)

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generation which might be responsible for mutations and DNA double strand breaks [80].

In addition, the HCV core protein binds to NBS1 protein, which inhibits Mre11–NBS1–Rad50 complex formation and thereby affects activation of ATM, a key element of the DNA damage response signaling pathway [81]. In the same line, NS3/4A protease has been recently demonstrated to downregulate activity of the Checkpoint kinase 2 (CHK2), a key sensor to DNA damage [75].

Overall, reduced ability of HCV-infected cells to efficiently repair DNA damage, coupled with the ability of HCV to induce DNA damages, would introduce random rearrangements into the genome, leading to predisposition to cancer (Figure 2).

Deregulation of BCR signaling. NS3/4A protease has been shown to induce CHK2 downregulation, which modulates posttranscriptional regulation of multiple mRNAs controlled by HuR. HuR, one of the CHK2 downstream targets, is a RNA-binding protein that binds to the 3'-UTR region of mRNAs and increases their stability [75]. In response to NS3/4A overexpression, the BCR signaling pathway was found to be the most affected pathways with the largest number of transcripts showing increased association with HuR, such as CD79A, CARD11, BTK, SYK, BCL6 etc. [75]. Overall, HCV infection in B cells may therefore contribute to B-cell transformation by upregulating host BCR signaling (Figure 2).

Deregulation of microRNAs network. A reduced expression of miR-26b has been found in HCV positive versus HCV negative patients with SMZL [82]. Diminished expression of miR-26b has demonstrated oncogenic potential *in vitro* and has been linked to a malignant tumor phenotype in hepatocellular carcinoma and lung carcinoma [83].

MiR-26b expression was also significantly down-regulated in B-cell lymphoma from HCV-transgenic mice [83], supporting a direct role of HCV infection in this process. Moreover, a recent analysis showed upregulation of miRNA-21 and downregulation of miRNA-26b in peripheral blood mononuclear cells from HCV-associated malignancies, i.e. hepatocellular carcinoma as well as NHL patients, when compared with controls [84]. An overexpression of miR-29a, miR-29b and miR-223 was also observed in patients with HCV-related nodal MZL [85]. Altogether, these findings suggest that deregulation of miRNAs network participates to HCV-related tumorigenesis.

Additional contributors to HCV-related lymphomagenesis

Genomics alterations in HCV-associated lymphoma. Given the latency for B-cell lymphoma development in HCV transgenic mice (>20 months) [70], it is most likely that additional genetic events are necessary for HCV-associated B-cell transformation.

NOTCH2, *NOTCH1* and *PTEN* mutations have been identified in, respectively, 20%, 4% and 2% of HCV-positive DLBCL patients. Mutations of the *NOTCH* pathway are associated with a shorter overall survival and correlate with coexistence in the diagnostic biopsy of a low-grade component along the large-cell component proper of DLBCL [86]. This finding is in agreement with the high frequency of *NOTCH2* mutations detected in MZL patients regardless of the HCV status [87]. Altogether, these data suggest that HCV-positive *NOTCH*-mutated DLBCL derive from previous (overt or not detectable) MZL.

Further studies are warranted to better characterize the mutational landscape of HCV-associated B-cell lymphoma.

Genetic predisposition in HCV-related diseases. More and more evidences argue for the role of host genetic factors in the development of HCV-related lymphoproliferative diseases.

Some specific SNPs in the MHC class II *HLA-DRB1* and -*DQA1* gene as well as in the *NOTCH4* gene have been demonstrated to be significantly associated with HCV-related benign and malignant lymphoproliferative diseases [88, 89].

The potential impact of the host HLA/KIR profile in HCVrelated disease progression has also been investigated [90]. *KIRDS2* and *KIRDL2* variants were significantly associated with HCV-related lymphoproliferative diseases including MC as well as malignant B-NHL. When analyzing specific combination of KIR and HLA haplotypes, it has been shown that the *HLA-Bw6/ KIR3DL1* combination was correlated with a higher risk of developing lymphoma than MC. In the other hand, reduction of *HLA-Bw4/KIR3DS1* was associated with an increased risk of developing an HCV-positive hepatocarcinoma [90].

In a French metacentric study including 87 patients with HCVassociated lymphoma of any histological subtype, a specific genetic variant of *TNFAIP3/A20*, the rs2230926G allele, was more frequently detected in patients with RF activity (20%) when compared with RF-negative patients (0%) (P = 0.01) [91]. It suggests that, in the context of chronic stimulation of RF+ B cells, a small constitutive A20 dysfunction leading to increased NF-kB activation may be sufficient for lymphomatous escape of autoimmune B cells.

Finally, specific TLR2-IL28B haplotypes have been recently reported to discriminate between HCV-positive patients more likely evolving toward liver damage (rs12979860 *IL28b*, *TLR2*-174 del variant) and those evolving toward a lymphoproliferative disorder (wild type haplotype) [92], supporting a role for innate immunity in HCV disease progression.

Models of lymphomagenesis associated with HCV infection

HCV-positive MZL and DLBCL: two distinct models of HCV-related lymphomagenesis

In view of the findings described above, two different routes of transformation leading to development of B-cell lymphoma in HCV infected patients may be considered.

It is now well established that chronic external stimulation leading to protracted stimulation of antigen-specific B-cell clones is likely to constitute the main driving mechanism in MZL and to some extent, in transformed DLBCL deriving from MZL [93–96].

In addition, we propose an alternative pathway of transformation based on direct HCV infection of B cells, especially in the HCV-positive *de novo* DLBCL subgroup [96]. Mixed cryoglobulinemia, RF and V_H1–69+ and V_K3–20/15 restriction usage are indeed unusual features of *de novo* large-B cells [7]. In addition, a fraction of HCV-positive DLBCL cases do not display associated

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Figure 3. Hypothetical model of HCV-positive follicular lymphoma pathogenesis. In the context of HCV infection, transformation of t(14; 18)positive memory B cells into FL could arise through GC reentries but may also involve actors outside mutagenic germinal center environment.

low-grade component or *NOTCH* pathways mutations [86]. Finally, the presence of viral proteins has been detected in tumor cells of HCV-positive DLBCL [78].

HCV-positive FL: a third pathogenetic pathway?

Although this model is highly speculative, we propose that a distinct pathway may be involved in HCV-positive FL (Figure 3).

When compared with normal healthy population [97], circulating IGH-BCL2 positive clones have been indeed observed with higher frequencies in patients with HCV infection, especially when associated with MC [15, 16] and in different subtypes of HCV-positive NHL [11, 17, 18]. We thus speculate that hepatitis C infection, through chronic inflammation, would favor GC reentries of opportunistic t(14; 18)-positive memory B cells, during a lifetime of recurrent immunological challenges, as previously shown in HCV-negative FL [98]. Stimulation and proliferation of GC/memory B cells may be triggered by a noncognate bystander T-cell help from T follicular helpers which is antigen independent and more based on CD40-CD40L interaction and/or cytokine production. Furthermore, t(14; 18)-positive GC/memory B cells may acquire secondary oncogenic events due to AID-mediated SHM and CSR, which promotes FL development and ultimately transformation into DLBCL.

However, in contrast to the other HCV-positive B-cell lymphoma subtypes, prevalence of HCV infection in FL patients is not different from those in the controls [3]. We therefore hypothesize that non-cognate bystander effect could occur outside germinal centers and involve other actors. Interaction between mannosylated Ig of FL cells and C-type lectins expressed by cells of innate immunity, such as macrophages and dendritic cells, has been indeed demonstrated to provide an activating antigen-independent signal for FL cells [99].

Discussion

Conclusion

HCV lymphomagenesis represents a fascinating model of cellular transformation involving several mechanisms, including chronic antigenic stimulation, interactions with infected microenvironment and cytokines and at least in some cases, direct transformation by virus proteins. Based on that, different models of HCVrelated lymphomagenesis have begun to emerge (Figure 4). In addition, DAA-based antiviral therapy has demonstrated promising results in HCV-positive B-cell lymphoma, especially in MZL, and should be integrated as part of the therapeutic arsenal. However, it

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Figure 4. Models of HCV-related lymphomagenesis. In addition to indirect chronic antigenic stimulation prevailing in marginal zone lymphoma (MZL) and transformed diffuse large-B-cell lymphoma (DLBCL), we propose two alternative mechanisms of transformation in HCV-positive *de novo* DLBCL and follicular lymphoma (FL).

is of prime importance to well define and understand the mechanisms of HCV-driven NHL pathogenesis in order to guide therapeutic decision. More biological evidence is still needed and appropriate studies should be conducted.

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Disclosure

The authors have declared no conflicts of interest.

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