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MINIREVIEWS

Hepatitis B in patients with hematological diseases: An update

Chiara Coluccio, Paola Begini, Alfredo Marzano, Adriano Pellicelli, Barbara Imperatrice, Giulia Anania, Gianfranco Delle Fave, Massimo Marignani

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Abstract

Hepatitis B virus (HBV) reactivation (HBVr) in patients undergoing immunosuppressive therapy is still a hot topic worldwide. Its prevention and management still represents a challenge for specialists dealing with immunosuppressed patients. Aim of this paper is to provide a critical review of the relevant information emerged in the recent literature regarding HBV reactivation following immunosuppressive treatments for oncohematological tumors. A computerized literature search in MEDLINE was performed using appropriate terms arrangement, including English-written literature only or additional relevant articles. Articles published only in abstract form and case reports not giving considerable news were excluded. Clinical manifestation of HBVr can be manifold, ranging from asymptomatic self-limiting anicteric hepatitis to life-threatening fulminant liver failure. In clusters of patients adverse outcomes are potentially predictable. Clinicians should be aware of the inherent risk of HBVr among the different virological categories (active carriers, occult HBV carriers and inactive carriers, the most intriguing category), and classes of immunosuppressive drugs. We recommend that patients undergoing immunosuppressive treatments for hematological malignancies should undergo HBV screening. In case of serological sign(s) of current or past infection with the virus, appropriate therapeutic or preventive strategies are suggested, according to both virological categories, risk of HBVr by immunosuppressive drugs

and liver status. Either antiviral drug management and surveillance and pre-emptive approach are examined, commenting the current international recommendations about this debated issue.

Key words: Reactivation; Lymphoma; Hematology; Immunosuppressive therapy; Prophylaxis; Hepatitis B virus; Chemotherapy; Occult/active/inactive carrier; Entecavir; Lamivudine

Core tip: Despite the increasing awareness regarding the issue of hepatitis B virus reactivation (HBVr) in patients undergoing immunosuppressive treatments, there are still some many debated items concerning this potentially fatal but preventable complication. Both hepatitis B surface antigen (HBsAg) patients and subjects with serological signs of previous resolved exposure to the virus (HBsAg negative/anti-core antibody positive patients) are at risk of HBVr. Purpose of our work was to analyze the current international literature and dedicate guidelines, providing evidences and strategies that have been proposed to manage these patients.

Coluccio C, Begini P, Marzano A, Pellicelli A, Imperatrice B, Anania G, Delle Fave G, Marignani M. Hepatitis B in patients with hematological diseases: An update. *World J Hepatol* 2017; 9(25): 1043-1053 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i25/1043.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i25.1043

INTRODUCTION

Hepatitis B virus (HBV) infection represents a significant global health problem, since almost one third of the world's population has serological signs of previous or present infection, and that 240 million individuals are chronic hepatitis B surface antigen (HBsAg) carriers[1]. Worldwide, low rates of serological HBsAg positivity (0.2%-0.5%) and signs of previous HBV contact [4%-6% HBsAg negative/anti-hepatitis B core antigen antibodies (anti-HBc) positive subjects] are registered in north western and central Europe, north America and Australia. On the contrary, the highest prevalences are reported in China, Southeast Asia and tropical Africa (chronic infection 8%-20%, and previous exposure 70%-95%, respectively)[2].

It is presently well known that medications such as glucocorticoids and anticancer treatments can interfere with the host immune system and blunt the control that it exerts over HBV replication, with the potential to cause viral reactivation (HBVr) in both HBsAg positive patients and individuals with serological signs of previous resolved HBV exposure. HBVr can assume various manifestations, spanning from asymptomatic hepatitis to life threatening fulminant liver failure. This risk is most common among patients undergoing treatment for hematological tumors or those receiving hematopoietic stem cell transplantation (HSCT). Nevertheless, also patients with solid tumors (such as breast cancer), immunological diseases and inflammatory bowel diseases are exposed to the risk of HBVr[1,3-5].

In this paper, we will critically review the relevant information emerged in the recent international literature regarding HBVr, focusing on patients undergoing immunosuppressive treatments for hematological malignancies.

LITERATURE SEARCH

A computerized literature MEDLINE search was done adopting several combinations of these terms: HBsAg, reactivation, lymphoma, hematology, immunosuppressive therapy, anti-HBc, occult carrier, including only papers in English language. Literature on hematopoietic stem cell transplantation recipients was not considered. Articles published only in abstract form were excluded. Case reports have been included only if adding significant contributions.

HBV INFECTION, HOST IMMUNE RESPONSE AND VIROLOGICAL PROFILES

When the HBV virus encounters the human host, in the presence of a competent immune system, three outcomes relevant to our discussion can be observed: (1) the infection can be rapidly cleared, as it is to be expected in most immunocompetent adults. However, in a part of these individuals, the covalently closed circular (ccc) viral DNA can integrate and persist indefinitely as an immune template in the host hepatocyte; (2) The host immune response might create a dynamic equilibrium in which viral replication either stops or is minimally active; and (3) the host immune system is unable to either eradicate or control viral replication and a state of chronic liver disease ensues, potentially leading to the development of liver cirrhosis and its consequences. These different immunological and clinical scenarios of host-virus interplay constitute the basis to define the corresponding virological HBV categories, summarized in Table 1[5].

Active carriers (AC) are those HBsAg positive patients in whom HBV replication prevails over the control of host immune system, and are characterized by elevated HBVDNA levels ($\geqslant$ 2000 IU/mL). On the other extreme are the occult HBV carriers (OBI), individuals in whom the immune system has successfully cleared the acute viral infection. These individuals however still harbor the viral DNA inside the hepatocytes, integrated in the form of cccDNA but under the effective replicative control of the immune

Table 1 Virological categories of hepatitis B virus infected patients (adapted from[5])

	AC	IC	pOBI
HBsAg	+	+	-
Anti-HBc	+	+	+
Anti-HBs	-	-	-/+
qHBsAg	⩾ 1000	< 1000	-
ALT	Increased	Normal	Normal
HBV DNA in the blood	⩾ 2000	< 2000	-
Liver stiffness (kPa)	> or < 6	< 6	< 6

HBsAg: Hepatitis B surface antigen; AC: Active carrier; IC: Inactive carrier; OBI: Occult hepatitis B virus (HBV) infection; Anti-HBc: Anti-hepatitis B core antigen antibodies; Anti-HBs: Antibodies to HBV surface antigen.

system, only showing serological signs of previous viral exposure (*i.e.*, presence of anti-HBc), very low (< 200 IU/mL) or absent circulating HBVDNA, positive or negative antibodies to HBV surface antigen (anti-HBs), and normal transaminases[6]. The third, more intriguing and elusive category, is currently that of inactive carriers (IC), HBsAg and anti-envelope antigen antibody (anti-HBe) positive patients with indosable or < 2000 IU/mL HBVDNA levels. Their classical definition is completed by the concurrent presence of persistently normal levels of serum transaminases, no signs of HBV-induced liver inflammation/fibrosis and a clinically benign course. The IC state was generally ratified by the stability of these parameters during the course of an extended (usually 12-mo) observation period[7]. However, this lengthy mandatory observation period is awkward in settings requiring a rapid categorization, such in those in which a decision regarding the start of antiviral drugs to protect from HBVr is to be taken.

In the Asian pacific region, the benignity of this entity has been debated, and the term of "low replicative chronic HBV infection" proposed, favored over the "inactive carrier" definition, as the latter can give the patients an incorrect sense of confidence. Considering that hepatitis B infection should be considered a dynamic interplay between the host and the virus, the activity profile can modify over time and virological category can change at different time points[8]. However, this scenario is based on the virological characteristics of the Asian population, while in the Mediterranean basin up to one third of IC individuals present levels of HBVDNA between 2000 and 20000 IU/mL with normal transaminases and absence of liver fibrosis during long term observation. To further sharp the definition of this virological HBV category, recent studies have focused their attention on the role of quantitative HBsAg testing (qHBsAg), HBVDNA cut-offs, and use of fibroelastometry[9-11].

Recent studies have in fact provided data to allow a timely identification of IC group of patients with an acceptable approximation, without the need of a prolonged observation.

In the study by Brunetto *et al*[7], 209 genotype D carriers were enrolled, and the capacity of qHBsAg testing to discriminate between active and inactive HBV carriers and patients with active chronic hepatitis B (CHB) was tested. It was demonstrated that a one-time (so called "spot") quantification of HBVDNA below 2000 IU/mL and HBsAg less than 1000 IU/mL was able to single out IC with good sensitivity, specificity, positive and negative predictive values (91.1%, 95.4%, 87.9%, 96.7% respectively) concluding that this single observation approach obtains the same results of long term monitoring with an acceptable approximation[7]. Raimondo *et al*[6] recently evaluated the reliability of serum HBVDNA and qHBsAg testing, along with liver stiffness measurements (LSM) in identifying the IC status at a spot point investigation among 147 HBsAg and anti-HBe positive patients, including 57 IC and 90 individuals with CHB. The overall evaluation of all parameters allowed to recognize 23 out of 57 (40.3%) ICs, with good specificity, sensitivity, positive and negative predictive values, and diagnostic accuracy (100%, 96%, 100%, 92% and 97%respectively). Even removing from the analysis CHB or cirrhotic patients, the results were similar. It was concluded that combined assessment of HBVDNA level, liver stiffness along with quantitative surface antigen measurements, provide a dependable working instrument, correctly identifying a large portion of IC with a spot assessment only[12]. In genotype B and C patients the validation of a one-time dosage of qHBsAg and HBV DNA to predict IC state was performed in a population of 1529 subjects. When HBsAg < 1000 IU/mL was associated with HBVDNA < 2000 IU/mL, the one-time evaluation was able to discriminate IC from patients with chronic hepatitis B with slightly lower diagnostic accuracy[13]. Thus, it can be concluded by these observations that by using serological and elastographic testing, IC can be currently identified with an acceptable approximation in those instances when prolonged observation is unfortunately not an option.

HBV REACTIVATION AND FACTORS INFLUENCING ITS OCCURENCE

HBVr during immunosuppressive treatments can occur as the result of a loss of control over viral replication induced by these drugs, since they can modify the competence of the host immune system[3]. In this setting, the virus rapidly replicates infecting multiple hepatocytes, however in this phase usually no damage occurs since the immunological response is blunted by immunosuppressive medication. When the immunosuppressive therapy is concluded, a progressively restored immune system can activate the search, destroy and eradication of the HBV infected hepatocytes, and this can cause massive liver necrosis and acute liver failure. This event process can occur at different time points, usually ranging from a few months but also potentially developing years after the end of the immunosuppressive therapeutic cycle, after

Table 2 Incidence of hepatitis B virus reactivation without prophylaxis (adapted from[21])

Disease	HBsAg+ (%)	HBsAg-/anti-HBc+ (%)
Lymphoma	18-73	34-68
Acute leukaemias	61	2.8-12.5
Multiple myeloma	Not available	6.8-8
Breast cancer	21-41	Not available
Hepatocellular cancer (systemic chemotherapy)	36	11
Inflammatory bowel disease	36	0-7
Autoimmune diseases	Not available	17

HBsAg: Hepatitis B surface antigen; Anti-HBc: Anti-hepatitis B core antigen antibodies.

immune response is completely restored[14,15].

HBVr has been variably defined overtime and a consensus has not been reached. According with the American Gastroenterological Association (AGA) guidelines, in HBsAg carriers reactivation occurs when there is either a *de novo* detection of viremia or a one $\log_{10}$ or greater increase in HBVDNA as compared to baseline levels (obtained before starting therapy). Hepatitis flare is considered when there is at least a two-three fold rise of ALT above baseline or a predetermined multiple of the upper normal limit. In HBsAg negative/anti-HBc positive patients reactivation is defined by the reverse seroconversion to HBsAg-positive condition[16]. Similar definitions are also suggested by the Italian association for the study of the liver (AISF).

Since different levels of baseline HBVDNA influence the occurrence of HBVr, the different virological classes, proceeding from OBI to IC and then to AC, are at a progressively higher risk of reactivation. It is in fact widely accepted that subjects with high level of viremia before immunosuppressive therapy are at an increased risk for the development of HBVr as compared to those with undetectable or low levels of HBVDNA[17-19].

Accordingly, many studies have estimated that the risk of HBVr is 5- to 8-fold higher among HBsAg positive patients[20] and that HBeAg positive patients are at higher risk of developing HBVr as compared to HBeAg negative ones[17]. Compared to other diseases groups, patients with hematological malignancies are reported to be those characterized by the highest risk of experiencing HBVr (Table 2)[21] with figures ranging between 24%-88%[22]. It is speculated that this difference could be due to the intrinsic immunosuppression typical of hematological malignancies and to the treatments used to cure them. Interestingly, the first cases of HBVr were actually recorded among patients with lymphoma[23]. In a large multicenter case-control study conducted in Italy, the prevalence of HBsAg positivity among 400 B-cell non-Hodgkin's lymphoma (NHL) cases was higher than in 392 controls (8.5% *vs* 2.8%, respectively)[24]. Thirty-eight to 73% of HBsAg positive NHL cases undergoing chemotherapy for NHL can experience HBVr[25,26].

Also multiple myeloma patients are at risk of HBVr as reported in several recent papers, since in the advanced stages of this disease the occurrence of a more critical immune dysregulation might predispose to the development of viral reactivation[27].

A substantial risk of HBVr, not different from that of lymphoma patients, has also been described among patients undergoing treatment for acute myeloid leukemia. Recently Chen *et al*[28] observed that HBVr and HBV-related hepatitis occurred in 9.5 and 8.3 per 100 person-years, respectively. There is now clear evidence that different classes of immunosuppressive drugs are characterized by different risks of inducing HBVr. Medications used for hematological malignancies are frequently marked by a severe immunosuppressive effect, as the case of rituximab (RTX), an anti-CD20 monoclonal antibody acting as a potent B-cells depleting agent, mostly used in hematological malignancies during the last two decades[29] and well known to increase the chance of HBVr of more than five-fold[30]. This high risk is justified by the marked B-cell reduction, which interferes with the production of anti-HBs and their neutralizing effect on serum HBsAg. Moreover, RTX worsens the imbalance of antigen-presenting B-cells typical of chronic HBV infection, determining a lower activity of CD4 T-cell in generating an adequate immune response[31].

The rate of HBVr inherent to these B-cell depleting agents (RTX, but also ofatumumab) is roughly 16.9% among patients with serological signs of previous HBsAg exposure, and their seroreversion percentage is 20%-40%. With these drugs HBVr can be a late event, even up to 60 mo after the cessation of immunosuppressive therapy, further marking the strong and lengthened influence of these drugs on the recovery of immune competence[16,32,33].

Considering these evidences antiviral prophylaxis of these patients have to be prolonged up to 10-24 mo after the discontinuation of the B-cell depleting agents and a careful surveillance has to be activated after the antiviral therapy withdrawal[3,5]. Among the B-cell depleting agents, more drugs are or will soon be available. A possible example is Obinutuzumab, a new humanized monoclonal antibody to CD20[34] which, in association with other chemiotherapics, has been shown to be more effective than RTX in the treatment of chronic lymphatic leucemia (CLL)[35], but at the cost of determining a more profound immunosuppression than RTX. Even though no HBVr cases have been registered following the use of this drug, it is conceivable that the concerns developed during the experience with RTX should also be extended to the other members of this class of drugs.

Corticosteroids are also widely used in the treatment of hematological malignancies and combined to cytotoxic agents in several therapeutic schedules for the treatment of lymphoma and multiple myeloma. These dugs are able to influence the activity of T-cells but also to directly intensify HBV replication[36]. It has in

Table 3 Risk of hepatitis B virus reactivation according to different immunosuppressive drug classes (adapted from[21])

Risk	Drug class
High (> 10%)	B-cell depleting agents
	Anthracycline
	Corticosteroids high dose
Moderate (1%-10%)	TNFα inhibitors
	Cytokine and integrin inhibitors
	Tyrosine kinase inhibitors
	Corticosteroids moderate dose
Low (< 1%)	Corticosteroids low dose
	Traditional immunosuppression (*e.g.*, azathioprine or methotrexate)

fact been demonstrated that the prolonged assumption of prednisolone increases HBsAg and HBVDNA levels in liver cells, and that the withdrawal of corticosteroid seems to determine a rebound in immune T-cell function resulting in hepatocyte destruction[37]. Corticosteroids have the potential to cause HBVr, but with different percentages of risk depending on dosage, duration of treatment and route of administration; in fact high-dose (> 20 mg/d) prednisolone, and prolonged treatment extension (> 1 mo), correlate with higher risks of reactivation.

AGA evaluated the risk of HBVr according to distinct drug categories, basing its conclusions on an extensive systematic review of the available studies. However, on some medication, data were limited and extrapolated only from either case series or caser reports. This risk stratification is reported in Table 3[21]. A gradation of the HBVr risk (high > 10%, moderate 1%-10% and low < 1%) has been proposed and currently accepted in the western countries[1,3,5].

PREVENTION OF HBVr

To prevent HBVr, it is crucial to identify patients at risk for the development of this potentially severe event before starting immunosuppressive drugs. Most international scientific associations such as the European Association for the Study of the Liver (EASL), AGA, the Asian-Pacific Association for the Study of the Liver (APASL) and AISF suggest to screen for HBV all patients scheduled to undergo immunosuppressive treatment by testing HBsAg, anti-HBc and anti-HBs[1,3,5,8].

On the other hand the American Association for the Study of Liver Diseases (AASLD) and the American Society of Clinical Oncology (ASCO) recommend to limit HBV screening to patients with high or moderate risk of HBVr risk factors[38,39]; for patients at low risk, screening strategies should follow instead the indications produced by the Center for Disease Control and Prevention[40] and the United States Preventive Services Task Force[41,42].

It has been demonstrated in various studies that HBsAg positive patients should undergo antiviral treatment started before (2-4 wk) and continued during chemotherapy, regardless of baseline HBVDNA level, and not on a pre-emptive based strategy, considering that if hepatitis has already developed, it could be more difficult to control the extent of the reactivation process[32,43].

Currently, guidelines worldwide indicate treatment with nucleot(s)ide analogs (NA) for patients with hematological malignancies, positive for the HBsAg and receiving cytotoxic chemotherapeutic drugs[1,3,5,8]. The duration of the antiviral treatment in these patients has been the matter of long debates in the last decade, but actually a higher concordance is registered. In patients with CHB or cirrhosis antiviral therapy has not to be discontinued. However in IC it should be continued during the immunosuppressive treatment and for 12 mo after its discontinuation. Patients with serological signs of resolved past exposure to the virus and detectable viremia should be managed as surface antigen positive subjects, while those with undetectable serum HBVDNA should be carefully followed by ALT, HBsAg and/or HBVDNA testing (regardless of anti-HBs status), and promptly treated with nucleoside analogues upon confirmation of HBV reactivation before ALT elevation. However, when patients with this serological pattern (HBsAg negative/anti-HBc positive) are treated with RTX or similar immunosuppressive drugs, especially when low/absent serum hepatitis B surface antibodies are detected or if close HBVDNA surveillance is not feasible, many experts acknowledge their higher risk of viral reactivation and recommend prophylaxis[8]. In case of monitoring aimed at the prompt activation of pre-emptive therapy, ALT, HBsAg and/or HBVDNA testing is performed every 1-3 mo during the immunosuppressive treatment in the early phase, depending on the type of immunosuppressive drug and comorbidities. When prophylaxis is instead chosen, lamivudine (LAM) is usually suggested[44]. The 2007 Italian AISF guidelines and its recent implementation are in agreement with the international indications previously reported. In particular, among HBsAg-positive patients, AC are treated as their immunocompetent counterparts with the more potent antivirals available, while viremic IC, which received LAM in the past, are now preferentially treated with entecavir (ETV). In these patients monitoring of drug efficacy was performed by HBVDNA and ALT testing. In hematological anti-HBc positive subjects undergoing severely immunosuppressive regimens of various kind (see[4] for a complete list), universal prophylaxis with LAM has been advocated and recently confirmed. In these patients monitoring in prospective of pre-emptive therapy or of response to treatment is advised with ALT and HBsAg testing for their high specificity and maneuverability during the very long period at risk after the immunosuppressive treatment[4,5].

CHOICE OF ANTIVIRAL AGENTS

Regarding the antiviral to use in HBsAg positive sub-

jects, the 2017 American and European guidelines suggest the use of a NA with high potency and high genetic barrier (ETV or tenofovir disoproxil or alafenamde, respectively TDF and TAF)[1,3]. In these patients the role of LAM remains marginal in the very few IC patients without detectable viremia or in developing countries[3,5]. In HBsAg negative/anti-HBc positive subjects with hematological diseases and/or treated with B-cell depleting drugs high barrier antivirals can be obviously considered but the antiviral treatment with LAM is yet accepted[1,5].

In the face of such indications, the most part of data derived from the historical experience with LAM. Seminal papers considered LAM prophylaxis as an efficient agent to decrease the event of reactivation and hepatitis flare, to reduce the risk of HBV-related liver failure, and prevent the delay or discontinuation of chemotherapy as a consequence of HBVr[45]. The influential systematic review by Loomba demonstrated that LAM prophylaxis exerted a protective role against HBVr and death attributable to hepatitis B (relative risk 0.0-0.21 and 0.0-0.2 respectively)[22].

A later review concluded that antiviral LAM prophylaxis during cytotoxic treatment influenced HBVr, determining both a 87% reduction of this event, and a 92% decrease in treatment delay/early interruption of chemotherapy as compared to patients not given prophylaxis[45].

The systematic review and metanalysis of five randomised controlled trials contained in the recent AGA technical review, compared LAM prophylaxis to treatment at the beginning of viral reactivation (pre-emptive strategy)[16,43,46-49]. Antiviral prophylaxis was more effective than the pre-emptive strategy [overall risk ratio (RR) = 0.13], and also determined a significant decrease of hepatitis flare risk (RR = 0.16)[16]. Nevertheless, it has currently been suggested that LAM prophylaxis provides a suboptimal protective action for IC with detectable HBVDNA. The supposed superior efficacy of ETV as compared to LAM in the prevention of HBVr among patients undergoing treatment for hematological malignancies is supported by the results of the registrative studies in patients with CHB[50,51], in which ETV was shown to be more powerful than LAM in terms of histological amelioration, control of viremia, and reversal of ALT values to normal range in either HBeAg positive or negative chronic active hepatitis patients.

Additionally, in patients with NHL has been suggested that LAM provides a suboptimal preventive approach also in low viremic patients. A randomized multicenter study compared the efficacy of prophylactic therapy with LAM and ETV among HBsAg positive subjects and diffuse large B-cell lymphoma treated with RTX-CHOP (Cyclophosphamide, Hydroxydanorubicin, Oncovin, Prednisone); in low viremic (HBVDNA < 2000 IU/mL) patients it was demonstrated that the virological events were significantly lower in the ETV group considering hepatitis (8.2% *vs* 23.3%), HBVr (6.6% *vs* 30%) delayed hepatitis B (0% *vs* 8.3%) and chemotherapy disruption (1.6% *vs* 18.3%). However, at the moment this is the only available prospective study, burdened by some relevant limitations, such as the high prevalence of low viremic HBeAg positive patients in the Asiatic population evaluated[52]. However, a recent systematic review with network meta-analysis has suggested that prophylactic therapy with tenofovir or ETV may represent the most potent intervention to prevent HBVr and HBV-related morbidity and mortality in HBsAg-positive patients undergoing chemotherapy[53]. In two meta-analysis aimed to HBsAg-negative/anti-HBc-positive patients treated with RTX without antiviral prophylaxis, HBVr developed in 6.3%-16.9% of cases[16,54].

LAM was the drug most used for the universal prophylaxis in antiHBc-positive patients with hematological disease. In this setting viral breakthrough and loss of response during the antiviral treatment is very rare, while the risk of HBVr is significant during the first 6-12 mo after the discontinuation[5,16].

A unique randomized prospective study was performed in anti-HBc positive patients treated with RTX, comparing 3 mo of prophylaxis with LAM or ETV. HBVr was significantly higher in the LAM group (P = 0.19); however all the clinical events developed after (0.5-14 mo) the discontinuation of the drug without demonstrating a higher protective effect of ETV during the therapy[46].

LATEST NEWS AND COMPARISON BETWEEN THE MOST RECENT INDICATIONS

As previously reported, in the last few months some relevant indications on the management of HBV reactivation among immunosuppressed patients have emerged and published.

The Italian guidelines[5] are the result of the continuously updated work produced by a team of hepatologists dedicated to the management of immunosuppressed patients at risk for HBV reactivation. Its contents have been widely cited in this paper, as for instance the controversies regarding the best strategies to manage inactive carriers. Guidelines are discussed and developed in single topic events endorsed by AISF. Statements are produced after revision and discussion of the specific literature by hepatologists and other specialists such as hematologists, oncologists, immunoreumathologists, nephrologists and transplantologists. Virological classes and their relative diagnostic criteria are addressed as are screening and diagnostic approaches. Definitions of clinical and virological events are provided. Management and follow up strategies are also thoroughly scrutinized with the aim to promote the awareness regarding this issue, and collaboration among specialists. HBV screening is recommended in all patients undergoing

treatment for hematological malignancies with the use of HBsAg, anti-HBs, and anti-HBc. HBVDNA is then tested to both distinguish AC and IC and to identify potential false OBI. The different classes of risk for HBV reactivation proposed by the 2015 AGA guidelines have been incorporated. For patients with a high risk of reactivation, evaluation by an expert in liver disease is required. For HBsAg positive patients with hematological malignancies the risk of reactivation emerges to be clearly significant (24%-88%, median 50%), and the particular increase of HBVr associated with the use of RTX has been definitely stated. Also, the increased risk of HBVr due to the use of RTX in the OBI group has also been clearly recognized. In this latter virological category, the actual risk of reactivation as the result of treatment with several recently introduced biologics (imatinib, bortezomib, mogamolizumab, ofatumumab, carfilzomib, romidepsin, *etc*.) remains debated. As far as the treatment of HBsAg-positive patients is concerned, even if most available data came from the experience developed with LAM, the presence of newer drugs with greater potency and high genetic barrier, has imposed ETV and tenofovir (especially in the new form to be commercialized in Italy, TAF, with an improved safety profile) as the drugs of choice in viremic patients. In OBI treated with RTX for lymphoma, or with detectable HBVDNA LAM still maintains its role, in the absence of a proven greater protective effectiveness over other antivirals. Antiviral treatment with either ETV or TDF (TAF) is recommended indefinitely for AC patients, while in IC patients, LAM (HBVDNA negative) or ETV (HBVDNA positive) prophylaxis is indicated for at least 12 mo from the end of the immunosuppressive treatment. In OBI subjects duration of LAM prophylaxis it is indicated to extend prophylaxis for at least 18 mo after immunosuppressive regimen has been stopped. In LAM treated pOBI, the monitoring of ALT and HBsAg is indicated every three months. Monitoring in AC during and after the immunosuppressive treatment is similar to that of immune-competent; for IC in prophylaxis, monitoring should be performed dosing ALT and HBVDNA, every 12 wk in the case of LAM; every 6-12 mo, after virological response, with ETV and TDF(TAF). In case of viral breakthrough during prophylaxis or therapy with LAM or ETV, the prompt activation of a rescue therapy with either TDF or TAF is advised; during therapy with TDF/TAF or ETV a partial virological response requires a combined therapy with a nucleoside and a nucleotide. A similar monitoring (HBsAg in OBI and HBVDNA in AC) is recommended in the first month and every three months after the discontinuation of prophylaxis for the first year and every six months thereafter.

Another goal of the team is to provide practical indications for the working physician. To this purpose, statements are then published as a full report illustrating the management of the different subclasses of immunosuppressed patients. These indications have been published for the first time in 2007 and have been constantly updated thereafter during the course of the years. The most recent paper has been published online on the AISF web site in February 2017, and a further meeting is scheduled by the end of this year, with the aim of producing and English version of the newly discussed statements.

The EASL has published in April 2017 the updated guidelines on the management of hepatitis B infection[1]. In this paper, as in its previous 2012 version, the issue of immunosuppressed patients with signs of current or past infection with the HBV are addressed in the section dedicated to the treatment of various special patients groups with HBV infection. Also in this paper, the different classes of risk for HBV reactivation proposed by the 2015 AGA guidelines have been accepted. Vaccination of HBV seronegative immunosuppressed individuals is endorsed. Similarly to the AISF guidelines, it is suggested that all patients scheduled to undergo cytotoxic and/or immunosuppressive treatments should firstly perform a serological screening based on HBsAg, anti-HBs and anti-HBc testing. Evidence and grade of recommendation are very strong. All HBsAg-positive candidates for immunosuppressive therapies should undergo evaluation by a specialist to define their virological class. All HBsAg positive patients should start potent NA as a treatment or prophylaxis. A clear cut approach is proposed for AC, and they should be treated with ETV, TDF or TAF, similarly to the immunocompetent patients. Controversial remains the management of IC. Prophylactic LAM has been shown to obtain a reduction of both HBV reactivation risk and of associated morbidity and mortality. Nevertheless, a residual risk of HBV reactivation remains (approximately 10%) in patients with low viremia (HBV DNA < 2000 IU/mL). Thus, a simplified approach recommends ETV, TDF, TAF in all HBsAg positive patients, both as treatment and prophylaxis (Evidence level Ⅱ-2, grade of recommendation 1). The EASL guidelines also suggest long term prophylaxis (at least 12 mo, and 18 mo in case of rituximab-based regimens) after the cessation of the immunosuppressive treatment, and NA prophylaxis should be stopped only in case the underlying disease is in remission. During prophylaxis, liver function tests and HBVDNA should be tested every 3 to 6 mo. Testing should be performed with the same schedule also after NA withdrawal, since a relevant proportion of HBV reactivations develops after their discontinuation. It is not defined when testing should be stopped.

The risk of HBV reactivation in OBI varies widely according to underlying disease and the type and duration of immunosuppressive regimen. HBVDNA testing should be performed before immunosuppression. If viremic, they should be treated similarly to HBsAg-positive patients. As in the Italian guidelines, in patients at high risk (10%) of HBV reactivation (*i.e.*, anti-HBc

positive subjects undergoing treatment with rituximab in the oncohematological setting; those undergoing stem cell transplantation), antiviral (universal) prophylaxis is recommended. This should be continued for at least 18 mo after stopping immunosuppression and monitoring should continue for at least 12 mo after prophylaxis withdrawal. LAM may be used although cases of HBV reactivation due to LAM resistance have been reported. Interestingly, the EASL guidelines suggest that prophylaxis with ETV or TDF or TAF can also be considered in HBsAg-negative, anti-HBc positive patients receiving highly immunosuppressive regimens of extended duration. So it is concluded that these patients should receive anti-HBV prophylaxis if they are at high risk of HBV reactivation (Evidence level Ⅱ-2, grade of recommendation 1). In isolated anti-HBc positive subjects with either moderate (< 10%) or low (< 1%) risk of HBV reactivation, pre-emptive therapy and not prophylaxis is recommended. Also, the EASL guidelines consider HBsAg reappearance (seroreversion) the main virological event in these patients, constantly associated with hepatitis flare. As also indicated in the Italian guidelines, HBVDNA detection leads to seroreversion and hepatitis in only 50% of cases, thus being less specific as compared to HBsAg testing. However, with an apparent contradiction or a conservative prudence, both HBsAg and/or HBVDNA are monitored every 1-3 mo during and after immunosuppression, and therapy with ETV, TDF or TAF started in case of detectable HBVDNA or HBsAg seroreversion following a pre-emptive strategy. Since after HBsAg seroreversion a severe, even fatal, acute hepatitis could ensue, NA should be started as early as possible, independently of ALT levels. Interestingly the opportunity of using universal prophylaxis rather than pre-emptive therapy is recommended for selected clinical settings, characterized by long duration of immunosuppression, limited compliance to monitoring or unknown risk of viral reactivation for new biological. Limited are the indications on how and when follow-up should be performed after NA withdrawal.

A very recent review by Loomba and Liang[3] also needs to be mentioned. It further stresses and perfects the 2015 position of the AGA regarding patients with signs of current or past HBV infection undergoing immunosuppressive treatments at risk for viral reactivation. The authors accurately scrutinize the most recent data regarding this issue, updating the risk of reactivation associated to other immunosuppressive treatments such as cytokine and integrin inhibitors, immune checkpoint inhibitors such as ipilimumab (anti-CTLA4) and nivolumab (anti-PD-L1), and histone deacetylase inhibitors (HDIs). Complementary information is also provided on tyrosine kinase and proteasome inhibitors. Fine mechanisms of reactivation are reviewed. As in the AISF guidelines, a thorough baseline evaluation of liver status is recommended, and screening for HBV infection by testing HBsAg, anti-HBc and anti-HBs suggested for all patients who are receiving therapies that have either a high or moderate risk of reactivation. Evaluation by a HBV specialist is recommended. Even if LAM might be considered in resource-limited countries, especially in HBsAg-positive individuals with either undetectable or very low HBVDNA serum levels, high potency and high genetic barrier antiviral drugs such as ETV and tenofovir are preferred. Patients with CHB (HBsAg positive HBVDNA ⩾ 2000 IU/mL, elevated transaminases) should be treated as their immunocompetent counterpart. IC (HBsAg positive, HBV-DNA < 2000 IU/mL, normal transaminases) should undergo prophylaxis when exposed to high- and moderate-risk immunosuppressive therapy. Prophylaxis should ideally be initiated 14-30 d prior the initiation of immunosuppressive treatment and maintained for a minimum of 12 mo after its discontinuation.

For IC exposed to low-risk immunosuppressive treatments and OBI patients, surveillance with ALT and HBsAg (adding HBVDNA in those who are HBsAg positive) is recommended. To reduce the event of reactivation, OBI treated with RTX or other high risk treatments should undergo prophylaxis. For OBI at a moderate risk, anti-HBV prophylaxis should be considered, but they could also be monitored for serum ALT and HBsAg levels (and not by HBVDNA testing, similarly to the AISF indications) every 3 mo up to 6 mo after the discontinuation of immunosuppressive treatments. However, since HBV reactivation may occur up to 1-2 years after the last dose of RTX, patients treated with this medication may continue prophylaxis for up to 2 years after its discontinuation.

DISCUSSION AND CONCLUSION

Management of patients with HBV infection undergoing immunosuppressive therapy for hematological malignancies is still a challenge. It is necessary to be aware and vigilant about the risk of HBVr and its potential dire consequences and complications. Baseline screening for HBV infection before treatment initiation it is thus mandatory for these patients. HBV serum markers (HBsAg, anti-HBc and anti-HBs) must be checked, in order to stratify the risk of reactivation and decide which category of patients needs therapy and what is the best option for them.

Management with appropriate antivirals is indicated for their marked propensity to reactivate. Antiviral therapy is necessary in patients with moderate or high risk for reactivation. For HBsAg positive patients antiviral therapy is mandatory; for HBsAg negative/anti-HBc positive patients (OBI) it is possible to consider either prophylactic antiviral management (especially in patients undergoing high-risk therapies), or a pre-emptive approach monitoring ALT, HBsAg and/or HBV-DNA level and starting antiviral therapy as soon as it becomes detectable in the blood.

For several years LAM has been the only antiviral available to treat and manage hepatitis B and its reactivation, but during the last few years several

studies have been published to demonstrate the efficacy of antivirals with superior characteristics of potency and genetic barrier as ETV and TDF (waiting for the availability of TAF, a less nephrotoxic prodrug). Today in the setting of hematology these high barrier drugs have to be used in HBsAg-positive patients and it should be clear that LAM maintains a role only for the universal prophylaxis of HBVr in HBsAg-negative/anti-HBc positive (OBI) patients.

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MINIREVIEWS

Hepatitis B in renal transplant patients

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Abstract

Hepatitis B virus (HBV) poses a significant challenge for both dialysis patients and kidney transplant recipients despite its decreasing rates, especially in developed countries. The best preventive method is vaccination. Patients with chronic renal disease should ideally be vaccinated prior to dialysis, otherwise, reinforced vaccination practices and close antibody titer monitoring should be applied while on dialysis. HBV infected dialysis patients who are renal transplant candidates must be thoroughly examined by HBV-DNA, and liver enzyme testing and by liver biopsy. When needed, one must consider treating patients with tenofovir or entecavir rather than lamivudine. Depending on the cirrhosis stage, dialysis patients are eligible transplant recipients for either a combined kidney-liver procedure in the case of decompensated cirrhosis or a lone kidney transplantation since even compensated cirrhosis after sustained viral responders is no longer considered an absolute contraindication. Nucleoside analogues have led to improved transplantation outcomes with both long-term patient and graft survival rates nearing those of HBsAg(-) recipients. Moreover, in the cases of immunized HBsAg(-) potential recipients with concurrent prophylaxis, we are enabled today to safely use renal grafts from both HBsAg(+) and HBsAg(-)/anti-HBc(+) donors. In so doing, we avoid unnecessary organ discarding. Universal prophylaxis with entecavir is recommended in HBV kidney recipients and should start perioperatively. One of the most important issues in HBV(+) kidney transplantation is the duration of antiviral prophylaxis. In the absence of robust data, it seems that prophylactic treatment may be discontinued in selected stable, low-risk recipients during maintenance immunosuppression and should be reintroduced when the immune status is altered. All immunosuppressive agents in kidney transplantation can be used in HBV(+) recipients. Immunosuppression is intimately associated with increased viral replication; thus it is important to minimize the total immunosuppression burden long term.

Key words: Hepatitis B virus (+) donor; Hepatitis B virus (+) recipient; Renal transplantation; Viral reactivation; Immunosuppression; Nucleoside analogues; Antiviral discontinuation; Antiviral prophylaxis; Hepatitis B

Core tip: Though decreasing, hepatitis B still remains a considerable problem, especially in high-risk patient populations as kidney transplant recipients. The widespread use of new antivirals and the introduction of universal prophylaxis immediately after transplantation have changed the picture in hepatitis B virus (HBV) (+) transplantation. Long term survival rates of HBV(+) recipients are approaching those of HBV(-), altering HBV(+) kidney transplantation from a "high risk" procedure into routine practice. Furthermore, accumulating evidence confirms the safety of transplantation from HBsAg(+) donors into immunized recipients. All immunosuppressants can be used in HBV(+) transplantation and total immunosuppression must be kept at the lowest possible levels long term.

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HEPATITIS B PREVALENCE AND PREVENTION IN HEMODIALYSIS PATIENTS

Acute and chronic hepatitis are caused by a double stranded DNA type of virus, hepatitis B virus (HBV). Although a safe and effective vaccine has been available for at least twenty years now, infection of HBV remains an enormous problem of public health worldwide[1].

Because of increased skin breaching, significant exposure to blood products, the sharing of dialysis machines, the nature of the dialysis process that allows great access to the bloodstream and underlying immunodeficiency problems, hemodialysis patients are at a greater risk for HBV infection. Fortunately, a number of prevention measures have in the last thirty years effectively resulted in the significant reduction of HBV infection incidence amongst hemodialysis patients. These include but are not limited to stricter adherence to general hygiene rules, mandatory separation of these patients during dialysis, aggressive vaccination protocols as well as erythropoietin use. However, hepatitis B prevalence remains a challenge in dialysis[2]. USRDS data indicates that 1% of dialysis patients tested positive for hepatitis B surface antigen (HBsAg) while in a registry study of Asian-Pacific countries the prevalence of HBsAg in hemodialysis populations ranged from 1.3% to 14.6%[3,4]. In general the incidence of HBsAg positivity among dialysis patients ranges from 0%-7% in low-prevalence countries to 10%-20% in endemic areas.

As in most public health challenges, immunization is the most critical move in preventing HBV infection. It is preferable that chronic kidney disease patients are vaccinated at an early stage and certainly prior to going on dialysis, because vaccine immunogenicity is higher in the general population in comparison to dialysis patients (90% *vs* 70%). Still, dialysis patients should also be vaccinated against HBV infection and have an annual test regarding their hepatitis B antibody (anti-HBs) titer. If it is lower than 10 IU/mL, an intensified protocol should be followed vis a vis a booster vaccine dose should be administered. Such protocols have shown very good responses in hemodialysis patients[5].

HBV EVALUATION IN THE PRETRANSPLANTATION SETTING

HBsAg (+) kidney transplant candidate

All dialysis patients should be routinely checked for HBsAg. In case of seropositivity, additional serologic markers including anti-HBc (IgM and IgG), HBeAg/anti-HBeAb, anti-HbsAb, quantitative HBV-DNA PCR and liver biochemistry including transaminases, ALP, GGT and bilirubin are considered necessary in order to differentiate between active and inactive liver infection.

Active carrier state is defined as HBsAg(+) in the presence of HBeAg(+) or HBeAb, with HBV viral load above 20000 IU/mL with or without elevated alanine aminotransferase (ALT) levels whereas inactive carriers are HBsAg(+) and negative for HBeAg(-) with persistently low viral load, normal liver enzymes and low anti-HBc IgM or anti-HBc IgG levels[6]. The occult HBV carrier state refers to a rare subgroup of patients who are HBsAg(-), most often with detectable anti-HBc but low viral load without liver enzyme elevation[7].

According to these definitions, the most cost-effective strategy is to screen and monitor all dialysis patients with basic serology which includes HBsAg, anti-HBc and anti-HBs. HBV PCR should be performed in the few cases of isolated anti-HBc positivity in order to detect occult carriers, especially among those on the waiting list[8].

In active HBV carriers on hemodialysis, therapy with one of the available antiviral agents is indicated until HBeAg becomes negative and viral replication is suppressed. Inactive carriers should be monitored with HBV-PCR and liver enzymes.

By interpreting HBV serology and virology in hemodialysis patients, it is essential to take into consideration the altered natural history of hepatitis B in this patient setting. HBV infection is usually asymptomatic even in the acute phase, transaminase levels are lower compared to the general population and seroconversion

from HBeAg to anti-HBeAb or from anti-HBc IgM to IgG is delayed or does not occur, even after resolution of the active infection[9]. About 80% of HBV infected dialysis patients progress silently to a chronic carrier state[10].

While on the waiting list, dialysis patients should be monitored every 6-12 mo with HBV-DNA and transaminase levels. Wait-listed transplant candidates must be either inactive carriers or sustained viral responders (SVR) with persistently low, or undetectable HBV-DNA.

Kidney Disease Improving Global Outcomes (KDIGO) guidelines recommend performing a liver biopsy in hemodialysis patients that are candidates for a kidney allograft and are positive for HBsAg, so that hepatitis' severity is assessed. After baseline histological evaluation, candidates should repeat liver biopsy every 3-5 years, if there is ongoing viral replication[11].

Currently, non-invasive tools for the assessment of hepatitis stage are available. The biochemical indices as the APRI score, though useful in the general population, have a reported diagnostic accuracy of about 50% in dialysis patients[12]. The same applies for transient elastography, a routine applied noninvasive tool aiming to assess hepatic fibrosis by liver stiffness measurement (LSM). Unfortunately, both in HBV infected hemodialysis patients and kidney recipients it has not yet been validated. Liver stiffness measurement is influenced by the fluid volume of the patient, which complicates the interpretation of the results due to the discrepancy between pre- and postdialysis values[13]. In a single center cohort of 284 dialysis patients with hepatitis C transient elastography demonstrated high diagnostic accuracy without diminishing the need for further validation, especially in pre-transplant control[14]. Still, in regards to kidney transplant candidates, performing a liver biopsy continues to be considered the "gold standard".

Liver cirrhosis has been regarded for a long time as a definite contraindication for lone kidney transplant with a combination of kidney-liver transplantation being considered the established therapy option. On the other hand nowadays, using new nucleotide analogues often leads to sustained viral response, fibrosis regression and the eventual evolution to a stage of septal inactive cirrhosis. In such cases, a follow up biopsy - 12 mo after the original SVR-must be performed and if the disease remains inactive, the patient may move to the waiting list and possibly undergo lone kidney transplantation[15].

A recent single center study provided data of an excellent five-year survival rate (94%) in 12 cirrhotic patients with hepatitis B after kidney transplantation alone[16].

Routine evaluation for hepatocellular carcinoma (HCC) with liver ultrasound and alfa-fetoprotein values every 6 mo is recommended in all dialysis patients with advanced fibrosis or pre-cirrhotic stage[11].

HBsAg(+) prospective kidney donor

HBV transmission from donor to recipient may occur in kidney transplantation as in all solid organ transplantations. HBV-infected donors' kidneys may be safely used under certain conditions and thus avoid unnecessary organ discarding especially in countries with organ shortage and low HBV prevalence. The routine serologic evaluation of a potential living or deceased donor includes HBsAg, antiHBc and HBsAb. The risk of HBV transmission *via* donation depends on the donor's serologic status.

HBsAg(+)/antiHBc(-)/antiHBs(-): Kidney transplantation is not suggested when the donor is HBsAg(+) and the recipient is HBV naïve since it poses an increased chance of an acquired infection which in most cases has an aggressive progression[17]. Jiang *et al*[18], however, have shown that allografts from HBsAg (+) donors may safely be used in transplantation when the recipient is HBsAg(-) independent of immunity type. This applies to all HbsAg(+) patients with a titer count of more than 10 IU/mL simultaneously receiving hepatitis B hyperimmune globulin (HBIG) independently of whether they are receiving an additional vaccine dose. Even though the probability of transmission is relatively small, it is imperative in such cases to obtain a written informed consent after fully briefing the patient prior to moving along with kidney transplantation. Singh *et al*[19], describe a successful transplantation in 104 anti-HBs(+) patients. Twenty seven recipients received only the original vaccination whereas, the rest concurrently received additional vaccine dose, HBIG and other antiviral medication.

At Laiko hospital in Athens, this kind of renal transplantations from seropositive donors to seronegative or HBs antibody positive patients independent of immunization type (past infection, vaccine) are only allowed when the recipient's titers are at least 10 IU/mL. All recipients receive one booster vaccination dose combined with HBIG just before transplantation. After the introduction of Entecavir such recipients receive post transplantation antiviral prophylaxis for 6 mo. Following this protocol, we have performed 13 transplantations from HBsAg(+) donors to immunized recipients with excellent long term results (unpublished data).

Another safe way to avoid unnecessary organ discarding especially in endemic areas, is to transplant kidneys from HBsAg(+) donors into HBsAg(+) recipients, a practice which offered successful results. In Greece, the allocation policy allows such transplantations, which are also performed in our center with good results.

HBsAg(-)/antiHBc(+)/antiHBs(+): Kidney transplant donors with this serologic profile are considered safe, since there is no way to transmit HBV to the kidney recipient**.** A single case report describes HBV transmission from a multiorgan donor only to the reciepient of the liver graft[20].

HBsAg(-)/antiHBc(+)/antiHBs(-), *i.e.*, isolated

presence of anti-HBc: The risk of HBV transmission from donors with this serological profile, though very low, has not been completely clarified. A recent analysis that examined transplants from anti-HBc(+) donors to 1385 HBsAg(-) recipients found seroconversion to HbsAg-positivity only in four recipients (0.28%) and to anti-HBc-positivity in 32 patients (2.3%)[21]. These donors should preferably be checked for the presence of anti-HBcIgM in order to exclude recent infection. Unfortunately, in relation to deceased donors, such testing is due to time constraints practically impossible. Renal transplantation should however be at the very least considered, since transmission risk is significantly smaller than from HBsAg(+) donors[22,23]. If one selects the safer side, it is preferable to apply the protocol relevant to HBsAg(+) donors.

OUTCOMES OF HBV INFECTED PATIENTS AFTER KIDNEY TRANSPLANTATION

HBV infection is associated with worse survival rates for seropositive patients in comparison to seronegative ones. In a 2005 study with an overall population of 6050 seropositive renal transplant recipients, Fabrizi *et al*[24] calculated a relative death risk of 2.49. The respective graft loss risk was 1.44.

On histological level, the severity of chronic hepatitis B increases during the post-transplantation period and is characterized by higher rates of progression to cirrhosis and mortality due to liver failure. Moreover, HBV(+) renal transplant patients are at increased risk of hepatits B reactivation which may rarely manifest as fulminant hepatitis with massive necrosis or as severe cholestatic hepatitis[25].

The only study of renal transplant patients' liver biopsies did not detect histological worsening in only 15% of seropositive recipients. Following the kidney transplantation, 28% of the patients progressed to liver cirrhosis whereas none had developed it beforehand. Twenty-three percent of the cirrhosis patients also developed hepatocellular cancer[26].

Survival rates for HBV infected kidney transplant recipients have since 1986 significantly increased due to the extensive use of antiviral agents. In a small Italian study, the authors reported that 67% out of the 42 HbsAg(+) patients that received a renal transplant from 1976 to 1982, achieved a survival rate of 12 years[27]. Similarly, Yap *et al*[28], reported that 81% amongst 63 seropositive kidney allograft recipients that received nucleoside/nucleotide analogues therapy, achieved a survival rate of 10 years. Liver failure, however, is still the leading cause of death for this cohort.

ANTIVIRAL TREATMENT IN KIDNEY TRANSPLANTATION

Goal of antiviral treatment

The therapeutic aim is to effectively suppress viral replication, prevent hepatic fibrosis, and at the same time minimize drug resistance. In order to systematically measure the patients' response to therapy, we must measure HBV DNA levels because ALT has a low reliability as a marker of liver disease activity.

Antiviral treatment strategies in kidney transplant recipients: Preemptive administration or prophylaxis?

The introduction of antivirals after transplantation aims to prevent immunosuppression-induced increase of viral replication which may lead to hepatitis B reactivation. The latter is defined by high viral load and or biochemical hepatitis. Virus reactivation is diagnosed by redetection of previously negative HBV-DNA using a highly sensitive assay with a cut off level less than 20 IU/mL, while "hepatitis" diagnosis relies on > 3 fold increase of ALT levels or an absolute increase in ALT above 100 IU/mL. Reverse seroconversion means redetection of HBsAg or anti-HBcAg when previously negative[29].

Antiviral prophylaxis means that treatment is initiated in inactive carriers in order to prevent HBV reactivation. The term "universal prophylaxis" is used when treatment is applied to the entire population at risk as for example to all kidney recipients under treatment with immunosuppression. Preemptive treatment defines antiviral administration after the reappearance of viral load or after the occurrence of seroconversion. According to recent guidelines, universal prophylaxis is recommended for all patients of moderate to high risk for viral reactivation during immunosuppression[30].

Treatment initiation: When should antiviral prophylaxis start?

Antiviral prophylaxis must begin before or at worst immediately after transplantation. A study of 15 patients with normal transaminase levels before transplantation, showed that the 7 that started LAM therapy along with the procedure had undetectable HBV DNA levels for the duration of the observation period. Half of the patients that didn't receive early treatment presented transaminase elevation during the first post-transplantation year[31].

Currently available antiviral agents and their use in kidney transplantation

A number of antivirals agents are available to treat hepatitis B. They include: Pegylated interferon alfa 2a, interferon alfa-2b as well as the nucleoside analogues LAM, telbivudine, tenofovir, entecavir, and adefovir.

Interferon and PEG-INF

The use of interferons following kidney transplant procedure is no longer advised since these agents have led to immunomodulatory effects and ultimately either to graft rejection or to hepatitis reappearance at a rate of almost 80% after suspending treatment[32].

LAM

LAM is a nucleoside reverse transcriptase inhibitor

and has been considered the best therapeutic option and it was the first such agent to be approved for clinical use in HBV infected kidney allograft recipients. The prophylactic use of LAM post-transplantation has offered long-term efficacy. A meta-analysis of 14 clinical trials with a total of 184 recipients that received LAM, indicated in 91% of them untraceable viral cargo and normal liver enzyme in 81%, for a significantly long time[33].

Prolonged treatment with LAM, however, eventually leads to the treatment resistance. In most cases resistance occurs due to a mutation in the tyrosine-methionine-aspartate-aspartate (YMDD) locus of HBV DNA polymerase[34]. The clinical presentation of resistance varies. Some patients show only reappearance of serum HBV DNA while others present with HBV reactivation.

The rate of LAM resistance varies from 20% up to 60% in different studies[35,36]. Following 29 kidney allograft recipients for a mean period of 69 mo, Fabrizi *et al*[34], reported that 48% of them (14/29) developed LAM resistance, whereas all 14 of them had YMDD mutation. Out of these patients that presented resistance, 79% had a disease flare.

Prolonged period of therapy is positively linked with resistance to LAM with the cumulative probability reaching 60% after 69 mo of therapy[33,35]. Patients with LAM resistance should be treated preferably with adefovir or tenofovir, if renal function permits or alternatively with entecavir.

Even though LAM is not nephrotoxic, it is removed by the kidney, and therefore the dose ought to be adjusted to the patient's renal function. The recommended dose for patients with estimated GFR > 50 mL/min per 1.73 m^2 is 100 milligram per day and 100 milligram every second day for those that present kidney injury/failure.

Most importantly, after systematic use of LAM prophylaxis, survival rates in HBV infected kidney transplant recipients have increased progressively with 81% of them reaching a survival rate of ten years, which is very similar to that of seronegative patients[37].

Entecavir

Entecavir is an analog for guanosine and is considered to be much more effective compared to LAM. It has a high antiviral potency, a high genetic barrier for resistance, a good safety profile and is effective in treatment of naïve as well as of LAM treated patients without resistance.

There is significant evidence of its ability to successfully suppress the virus for a prolonged time. Hu *et al*[38] recently, in 2012, studied 18 (67% of total cases) naïve renal transplant recipients and 9 (33%) recipients that had been previously treated with LAM but without resistant mutations, entecavir was successful in clearing HBV DNA in 70%, 74%, 96% and 100% of patients after 12, 24, 52 and 104 wk respectively. Moreover, compared to LAM, entecavir reached at the same time of treatment higher rates of undetectable HBV DNA (32% *vs* 70%, 37% *vs* 74%, 63% *vs* 96% and 63% *vs* 100% of patients at 12, 24, 52 and 104 wk respectively; $P < 0.005$).

LAM resistant HBV patients, however, do not show similar results. Complete response to entecavir may take more than 6 wk and may not be achieved at all. Entecavir use in LAM or adevofir resistant kidney allograft recipients, was studied by Kamar *et al*[39], examining 10 patients with solid organ transplantation, that included eight renal transplant recipients. After 16.5 mo of therapy, there was a variable decrease in HBV DNA viral load with 50% succeeding in clearing HBV reporting no important unwanted reactions.

Between kidney allograft recipients there are no reported Entecavir-resistant cases. Similarly in the general population Entecavir-resistant patients after 5 years of therapy is minimal (1.2%) in naïve patients. On the contrary, in cases with LAM resistance the chance of entecavir-resistant cases increases annually from year 1 to year 5 (6%, 15%, 36%, 46% and 51% respectively)[40]. According to recent guidelines, entecavir has displaced LAM as first line prophylaxis in HBV(+) kidney transplant recipients[30].

Adefovir dipivoxil

Adefovir, an acyclic nucleoside, is an adenosine analog and is used both in a single agent therapy or combined to entecavir in HBV infected patients and LAM-resistant cases[41]. It is mainly used in LAM resistant HBV patients either as monotherapy or as "add on" therapy to LAM[42].

It is, however, potentially nephrotoxic. Research on HIV patients indicates that high daily doses of adefovir (60-120 mg) could result in renal tubular injury[43]. In a study of 11 renal transplant recipients with LAM resistance that were treated solely with adefovir by Fontaine *et al*[44], dosage was adjusted according to renal function. After 12 mo, serum HBV DNA declined satisfactorily and no hepatitis B reactivation was observed. There was no evidence of nephrotoxicity with no significant adverse events and the drug seemed to be well tolerated. In an analogous study of 11 kidney LAM resistant transplant recipients, adefovir was administered at very low doses according to GFR (2.5-10 mg/d) and showed good efficacy in terms of reducing HBV DNA viral load and normalizing liver enzymes after two years of therapy. Renal parameters were closely monitored and showed a slight increase in creatinine (from 125 ± 35 to 141 ± 32 mmol/L, $P = 0.02$), an increase in proteinuria as well as slight impairment of proximal tubular reabsorption[45]. In a series of 14 LAM resistant transplant recipients, adefovir was administered to 5 patients as monotherapy and to 9 as "add on" to LAM. Five out of 14 patients (29%) had a significant decline in GFR (loss of 10 mL/min or more after 32 mo therapy) which led to treatment

discontinuation in 4 of them[46].

Tenofovir disoproxil fumarate

Tenofovir DF as a nucleotide analog reverse-transcriptase inhibitor (NtRTI) selectively inhibits viral reverse transcriptase, a crucial enzyme in retroviruses such as human immunodeficiency virus and hepatitis B virus, while showing limited inhibition of human enzymes, such as DNA polymerases. Tenofovir has a strong antiviral effect, prevents viral replication and is used in the therapy of naïve patients and those that present LAM resistance[47,48]. In a study with HBV infected patients of the general population, this nucleotide analog had a strong effect when used to treat patients with LAM resistance, while no tenofovir-resistant cases appeared during a forty eight month post-therapy follow up[49]. Still, the shortage of data referring to kidney transplant recipients leads to concerns for potential kidney injury. In a pilot study by Daudé *et al*[50], 7 solid organ recipients - 3 with kidney transplantation - received tenofovir as rescue therapy after resistance to other nucleoside analogues. After 12 mo, there was effective suppression of viral replication with HBV clearance in 3 out of 7 patients.

Telbivudine

Telbivudine is ineffective in LAM resistant HBV renal transplant recipients, due to cross-resistance to entecavir and LAM. There is not enough information regarding telbivudine in the area of kidney transplant recipients.

Treatment duration: Is discontinuation of antivirals feasible?

In the general population the duration of antiviral treatment with nucleoside analogues still remains unclear, since nucleoside analogues cannot completely eradicate HBV[51]. The duration of antiviral therapy for renal transplant patients is even more difficult to assess, while data referring to long term outcomes after nucleoside analog withdrawal in immunosuppressed patients including kidney transplant recipients are lacking. The prophylactic or preemptive use of LAM initially and the newer nucleoside analogues later on, have indeed changed the picture in kidney transplantation, with HBV(+) recipients reaching significantly better long term outcome worldwide. Nevertheless, there are still unresolved issues concerning the use of antivirals in transplantation. Solid organ recipients including kidney, are receiving lifelong immunosuppression. Consequently, one logical assumption might be that they also need lifelong prophylaxis to prevent viral breakthrough or reactivation. On the other hand, "lifelong" antiviral prophylaxis, besides cost, is associated with various problems. The main issue is the development of resistance, primarily to LAM but *via* cross-resistance also to the newer agents as entecavir and to a lesser degree adefovir and tenofovir. Rates of LAM resistance increase with increased therapy duration and approach 60% after 5 years of treatment[35]. Therefore, the prophylactic use of entecavir as first line prophylaxis has already been implemented following recent guidelines. Unfortunately, entecavir is much more expensive and has not been widely approved, especially in developing countries. Furthermore, adefovir and tenofovir are both nephrotoxic[43,46] and with the lower doses used as prophylaxis in kidney transplantation, their long term therapeutic efficacy has not yet been proven.

After the development of resistance, combination therapies are indicated either by switching from LAM to entecavir and tenofovir or as "add on" to LAM. Combination therapies have the same adverse effects and are even more expensive than single agents. Last but not least, nucleoside analogues interfere with immunosuppressive agents as calcineurin inhibitors, making patient monitoring after transplantation even more complicated.

For all these reasons, the feasibility of treatment discontinuation remains one of the most important, yet unresolved issues in HBV(+) kidney transplantation. The first attempt for LAM discontinuation was published by Chan *et al*[52] in 2002. LAM was discontinued in 12 low-risk kidney recipients after stabilization. Withdrawal was successful in 5 patients (41.7%)[53]. Another study retrospectively followed a small cohort of of 14 HBsAg(+) renal transplant recipients. In six of them, antiviral therapy seized after a median of 14 mo. Each of them was on stable maintenance of immunosuppression without any sign of viral activity. After discontinuing antiviral treatment and following the patients for a median of 60 mo, 4 of them (67%) presented no sign of viral breakthrough or HBV re-activation. In the last 2 cases who presented HBV reactivation, antiviral treatment was subsequently reinstated leading to HBV clearance[54]. Despite the small number of cases in both studies, they provide promising results for further investigation.

To sum it up, post renal transplantation antiviral therapy could be withdrawn in cautiously chosen subsets of patients that fulfil certain criteria: Stable renal function, low immunological rejection risk, a minimum of 6-9 mo low-dose maintenance immunosuppression, no evidence for HBV activity and a minimum of 12 mo therapy with antiviral agent without developing resistance. Frequent measurement of HBV-DNA levels and 3-6 mo testing of liver enzymes are essential while antiviral treatment ought to be reinstated if immunosuppression grows, *i.e.,* in the case of antirejection therapy.

IMMUNOSUPPRESSION IN THE COURSE OF HBV AFTER KIDNEY TRANSPLANTATION

There is an association between immunosuppression and HBV reappearance, both in seropositive patients,

and in those positive for anti-HBc/anti-HBs, most frequently in a titer count that is quite low, *i.e.,* previously infected patient[55]. Most data derive from HBV infected patients that receive treatment for either solid organ or hematological malignancies[55,56].

Recipient's immunocompetence as well as the overall level of immunosuppression are highly associated with HBV reactivation after transplantation. Immunosuppression affects the relationship between the host and HBV possibly resulting in serious liver damage. Immunosuppressionmay lead to liver injury through two distinct routes. One pathogenetic pathway is virus hepatotoxicity due to unrestrained intracellular viral replication resuting from diminished host immunosurveillance. Such a risk is intimately associated with the initial phase, during which the overall burden of immunosuppression is elevated while the most severe clinical manifestations are fibrosing cholestatic hepatitis (FCH) and fulminant liver failure. FCH has been initially described as complication of HBV infection in liver grafts. A small number of FCH cases with dismal course have been reported in renal transplant recipients as well without differing histologically from FCH manifesting in liver allografts[57-59].

The second pathway involves secondary immune mediated liver injury occuring when immunosuppressants are withdrawn and immune efficiency is reconstituted. The host immune response destroys HBV infected hepatocytes leading to extensive parenchymal necrosis. This pathway has mainly been observed in solid organ and hematologic malignancies cases even after 6 to 12 mo having completed chemotherapy. In renal transplantation, this process may lead to accelerated liver damage after rapid reduction of immunosuppression, usually after tapering of the high corticosteroid-doses given for anti-rejection therapy[56].

Immunosuppressants

The traditional immunosuppressive agents that may be prescribed in different permutations for renal transplant recipients are: Corticosteroids, azathioprine, mycophenolate acid derivates (MMF/MPA), calcineurin inhibitors (cyclosporin, tacrolimus), and the well known inhibitors of mammalian target of rapamycin (mTORi's: Everolimus, sirolimus). There are two more groups of immunosuppressants; Monoclonal antibodies (anti-CD20 Rituximab, anti-IL2 Basiliximab) and polyclonal antibodies as ATG (antithymocyte globulin) that may be prescribed for either induction or rejection therapy.

According to the KDIGO guidelines all immunosuppressive agents currently used for induction and maintenance immunosuppression in kidney transplantation can be used in HBV(+) recipients[11]. They all increase replication of the virus and may lead to increased chances of HBV reactivation. The American Gastroenterological Association (AGA) has assessed the HBV reactivation risk depending on the use of particular immunosuppressants[30].

Rituximab

Rituximab is considered to have the most elevated risk for HBV reactivation (> 10%) from all immunosuppressive agents that are used in renal transplantation, according to AGA guidelines[30]. Furthermore, this risk may continue up to 12 mo, due to the prolonged duration of the antibody's immune reconstitution. Rituximab is linked to HBV reactivation in HBsAg(+) but also in recipients with anti-HBc possitive and those with anti-HBs positive (reverse seroconversion). In a retrospective analysis, 24.3% between 230 B-cell lymphoma patients, HBsAg-negative patients that received rituximab, were anti-HBc(+). Reactivation oft he virus was observed in 8.9% of patients. Entecavir use led to HBV DNA clearance and allowed for the re-administration of rituximab[60].

Polyclonal antibodies (Antithymocyte globulin)

After administering antithymocyte globulin to patients with severe aplastic anemia, increased rates of viral replication have been reported in HSV, EBV and CMV infections. More specifically, in those cases ATG was given concomitantly with cyclosporin[61]. There is a shortage of reliable data in relation to HBV reactivation after ATG therapy.

Corticosteroids

Corticosteroids (CS) are the most commonly used immunosuppressant in the world. They are, however, undeniably related to elevated viral replication. HBV reactivation risk is dependent upon the dose as well as on the duration of CS use. High CS doses increase viral load even though ALT may decrease. During steroid tapering, one finds the opposite effect with influenced liver enzymes four to six weeks following withdrawal[56,62]. As stated by American Gastroenterological Association, doses of prednisone of 20 mg per day or/and long periods of administration (> 3 mo) could increase the risk for reactivation of hepatitis B along with quick reduction, because of immune modification[30].

In relation to renal trnsplantation, increased doses of corticosteroids are used in the first wk post transplantation; the doses are reduced from that point on and for the next 3-6 mo, eventually leading to a prednisone standard of 5 mg every day or second day. Corticosteroids can totally be sidestepped (steroid-avoidance regimens) or at least could be retracted at four to six weeks or more (steroid-sparing regimens), in stable and low immunological risk cases with outstanding outcomes. In HBV renal transplant patients, CS should be administered at the lowest possible doses and ideally should be withdrawn or even totally abandoned in low immunological risk cases.

Calcineurin inhibitors

Tacrolimus and ciclosporine continue to be the mainstays of immunosuppressive regimens in renal transplant

recipients. There is enough evidence that cyclosporin leads to *in vitro* reduction of viral replication[63,64]. Today, most immunosuppressive treatments are based on tacrolimus. Despite the lack of definitive guidelines, many people support the use of cyclosporin instead of tacrolimus in HBV infected renal transplant patients. Some others prefer to withdraw steroids from a tacrolimus-based regimen. Due to the lack of definite guidelines, choosing between the two calcineurin inhibitors depends on each hospital's practice.

Antimetabolites

Even though, azathioprine is considered to be hepatotoxic, it has not been linked when administered as monotherapy to elevated HBV reactivation risk. Still, the use of more potent and more selective antimetabolites as MPA's, has limited azathioprine use in renal transplantation to patients with special indications[65].

Mycophenolate acid derivates

Azathioprine has been replaced by mycophenolate mofetil and its most recent derivate mycophenolate sodium in the majority of immunosuppressive treatments. There is no definite data about MPA's and HBV reactivation. They are, however, generally considered to be safe for HBV renal transplant patients.

Mamalian target of Rapamycin inhibitors

The reactivation of HBV under treatment with mamalian target of Rapamycin (mTOR) inhibitors has not been examined in kidney transplantation but normally their safety is not disputed. Everolimus when used as a chemotherapeutic agent has been reported to lead to HBV reactivation. The doses, in those cases, however were more elevated compared to accustomed ones prescribed as standard immunosuppressive regimen in renal transplant recipients[66].

Summarizing, all immunosuppressive agents used in renal transplantation could be administered in HBV positive patients. There is no evidence for any specific effect of a particular immunosuppressive agent on viral replication since it is associated with the total amount of immunosuppression. Efforts to minimize immunosuppression-induced viral reactivation should focus on minimization of the total immunosupression burden long term, which is more important than the choice of one single agent over another. Minimization protocols, especially corticosteroid-avoiding or sparing protocols, are preferable and should be applied to low-immunological risk HBV(+) recipients. Close HBV monitoring is mandatory whenever the total immunosuppression status is altered.

CONCLUSION

In the era of potent antivirals and with evolving knowledge and mounting evidence in the areas of both kidney transplantation and hepatitis B, HBsAg(+) renal transplant candidates and recipients can be monitored and successfully treated, reaching survival rates that are comparable to their HBsAg(-) counterparts. Furthermore, under certain conditions kidneys from HBsAg(+) donors can be safely transplanted into immunized recipients thus avoiding unnecessary organ discard.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Real-world efficacy of daclatasvir and asunaprevir with respect to resistance-associated substitutions

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Informed consent statement: All study participants provided informed written consent before study enrollment.

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Abstract

AIM

To investigate daclatasvir (DCV) and asunaprevir (ASV) efficacy in hepatitis C (HCV) patients, with respect to resistance-associated substitutions (RASs).

METHODS

A total of 392 HCV-infected patients from multiple centers were included in this study. We evaluated their clinical courses and sustained virologic responses (SVR) according to pretreatment factors (gender, age, history of interferon-based regimens, platelet counts, level of viremia, pretreatment NA5A:L31, and Y93 substitutions). We also analyzed the pretreatment and post-treatment major RASs of NS3:D168, NS5A:L31 and Y93 substitutions using a direct-sequencing method in 17 patients who were unable to achieve SVR at 12 wk after treatment completion (SVR12).

RESULTS

The overall SVR12 rate was 88.3%. Thirty-one patients discontinued treatment before 24 wk because of adverse events, 23 of whom achieved SVR12. There were no significant differences in SVR12 rates with respect to gender, age, history of interferon-based regimens, and platelet counts. The SVR12 rate in patients with viral loads of $\geqslant$ 6.0 log IU/mL was significantly lower than those with viral loads of < 6.0 log IU/mL (P < 0.001). The SVR12 rate in patients with Y93 substitution-positive was significantly lower than those with Y93 substitution-negative (P < 0.001). The L31 substitution-positive group showed a lower SVR12 rate than the L31 substitution-negative group, but the difference was not statistically significant. Seventeen patients who did not achieve SVR12 and had available pretreatment and post-treatment sera had additional RASs in NS3:D168, NS5:L31, and Y93 substitution at treatment failure.

CONCLUSION

Combination of DCV and ASV is associated with a high SVR rate. Baseline RASs should be thoroughly assessed to avoid additional RASs after treatment failure.

Key words: Hepatitis C; Asunaprevir; Combination therapy; Resistance-associated substitutions; Daclatasvir

Core tip: Hepatitis C - infected patients treated with daclatasvir and asunaprevir were evaluated for sustained virological response (SVR) according to pretreatment factors. The overall rate of SVR12 was 88.3%. The SVR12 rate in the $\geqslant$ 6.0 log IU/mL group was significantly lower than in the < 6.0 log IU/mL group. The SVR12 rate in Y93 substitution-positive patients was significantly lower than that in non-Y93 substitution patients. The L31 substitution-positive group had a lower SVR 12 rate than the L31 substitution-negative group. Seventeen patients who did not achieve SVR 12 had additional RASs in NS3:D168, NS5:L31, and Y93 post-treatment. Baseline RASs should be thoroughly assessed to avoid additional RASs after treatment failure.

Fujii H, Umemura A, Nishikawa T, Yamaguchi K, Moriguchi M, Nakamura H, Yasui K, Minami M, Tanaka S, Ishikawa H, Kimura H, Takami S, Nagao Y, Shima T, Itoh Y. Real-world efficacy of daclatasvir and asunaprevir with respect to resistance-associated substitutions. *World J Hepatol* 2017; 9(25): 1064-1072 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i25/1064.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i25.1064

INTRODUCTION

Hepatitis C virus (HCV) is one of the most important chronic infections worldwide. An estimated 170-200 million people are infected with HCV worldwide[1], with approximately 1.0-1.5 million infected people in Japan[2]. HCV treatments have dramatically changed recently. Pegylated interferon (PEG-IFN) and ribavirin (RBV) dual therapy has long been the standard treatment for genotype 1 chronic hepatitis C (CHC). Recently, however, newer anti-HCV drugs, termed direct-acting antiviral agents (DAAs), have become available[3].

Telaprevir (TVR) was the first nonstructural protein 3 (NS3) protease inhibitor (PI)[4] approved in Japan, followed by the second generation NS3 PIs, simeprevir (SMV) and vaniprevir[5-8]. These drugs were scheduled to be administered in combination with PEG-IFN and RBV, and could enhance treatment efficacy. However, both PEG-IFN and RBV can cause various side effects, and they are contraindicated in elderly patients and/or patients with certain comorbid conditions.

The combination of oral daclatasvir (DCV), a NS5A inhibitor, and asunaprevir (ASV), a second generation NS3 PI, has been the first drug combination approved in Japan for the treatment of HCV genotype 1-infected patients. This drug combination showed high rates of sustained virologic response (SVR) and better tolerability[9,10]. Thus, many patients for whom conventional IFN-based treatment was intolerable or incurable have been medicated. We performed a retrospective cohort study to evaluate the safety, tolerability, and effectiveness of DCV and ASV combination therapy in real-world clinical practice. Moreover, we evaluated the presence of pretreatment and post-treatment major resistance-associated substitutions (RASs) (NS5A: L31 and Y93 substitution, and NS3:D168 substitution) using a direct-sequencing method in 17 patients who did not achieve SVR12.

MATERIALS AND METHODS

Patients

Patients were enrolled at Kyoto Prefectural University of Medicine and seven affiliated hospitals in the Kinki area of Japan (Kyoto, Osaka, Nara, Shiga Prefecture)

from 2014 to 2015. Study protocols were approved by the ethics committee of each institution and conformed to the provisions of the Declaration of Helsinki. Eligible patients were those aged at least 20 years with HCV genotype 1 infection diagnosed by board-certified hepatologists. Patients with decompensated liver cirrhosis, chronic hepatitis B, HIV, autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, or Wilson's disease, were excluded. Patients with uncontrollable hypertension, those with a history of alcohol abuse or clinically significant medical conditions (severe renal disease, severe heart disease, active drug users, pregnancy, and those receiving drugs which interact with DCV or ASV) were also excluded. Patients were followed up monthly or every 2 wk to assess liver function and virological markers during treatment and until 12 wk after the completion of DCV and ASV therapy. All patients gave informed consent to participate in this study. Five patients were lost to follow-up or underwent extreme protocol deviation (*e.g.,* death by accident). Those lost to follow-up or had extreme protocol deviation were excluded from the analysis.

Study design

Patients received oral daclatasvir (Daklinza; Bristol-Myers Squibb Company) 60 mg once daily with oral asunaprevir (Sunvepra; Bristol-Myers Squibb Company) 200 mg twice daily, in accordance with prescribing information, for 24 wk. Patients were followed up until at least 12 wk after final treatment administration to assess SVR12.

HCV RNA responses during therapy were classified into the following groups: The non-response group (NR), patients whose HCV RNA remained detectable during treatment, resulting in treatment discontinuation; the breakthrough group (BT), patients whose HCV RNA was once undetectable but reappeared during treatment; and the relapse group (REL), patients whose HCV RNA was undetectable at the end of the 24-wk treatment but became detectable again during follow-up. SVR12 was defined as undetectable serum HCV RNA levels at 12 wk after the end of treatment (EOT). Therapeutic effects were evaluated using per-protocol analysis and included patients who received at least 2 wk of this therapy.

Continuation of treatment was decided by board-certified hepatologists. In general, patients whose serum HCV RNA was positive at 8 or 12 wk were judged as NR, and the treatment was terminated at that time. Patients whose serum HCV RNA reappeared were diagnosed as belonging to the BT group, and the treatment was stopped at the time. Dose reduction or discontinuation of DCV or ASV was determined by board-certified hepatologists. Discontinuation of the treatment was generally considered when grade 3-4 adverse events according to Common Terminology Criteria for Adverse Events v4.0 occurred.

Laboratory assessments

Blood samples were obtained for routine biochemical and hematological assessments at treatment initiation, on treatment weeks 4, 8, 12, 16, 20, 24, at EOT, and at 12 wk after EOT. Antiviral effects were mostly assessed by measuring serum HCV RNA levels using the COBAS TaqMan HCV test (Roche Molecular Diagnostics, Tokyo, Japan) with a lower limit of quantitation (LLOQ) of 15 IU/mL (with a quantitation range of 1.2-7.8 $\log_{10}$ IU/mL). Missing data points were deemed a success if the immediately preceding and subsequent time points were successful; otherwise, data points were termed as failures. Patients who had missing data because of premature discontinuations were considered failures from the point of discontinuation.

Pretreatment major RASs of NS5A, L31 or Y93 substitutions were assessed using commercially available assays of direct-sequencing method, the cycleave probe method, or invader assays. Furthermore, the pretreatment and post-treatment major RASs of NS3:D168, NS5A:L31, and Y93 substitution were investigated in 21 patients with virological failure by using a direct-sequencing method. In brief, HCV RNA was extracted from blood serum using a commercially available kit (QIAamp viral RNA kit; QIAGEN, Valencia, CA, United States). This sample was used for reverse transcription with random hexamer primers (SuperScript Ⅲ First-Strand Synthesis System for RT-PCR cDNA synthesis kit; Invitrogen, Carlsbad, CA, United States). The NS3 and NS5A regions were amplified by nested PCR using Takara Ex Taq HS (Takara Ex Taq, Otsu, Japan). The PCR primer sequences were NS3 forward primer: gccgcgatgccatcatcctcc, gtccaaatggccttcatgaagctgg, caatgtagaccaggacctcgtcgg and reverse primer: tggtgaaggtgggatccaagctgaa; or NS5A forward primer: atcctctccagccttaccatcact and reverse primer: ccatgaccaactcgggctggacctt. The PCR products were separated by electrophoresis on 1% agarose gels. These were purified using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and sequenced with second-round PCR primers using a dye terminator sequencing kit (BigDye Terminator v 1.1 cycle sequencing kit; Applied Biosystems, Foster City, CA, USA) and ABI PRISM 310 genetic analyzer (Applied Biosystems).

Statistical analysis

Baseline continuous data were expressed as median with interquartile ranges in parentheses, and categorical variables were expressed as numbers. Some baseline data were categorized, and univariate analyses were performed using the χ^2 or Mann-Whitney *U*-tests as appropriate. All *P*-values of < 0.05 of two-tailed tests were considered significant. All statistical analyses were performed using the SPSS 22.0 statistical package

Table 1 Baseline patient characteristics

No. of patients	*n* = 392	
Gender (male/female)	159/233	
Age, yr	71.0	(64.0-77.0)
< 65 yr *vs* ⩾ 65 yr	99/293	
Laboratory data		
Level of viremia (log IU/mL)	6.2	(5.8-6.5)
< 6.0 *vs* ⩾ 6.0	137 *vs* 255	(35.1% *vs* 64.9%)
Platelet count (× 10^4/mm^3)	12.6	(9.2-16.7)
10 < *vs* ⩾ 10	114/278	(29.0% *vs* 70.4%)
ALT (IU/L)	41	(29-65)
γ-GTP(IU/L)	34	(22-57)
Other data		
Prior treatment		
IFN *vs* PEG plus RBV *vs* TVR *vs* SMV	70/147/13/9	
NS5A polymorphisms, *n* (%)		
L31 substitution, *n* = 288	10 (3.5)	
Y93 substitution, *n* = 321	27 (8.4)	
L31 and/or Y93, *n* = 321	35 (10.9)	

Data are presented as numbers. Percentages or medians with interquartile ranges are presented in parentheses. ALT: Alanine aminotransferase; γ-GTP: Gamma-glutamyltransferase; IFN: Interferon; PEG plus RBV: Pegylated interferon plus ribavirin; TVR: Pegylated interferon plus ribavirin plus telaprevir triple therapy; SMV: Pegylated interferon plus ribavirin plus simeprevir triple therapy; L31: NS5A: L31 substitution patients. A total of 288 patients were assessed at pretreatment; Y93: NS5A: Y93 substitutions. A total of 321 patients were assessed at pretreatment.

(SPSS Incorporated, Chicago, IL, United States).

RESULTS

Baseline characteristics

The baseline patient characteristics are shown in Table 1. In total, 392 patients were included in this study. Female patients were predominant. Enrolled patients were generally older (median, 71.0 years) and had lower platelet counts. As for prior treatments, 70 patients had received IFN, 147 patients had received PEG-IFN or IFN plus RBV, 13 patients had a history of PEG-IFN plus RBV plus TVR, and 9 patients had a history of PEG-IFN plus RBV plus SMV. Concerning RASs, L31, and Y93 substitutions at pretreatment were seen in 3.5% (10/288) and 8.4% (27/321) of patients, respectively (Table 1). Two patients had both L31M and Y93H RASs.

Virological response to therapy and loss of HCV RNA during treatment

Undetectable HCV RNA levels were achieved in 79.7% (299 of 375), 94.1% (367 of 390), 94.1% (369 of 392), 92.8% (363 of 391), 92.2% (355 of 385), 91.3% (358 of 392), and 88.3% (346 of 392) of patients at treatment weeks 4, 8, 12, 16, 20, 24, and at 12 weeks' post-treatment, respectively (Figure 1). Because two of the SVR12 patients experienced late relapse of chronic hepatitis C and two additional patients were lost to follow-up, the final SVR24 resulted in 87.2%. Treatment was discontinued in 8 NR patients (2.0%) because the therapeutic effect was hardly expected, and in 18 BT patients (4.6%). Twelve patients (3.0%) received 24 wk of treatment but ended in REL. Thirty-one patients discontinued treatment before 24 wk because of adverse events. Reasons for discontinuation included liver dysfunction (15 patients), fever increase (6), detection of HCC (2), edema or ascites (2), and other reasons (6). Of these, 23 patients (15 liver dysfunction, 2 fever increase, 2 HCC, 1 edema and ascites, and 3 with other reasons) eventually achieved SVR12. Eight patients who received treatment less than 8 wk due to adverse events achieved SVR12. There were no treatment-related deaths.

SVR12 rates according to age, platelet counts, level of viremia, and substitutions in NS5A:L31 and Y93

We assessed the SVR12 rate according to gender, age (< 65 *vs* ⩾ 65), history of IFN-based treatment, platelet counts (< 10 × 10^4/mm^3 *vs* ⩾ 10 × 10^4/mm^3), level of viremia (< 6.0 log IU/mL *vs* ⩾ 6.0 log IU/mL), and pretreatment L31 and Y93 substitution (negative or positive). The SVR12 rate in the ⩾ 6.0 log IU/mL group was significantly lower than in the < 6.0 logIU/mL group (P < 0.001). As for Y93 substitutions, the SVR12 in the Y93 substitution-positive group was significantly lower than that in the Y93 substitution-negative group (P < 0.001). As for L31 substitutions, the L31 substitution-positive group showed a lower SVR 12 rate than their negative counterparts, but without statistical significance (P = 0.28). Other parameters were similar between the two groups (Figure 2).

Pretreatment and post-treatment RASs and fibrosis-4 index

We investigated the pretreatment and post-treatment major RASs (NS5A:L31 and Y93 substitutions and NS3:D168 substitution) using a direct-sequencing method in 21 patients who did not achieve SVR12. The results of direct-sequencing were of poor quality in four patients, leaving 17 patients who could be investigated completely. Five patients had NS3:D168 substitution, three had NS5A:L31 substitution, and six had NS5A:Y93 substitution before treatment. Five patients had neither NS3:D168 nor NS5A:L31 or Y93 substitutions before treatment. Analysis at the time of virological failure revealed that 14, 14 and 13 patients had NS3:D168, NS5A:L31, and NS5A:Y93 substitutions, respectively. All 17 patients whose pretreatment and post-treatment sera were available had one of the major RASs at the time of virological failure. Moreover, many patients had additional amino acid substitutions like NS5A Q54H (Table 2).

We compared the Fibrosis-4 (FIB-4) index[11] at baseline and at 12 wk after the end of treatment. Baseline FIB-4 index was 4.14 and it remarkably decreased to 3.78 at 12 wk after the end of treatment in the SVR12 group (P < 0.001). Meanwhile, bseline FIB-4 index was 3.84 and it slightly decreased to 3.57 in the non-SVR12 group (P = 0.03) FIB-4 index was

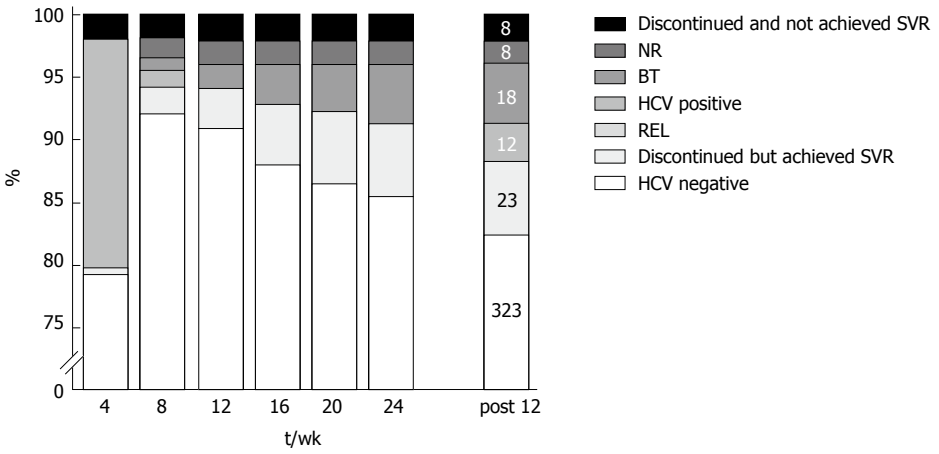

Figure 1 Virological response and treatment outcomes. Black closed squares indicate the proportion of patients who discontinued treatment because of adverse events and unachieved SVR12. Non-response (NR), where HCV RNA remained detectable during treatment, prompting treatment discontinuation. Breakthrough (BT), where HCV RNA was undetectable but reappeared during treatment. Relapse (REL), where HCV RNA was undetectable at the end of the treatment but became quantifiable again during follow-up. Gray closed squares indicate the proportion of patients with HCV RNA detected at the time of measurement. Light gray square indicate the proportion of patients who discontinued treatment because of adverse events but nevertheless achieved SVR12. White closed squares indicate the proportion of patients whose HCV RNA viral loads were undetected at the time of measurement. The Post 12 wk bar indicates the number of patients in each square.

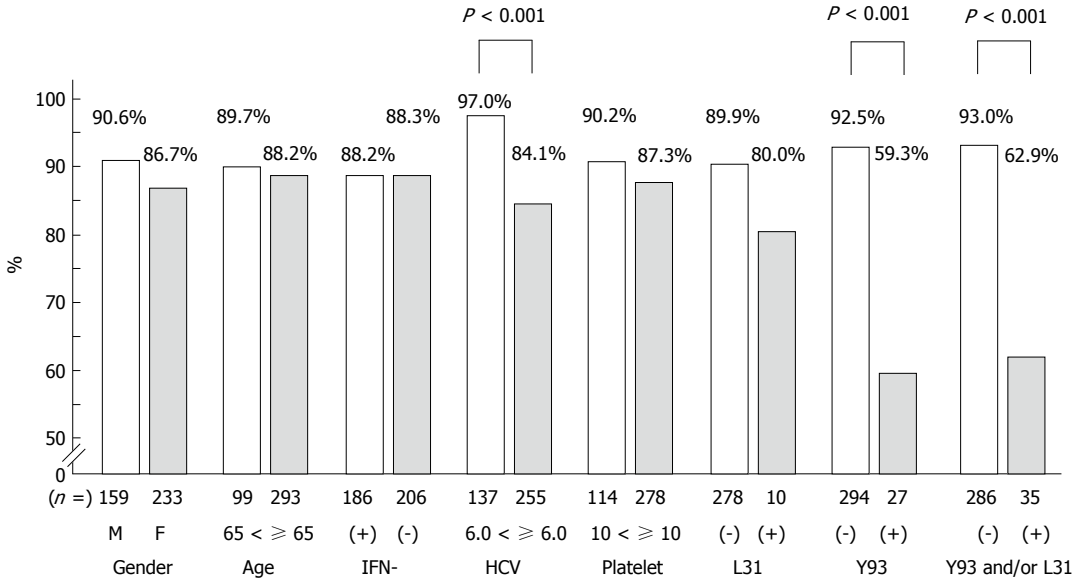

Figure 2 Bars in this graph indicate SVR12 rates according to gender (male, female), age (< 65 year *vs* ⩾ 65 year), history of interferon-based regimen treatment (+ *vs* -), level of viremia (< 6.0 logIU/mL *vs* ⩾ 6.0 logIU/mL), platelet counts (< 10 × 10^4/mm^3 *vs* ⩾ 10 × 10^4/mm^3), pretreatment existing L31 substitution [(-): substitution negative, (+): substitution positive], pretreatment existing Y93 substitution [(-): substitution negative, (+): substitution positive], and Y93 and/or L31 [(-): both L31 and Y93 substitution negative, (+): either L31 or Y93 substitution positive, or both L31 and Y93 substitution positive]. M: Male; F: Female; IFN: Interferon.

more markedly reduced in the SVR12 group.

DISCUSSION

In the present study, we presented the efficacy of DCV and ASV in real-world clinical practice. Most patients were rapidly cleared of HCV and achieved SVR12 with this combination of DAAs. Interestingly, some patients who discontinued treatment after a short duration because of side effects also achieved SVR. Resistance analyses revealed that patients who did not carry both Y93H and L31 mutations before treatment achieved 93.0% SVR12. All 17 patients who could not achieve SVR 12 and who were investigated for major RASs had either NS3-D168 substitution, or NS5A L31 and/or Y93 substitutions, including five patients who did not carry any RASs before treatment.

The high SVR rate of 88.3% in our present study is consistent with previous reports. Serum HCV RNA levels decreased rapidly, and was undetectable by 12 wk in the majority of patients[9,10]. In general, the therapeutic efficacy of a novel IFN-based HCV therapy

Table 2 Pretreatment and post-treatment major RAVs of 17 patients who did not achieve SVR12 (NS3:D168, NS5A:L31, and Y93 substitution)

No.	Pretreatment						Post-treatment				
	C.C.	D168	L31	Y93	Other NS3	Other NS5A	D168	L31	Y93	Other NS3	Other NS5A
1	BT	E	-	-	-	-	E	-	-	-	R30H
2	REL	-	L/I	-	-	A92T	-	M	-	-	A92K
3	NR	-	-	H	V170I	Q54Y	-	I	H	V170I	Q54Y
4	BT	Y	-	-	-	Q54H	Y	F	H	-	Q54H
5	NR	E	-	-	Q80R, V170I	Q54H	E	V	H	Q80R, V170I	Q54H
6	BT	E	-	-	-	Q54H	E	V/M	H	V170I	Q54H
7	BT	-	M/L	-	-	Q54V	V	M/V	H	-	Q54V
8	BT	-	-	H	-	Q54H	V	M	H	-	Q54H
9	REL	-	-	H	-	Q62E	V	I	H	-	Q62E
10	BT	-	-	H/Y	-	-	T	M	H	-	-
11	BT	-	-	H/Y	-	-	V	V/F	H	V170I	-
12	BT	Y	F	H	-	-	D	F	H	-	Q54H
13	BT	-	-	-	-	Q54H, A92T	V	-	-	-	Q54H, A92K
14	REL	-	-	-	-	Q54H	E	-	-	-	P32L, Q54H, A92K
15	BT	-	-	-	-	Q62N	V	V	H	-	Q62N
16	BT	-	-	-	-	-	-	V	H	-	-
17	REL	-	-	-	-	-	E	M	H	-	-

C.C.: Virological clinical course; NR: Non-response, HCV RNA was still detectable during treatment so treatment was discontinued; BT: Breakthrough, HCV RNA became undetectable but reappeared during treatment; REL: Relapse, HCV was undetectable at the end of the 24-wk treatment but became quantifiable again during follow-up; D168: NS3:D168 substitution; L31: NS5A:L31 substitution; Y93: NS5A:Y93 substitutions. Other NS3, other NS3 substitution except D168 substitution; Other NS5A, other NS5A substitution except L31 and Y93 substitution. Analyses were performed by using a direct-sequencing method.

in real-world clinical practice demonstrates lower SVR rates and higher rates of adverse events than observed in clinical trials. The stable therapeutic effect in a real-world setting is one of the notable benefits of the DCV and ASV combination therapy.

Baseline characteristics of gender, advanced age, history of IFN-based regimens, and low platelet counts (suggestive of advanced fibrosis or cirrhosis) did not influence SVR12 rates. An important finding was that patients with pretreatment viral loads of $\geqslant$ 6.0 log IU/mL showed a significantly lower rate of SVR12 than patients with pretreatment viral loads of < 6.0 log IU/mL. As for DCV and ASV treatment, Wang *et al*[12] showed that patients with lower viral loads (< 8×10^5 IU/mL: 8.0×10^5 IU/mL = 5.9 log IU/mL) seemed to have higher SVR rates than those with higher viral loads ($\geqslant 8 \times 10^5$IU/mL) in their meta-analysis[12]. Comparing the background characteristics of patients with viral loads of $\geqslant$ 6.0 logIU/mL and < 6.0 logIU/mL in this study showed that they were not significantly different with respect to gender, age, platelet counts, number of treatment discontinuations, and preexisting major RASs (L31 substitution or Y93 substitution). The number of patients with a history of IFN-based treatment was significantly greater in patients with viral loads of $\geqslant$ 6.0 log IU/mL than those with < 6.0 log IU/mL (P = 0.04). PEG-IFN and RBV-based treatment was covered by public health insurance only for high viral loads ($\geqslant$ 5.0 log IU/mL) in Japan. This might have caused the background difference in our study. The HCV RNA loads alone may have influenced treatment efficacy.

Twenty-three patients who experienced adverse events discontinued the treatment but nevertheless achieved SVR12. It is notable that eight patients who were treated for < 8 wk still achieved SVR12. The shortest treatment duration was only 2 wk. The factors that contributed to HCV clearance in such a short treatment duration are unknown. Because alanine aminotransferase (ALT) elevation was the main cause (15 in 23 patients) of stopping treatment early, elevated blood levels of ASV may be both a cause of ALT elevation and an enhanced treatment efficacy[13]. Interestingly, in our study, patients who discontinued treatment because of ALT elevation tended to have lower viral loads. This background factor of low viral load might influence treatment effectiveness. Patients with adverse events such as ALT elevation can still achieve SVR even with short treatment periods.

Resistance analyses before treatment revealed that patients who did not carry both Y93 and L31 substitutions using commercially available tests achieved 93.0% SVR12 (Figure 2). The SVR ratio of the Y93 substitution-positive group was significantly lower than in the Y93 non-substitution group. However, the SVR ratio of the L31 substitution-positive group was not significantly different from that of the L31 non-substitution group. The baseline prevalence of L31 substitution in our study was lower than that in other studies and this might have affected our statistical analyses.

We investigated RASs before treatment and after failure in 17 patients who failed to achieve SVR12. All 17 patients had major well-known substitutions (either NS5A L31 substitution and/or Y93 substitution, or NS3 D168 substitution) at the time of failure. The

appearance pattern of these RASs was different in each patient, but can be classified into one of two patterns. The first pattern (Cases 13-17) had no major substitutions before treatment, but carried more than one major variant after treatment. The other (Cases 4-11) had one major substitution before treatment but carried three major substitutions after treatment. The mechanism of occurrence for the first pattern is obscure, but it might have been influenced by the detection sensitivity of direct sequence methods. The mechanism of the second pattern is also obscure, but it revealed an important problem of this therapy for the next generation of DAA treatments from the viewpoint of drug resistance. At any rate, there are still many problems to be solved concerning RASs.

The first problem of pretreatment RAS analysis is that there are no available commercial assays for NS3 mutations in the real world. We did not check the RASs in NS3 for two reasons. One reason is that naturally occurring NS3 RASs are reported to be rare[14,15]. Another reason is that the guideline for the treatment of hepatitis C edited by the Japan Society of Hepatology do not recommend to check NS3 RASs, but recommend to check NS5A RASs before starting DCV/ASV treatment. The second problem is the difference in sensitivities of available assays[16,17]. We could measure NS5A variants using three different methods: Direct-sequencing, the cycleave probe method, and invader assay. Although ultra-deep sequencing is the most sensitive method and can detect minor variants with frequencies of < 1% (Miura *et al*[18]), this method is too expensive and complicated. The appropriate proportion of RASs to predict this treatment's efficacy has been reported in several studies. Ide *et al*[19] reported that SVR rates were clearly altered by the proportion of Y93 substitutions. In our unpublished data, patients who had > 10% pretreatment Y93 substitutions by the cycleave probe method tended to experience virological failure. Thus, this may be the appropriate cutoff value in our study (data not shown). Except for L31 and Y93 substitutions which can be checked commercially, other rare NS5A RASs were reported to have a rather small influence on therapeutic effect[20]. After all, although RASs have a great influence on the therapeutic efficacy of DCV and ASV combination treatment, we cannot determine the best method at present. Further larger-scale studies are needed to clarify this point.

The eradication of HCV can ameliorate liver inflammation as well as liver fibrosis[21]. We calculated the values of FIB4 index both at baseline and after SVR12. We found that FIB-4 index was more markedly reduced in the SVR12 group. This data indicate that liver fibrosis is improved in SVR12 group in the future

A major limitation of the present study was the inability to evaluate several factors known to influence HCV treatment efficacy. We did not examine IL28B[22], amino acid substitutions of the HCV core region 70 and 91[23], NS5A interferon sensitivity determining region[24], interferon/ribavirin resistance determining region[25]. These factors were mainly related to the efficacy of IFN based therapy and were not easily available in real-world.

DCV and ASV combination therapy is associated with a high SVR rate in real-world clinical practice. The SVR12 rate in patients with viral loads of HCV RNA $\geqslant$ 6.0 log IU/mL was significantly lower than that in patients with HCV RNA < 6.0 log IU/mL. The ratio of SVR12 in the Y93 substitution-positive group was significantly lower than that in the Y93 substitution-negative group. The pretreatment and post-treatment NS3:D168 substitution, and NS5A:L31 and Y93 substitutions were evaluated in 17 patients who did not achieve SVR 12 using direct-sequencing method. All 17 patients had increased RASs after treatment failure. Baseline RASs should be thoroughly assessed to avoid additional RASs after treatment failure.

COMMENTS

Background

The modality for treating hepatitis C has rapidly progressed in a recent years. The usage of directly acting antiviral (DAA) has changed the treatment dramatically. The combination of oral daclatasvir (DCV), a NS5A inhibitor, and asunaprevir (ASV), a second generation NS3 PI, is the first drug combination approved in Japan for the treatment of hepatitis C (HCV) genotype 1-infected patients. This drug combination showed high rates of sustained virologic response (SVR) and better tolerability. They performed a retrospective cohort study to evaluate the effectiveness of DCV and ASV combination therapy in real-world clinical practice. Moreover, they evaluated the presence of pretreatment and post-treatment major resistance-associated substitutions (RASs) (NS5A:L31 and Y93 substitution, and NS3:D168 substitution) using a direct-sequencing method in 17 patients who did not achieve SVR12.

Research frontiers

In the era of DAA treatment for hepatitis C, drugs are designed targeting NA3, NA5A, and NS5B of HCV. Evaluation of the efficacy/tolerability of the DAAs regimens as well as the acquisition of RASs in DAAs treatment are major interest in the field of hepatology. Above all, attention is paid for RASs (NS5A: L31 and Y93 substitution, and NS3:D168 substitution) in DAC/ASV treatment.

Innovations and breakthroughs

All 17 patients who failed to achieve SVR12 in DAC/ASV treatment had major well-known RASs (either NS5A L31 RAS and/or Y93 RAS, or NS3 D168 RAS) after treatment failure. The appearance pattern of these RASs was different, but can be classified into two patterns. The first pattern: No major RASs before treatment, but more than one major RASs after treatment failure. The second pattern: One major RAS before treatment but three major RASs after treatment failure.

Applications

DCV/ASV combination therapy is associated with a high SVR rate in real-world clinical practice, but appearance of RASs were seen in patients with treatment failure. Baseline critical RASs should be checked to avoid additional RASs after treatment failure.

Peer-review

Very interesting paper on DCV and ASV efficacy in HCV patients.

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ORIGINAL ARTICLE

Observational Study

CD4$^+$ T cells and natural killer cells: Biomarkers for hepatic fibrosis in human immunodeficiency virus/hepatitis C virus-coinfected patients

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Abstract

AIM

To characterize peripheral blood natural killer (NK) cells phenotypes by flow cytometry as potential biomarker of liver fibrosis in human immunodeficiency virus (HIV)/hepatitis C virus (HCV) coinfected patients.

METHODS

Peripheral mononuclear cells from 24 HIV/HCV (HBV

negative) coinfected and 5 HIV/HCV/HBV seronegative individuals were evaluated. HIV/HCV coinfected patients were divided in to groups: G1, patients with METAVIR F0-F2 and G2, patients with METAVIR F3-F4. NK surface cell staining was performed with: Anti-CD3(APC/Cy7), anti-CD56(PE/Cy5), anti-CD57(APC), anti-CD25(PE), anti-CD69(FITC), anti-NKp30(PE), anti-NKp46(PE/Cy7), anti-NKG2D(APC), anti-DNAM(FITC); anti-CD62L (PE/Cy7), anti-CCR7(PE), anti-TRAIL(PE), anti-FasL(PE), anti CD94(FITC). Flow cytometry data acquisition was performed on BD FACSCanto, analyzed using FlowJo software. Frequency of fluorescence was analyzed for all single markers. Clinical records were reviewed, and epidemiological and clinical data were obtained.

RESULTS

Samples from 11 patients were included in G1 and from 13 in G2. All patients were on ARV, with undetectable HIV viral load. Liver fibrosis was evaluated by transient elastography in 90% of the patients and with biopsy in 10% of the patients. Mean HCV viral load was (6.18 ± 0.7 $\log_{10}$). Even though, no major significant differences were observed between G1 and G2 regarding NK surface markers, it was found that patients with higher liver fibrosis presented statistically lower percentage of NK cells than individual with low to mild fibrosis and healthy controls (G2: 5.4% ± 2.3%, G1: 12.6% ± 8.2%, P = 0.002 and healthy controls 12.2% ± 2.7%, P = 0.008). It was also found that individuals with higher liver fibrosis presented lower CD4 LT count than those from G1 (G2: 521 ± 312 cells/μL, G1: 770 ± 205 cells/μL; P = 0.035).

CONCLUSION

Higher levels of liver fibrosis were associated with lower percentage of NK cells and LTCD4$^+$ count; and they may serve as noninvasive biomarkers of liver damage.

Key words: CD4$^+$ T cell; Human immunodeficiency virus/hepatitis C virus-coinfection; Fibrosis; Biomarker; Natural killer cells

Core tip: Approximately 2.3 million individuals with human immunodeficiency virus are coinfected with hepatitis C virus (HCV). The high cost of HCV treatment restricts its use. It is crucial to identify patients with advanced liver fibrosis with an urgent need of treatment. The aim of this study was to identify natural killer (NK) phenotypes as a biomarker for liver fibrosis. We observed that those subjects with higher fibrosis are those with lower percentage of NK cells and also with lower LTCD4$^+$ count. These constitute two simple parameters that might be performed in a routine laboratory test and used in clinical practice as biomarkers for liver fibrosis.

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INTRODUCTION

Chronic hepatitis C virus (HCV) infection affects 115 million individuals worldwide and is a common cause of chronic hepatitis, which may eventually progress to cirrhosis and hepatocellular carcinoma[1]; whereas currently 36.9 million people are living with human immunodeficiency virus (HIV)/aids [2]. Because of overlapping pathways of transmission, approximately 2.3 million individuals worldwide are estimated to be coinfected with both viruses[3]. Direct antiviral agents (DAA) are a major development in the treatment of HCV infection, with cure rates higher than 90%[4]. However, the high cost of DAA regimens and competing public health priorities have prompted a worldwide discussion whether all patients should have access to the new therapies without restriction. In many countries, new DAA regimens are therefore reserved for patients with advanced fibrosis or cirrhosis[5,6].

Liver fibrosis is a response to a wound-healing process triggered by various types of chronic liver injuries, among them HCV infection[7]. Liver fibrosis is well characterized by abnormal accumulation of extracellular matrix, and hepatic stellate cells (HSCs) are considered to be the major type of cells responsible for liver fibrosis. Such profibrotic role might be down-regulated by natural killer (NK) cells either directly through induction of HSC apoptosis or indirectly *via* production of IFN-γ. Increased peripheral NK cell-mediated cytotoxicity has been associated with less liver fibrosis during HCV infection and likely reflects this mechanism[7]. HIV infection *per se* has a strong suppressive effect on anti-HCV activity of NK cells[8].

NK cells are lymphoid cells that are primary responders to microbial infections and tumor cells[9]. Phenotypically, NK cells are defined as CD3$^-$CD56$^+$cells with variable expression of CD16, depending on cell subpopulation of NK cells. They comprise approximately 5%-20% of peripheral lymphoid cells, but up to 30%-50% of intrahepatic lymphoid cells. NK cell activation is regulated by cell surface receptors that become engaged by cognate ligands expressed on target cells by cytokines, and by Toll-like receptors (TLRs)[9-11].

Different techniques to asses liver fibrosis have been developed, from liver biopsy (gold standard) to non-invasive studies (transient liver elastography; patented and nonpatented biomarkers - FIB4, FibroTest, APRI, *etc*). Liver biopsy is invasive and has risk of complications[12]. In addition, liver biopsy may be limited by the size of the specimen obtained as well as sampling,

Table 1 Fluorochrome-conjugated antibodies panels

Antibody	Fluorochrome	Clone	Provider
All panels			
Anti-CD3	APC/Cy7	SK7	BioLegend
Anti-CD56	PE/Cy5	679.1Mc7	Beckman Coulter
Panel 1			
Anti-CD57	APC	HNK-1	BioLegend
Anti-CD25	PE	BC96	BioLegend
Anti-CD69	FITC	FN50	BioLegend
Panel 2			
Anti-NKp30	PE	P30-15	Biolegend
Anti-NKp46	PE/Cy7	9E2	Biolegend
Anti-NKG2D	APC	1D11	Biolegend
Anti-DNAM	FITC	TX25	Biolegend
Panel 3			
Anti-CD62L	PE/Cy7	DREG-56	Biolegend
Anti-CCR7	PE	G043H7	Biolegend
Panel 4			
Anti-TRAIL	PE	S35-934	BD Bioscience
Panel 5			
Anti-FasL	PE	NOK-1	Biolegend
Anti CD94	FITC	DX22	Biolegend

BioLegend, San Diego, California United States. BD Bioscience, San Jose, California, United States. NK: Natural killer.

intraobserver, and interobserver variability[13]. On the other hand, there are many unresolved issues regarding the accuracy (especially in HIV/HCV-coinfected patients) of noninvasive studies[14], and in low-resource countries there is an important barrier to access to these methods (in particular liver elastography and patented biomarkers).

The identification of noninvasive liver fibrosis biomarkers is still an open research area. In this context, we reasoned that the study of the phenotype of peripheral blood cells may unravel interesting clues towards the identification of such biomarkers. Some evidence indicates that the characteristics of the immune cells, including NK cells, observed in peripheral blood are similar to those seen in liver with relatively lower levels of magnitude. Accordingly, the aim of this study was to characterize peripheral blood NK cell phenotypes by flow cytometry as potential biomarker for liver fibrosis in patients chronically coinfected with hepatitis C and HIV.

MATERIALS AND METHODS

Study cohort

Informed consent was obtained from each subject. The study protocol is in line with the ethical guidelines of the Declaration of Helsinki and was approved by the ethics review committee of Fundación Huésped (Buenos Aires, Argentina).

Cryopreserved peripheral blood mononuclear cells (PBMC) from 24 HIV/HCV-coinfected individuals and 5 HIV/HCV-seronegative individuals (healthy controls, HC) were used in this study. HIV/HCV-coinfected patients and healthy control individuals enrolled in this study were not acutely or chronically infected with HBV; they denied current use of recreational drugs or alcohol intake. HIV/HCV-coinfected patients were divided into two groups based on their level of liver fibrosis (group 1: Patients with METAVIR score F0 to F2 on liver biopsy or transient elastography - FibroScan®-; and group 2: Patients with METAVIR score F3-F4). Hepatic fibrosis was evaluated by liver biopsy in 10% of patients and by transient hepatic elastography in 90% of patients. All healthy control individuals presented F0-F1 fibrosis according to transient liver elastography (less than 5 kPa). Clinical records were reviewed, and epidemiological and clinical data were obtained.

Multicolor flow cytometry

Cryopreserved PBMC were thawed and stained with fluorochrome-conjugated antibodies distributed in five different panels (depending on PBMC availability) to evaluate expression of different markers on NK cells detailed in Table 1. Staining was performed for 30 min at 4 ℃. Samples were washed, fixed in 1% paraformaldehyde and acquired in a FACS Canto flow cytometer (BD Biosciences). Data were analyzed using the FlowJo software (TreeStar, Ashland, Oregon, United States). NK cell populations were defined according to the corresponding isotype control.

Plasma viral load levels (Abbott RealTime HIV-1 RNA version 3; Abbott Molecular, Inc., Des Plaines, IL, United States) were assessed in HIV-infected subjects and $CD4^+$ T-cell counts (flow cytometry double platform, BD FACSCanto; BD Biosciences, San Diego/California, United States) were assessed in HIV and HIV-negative individuals.

Statistical analysis

For categorical variables, both χ^2 and Fisher's exact test were applied. For continuous variables, the nonparametric Kruskal-Wallis and Mann-Whitney test were used. Area under the receiving operating curve (ROC) was used to calculate the cut-off point in NK cell percentage with the best sensitivity of high liver fibrosis. Statistical analyses were performed using the Statistical Package for the Social Sciences software version 19.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Patient characteristics at the time of liver fibrosis assessment are shown in Table 2. Individuals from Group 1 (n = 11, 46%) presented low to mild liver fibrosis (METAVIR F0-F2) whereas patients included in Group 2 (n = 13, 54%) had severe fibrosis (METAVIR F3-F4). Forty percent of patients had previously received HCV treatment with pegylated interferon and ribavirin (with no differences between groups); a median of 6.25 ± 1.48 years before sample collection; none of them achieved sustained virological response. The mean age was 46.9 years (± 8.4); 83% were male. Patients from group 2 were older than those with lower METAVIR score (P = 0.028). No differences were

Table 2 Characteristics of the population and divided according the level of fibrosis

Characteristics	Group 1 *n* = 11	Group 2 *n* = 13	Control *n* = 5	*P* value
Age (yr)[1]	46.3 (3.9)	52.2 (4.5)	31 (4.8)	0.02
Male/female	9/2	11/2	3/2	0.85
CD4 cell count[1]	770 (205)	521 (312)	910 (251)	0.03
CD8 cell count[1]	1079 (475)	657 (339)	NA	0.24
METAVIR F0-F2	100%	0	100%	NC
METAVIR F3-F4	0	100%	0	NC
TGP[1]	79.7 (74.2)	99 (71.4)	NA	0.41
Time of known HIV infection[1]	18 (6.52)	17.3 (3.8)	NC	0.60
Time of known HCV infection[1]	14.3 (7.0)	13.8 (4.6)	NC	0.65

[1]mean ± SD. Beckman Coulter, Marseille, France. *P* values, correspond to comparison between group 1 and 2. NA: Not available; NC: Not correspond; HIV: Human immunodeficiency virus; HCV: Hepatitis C virus.

found between groups regarding gender or mean time of known HIV and HCV infection. The mean HCV viral load was 6.18 ± 0.70 log, with no differences between the two groups (G1: 6.54 ± 0.24; G2: 6.18 ± 0.7). HCV genotype 1 was identified in 90% of the patients, the rest presented infection by genotype 3. All patients were on antiretroviral treatment with undetectable HIV viral load, with no differences in the time on ARV therapy between groups. Patients with higher fibrosis presented lower $CD4^+$ T cell count (521 ± 312 cells/μL) than those from group 1 (770 ± 205 cells/μL, P = 0.035) There was no difference in the $CD4^+$ T cell count between group 1 and healthy controls (P = 0.49).

Regarding NK cells, a lower percentage was found in samples from patients of group 2 (5.4% ± 2.3%) compared both with patients from group 1 (12.6% ± 8.2%, P = 0.002) and healthy controls (12.2% ± 2.7%, 0.008) (Figures 1 and 2). With ROC curve analysis a cut-off of a NK cell percentage lower than 6.6% was determined to have 90% sensitivity and 77% specificity to predict the presence of METAVIR F3-F4 (Figure 3).

The percentage of $CD56^{bright}$ NK cells (G1: 11.7% ± 8.0%, G2: 7.1% ± 4.0%, HC: 6.8% ± 3.6%) and $CD56^{dim}$ NK cells (G1: 88.2% ± 7.6%, G2: 73.7% ± 40.1%, HC: 92.9% ± 3.6%) did not present differences among the three groups studied.

As the function of NK is regulated by an array of activating and inhibitory receptors, we also evaluated the NK cell activating receptors[15] NKp46 (CD335), NKp30 (CD337), NKG2D (CD314) and DNAM (CD226), the activation markers CD69 and CD25, and other molecules involved in NK cell effector functions, terminal differentiation and cytotoxicity such as CD94, TRAIL, CD57[16], Fas-L (CD178), CCR7 (CD197) and CD62L.

When compared with healthy controls, samples from patients included in group 2 presented a higher frequency of $CD56^{bright}$ $DNAM\text{-}1^+$ NK cells (76.2% ± 18.5% *vs* 4.6% ± 8.5%, P = 0.008) and $CD56^{dim}$ $DNAM\text{-}1^+$ NK cells (42% ± 29% *vs* 6.0% ± 8.1%, P = 0.018). The same differences were observed between group 1 and healthy controls, both in the percentage of $CD56^{bright}$ $DNAM\text{-}1^+$ NK cells (71.2% ± 23% *vs* 4.6% ± 8.5%, P = 0.003) and in the percentage of $CD56^{dim}$ $DNAM\text{-}1^+$ NK cells (41% ± 28% *vs* 6.0% ± 8.1%, P = 0.013).

Additionally, samples from group 1 exhibited higher percentage of $CD56^{bright}$ $CD25^+$ NK cells (53.1% ± 16.6% *vs* 19.4% ± 18.9%, P = 0.029) and $CD56^{dim}$ $CD25^+$ NK cells (28.3% ± 10.2% *vs* 7.1% ± 5.6%, P = 0.001) than healthy controls. These results show the possible consequence of a higher activation degree in NK cells from subjects with chronic infection. Of note, there were no differences in the frequency of these NK cells subsets between group 1 and 2. Moreover, no differences were observed in the other activator molecules evaluated (NKp46, NKp30, NKG2D, CD69) neither between group 1 and 2 nor between controls and HCV-infected subjects.

No differences in surface expression of CD94 were observed between the 3 groups. The frequency of these molecules was very high in $CD56^{bright}$ NK cells in all the samples evaluated (G1: 77.1% ± 28.2%, G2: 91.7% ± 1.2%, controls: 77.4% ± 36.7%), whereas in $CD56^{dim}$ NK cells this molecule was stained in less than 50% (G1: 48.4% ± 21.4%, G2: 36.9% ± 19.7%, controls: 31.6% ± 16.9%).

The frequency of $CD56^{dim}$ $TRAIL^+$ NK cells was higher in samples from group 2 than those from group 1 (29.4% ± 31.7% *vs* 7.5% ± 3.1%, P = 0.04), while no differences were observed between coinfected patients and healthy controls.

Nevertheless, the percentage of $CD56^{dim}$ $FasL^+$ NK cells was lower in samples from HCV/HIV-coinfected patients (G1: 27.2% ± 19.8%, P = 0.001; G2: 36.9% ± 19.7%, P = 0.01) than those from healthy controls (69.3% ± 18.2%), without detecting differences between groups 1 and 2.

In addition, there was a trend towards a higher percentage of $CD56^{dim}$ $CCR7^+$ NK cells in samples from patients with advanced fibrosis than in samples from patients with lower fibrosis (G2: 56.4% ± 36.2% *vs* G1: 24.4% ± 14.6%; P = 0.05). Regarding the CD62L expression, there were no differences in $CD56^{bright}$ NK cells between groups (G1: 61.8% ± 24.9%; G2: 87% ± 15.1%; P = 0.09).

DISCUSSION

In this study we found that patients with advanced fibrosis presented lower LT $CD4^+$ cell counts than subjects with low to mild fibrosis. All the patients were on successful antiretroviral treatment. Even though there are controversial data whether the presence of HCV is a factor that alters LT CD4 recovery with ARV, it can be hypothesized that patients with higher chances to develop liver fibrosis are those with lower LT $CD4^+$ cell recovery after HIV treatment. Such a poor HAART-mediated LT $CD4^+$ cell recovery may

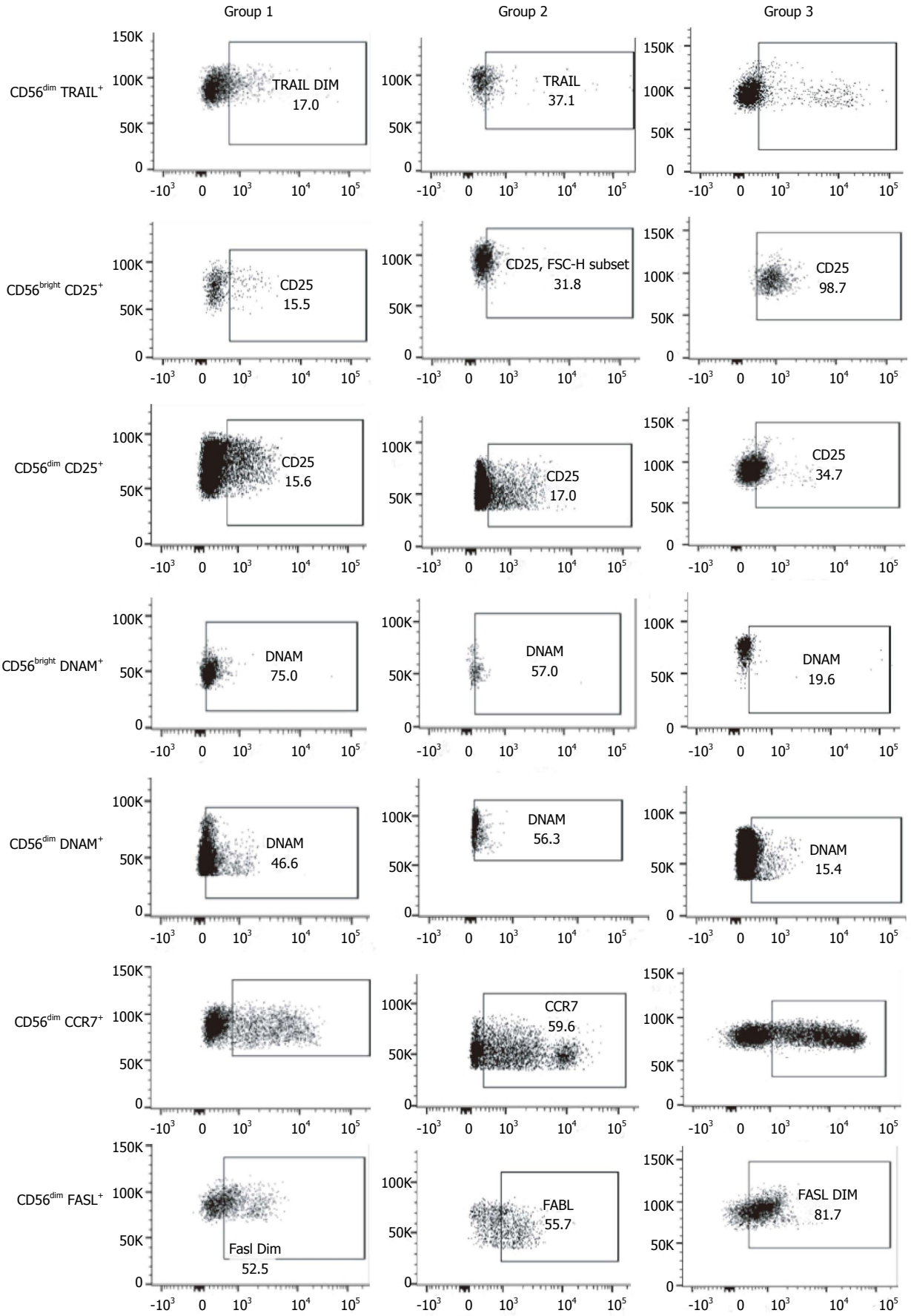

Figure 1 Representative dot plots of each of groups evaluated.

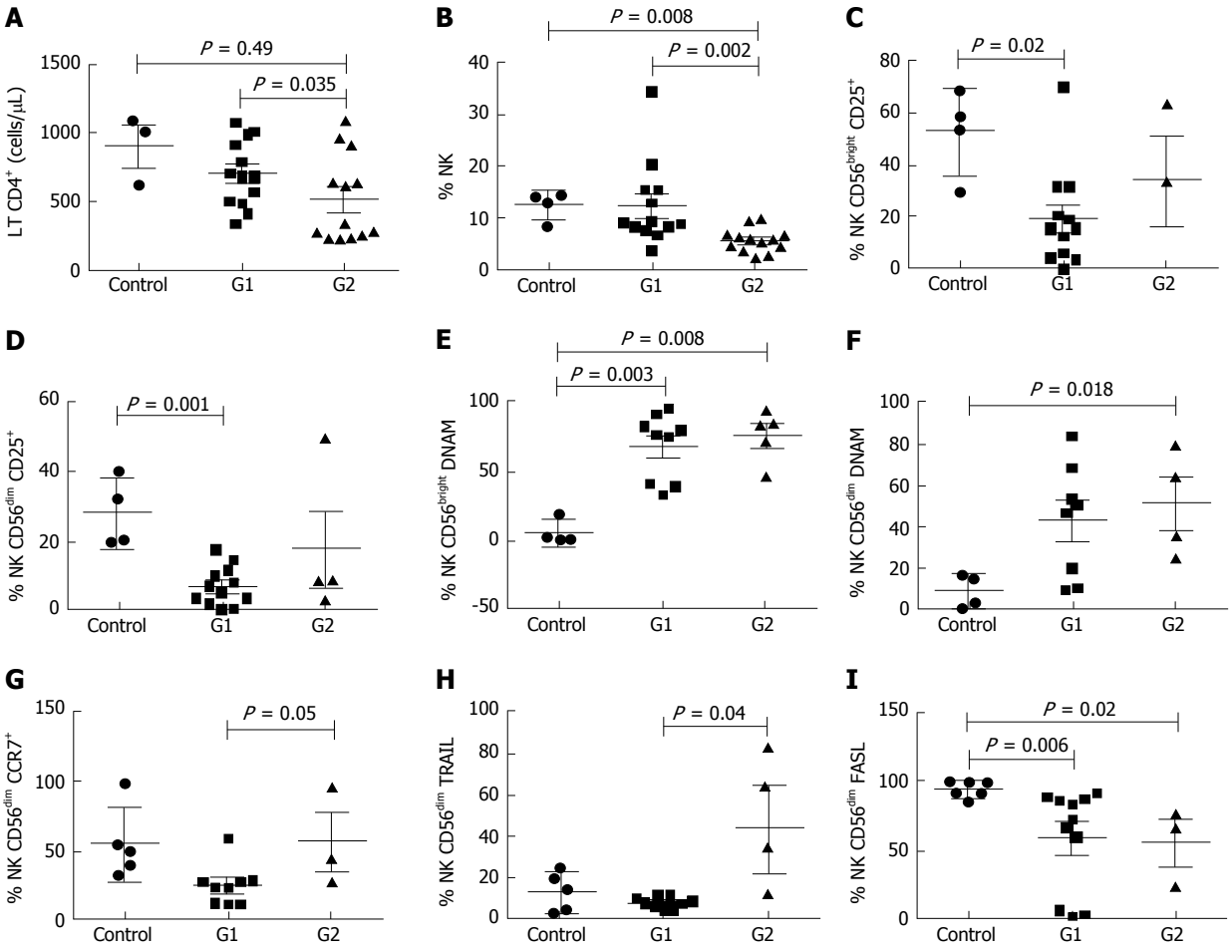

Figure 2 Differences between groups 1, 2 and controls regarding. A: Total CD4 cell count; B: Percent of NK cells; C: Percent NK CD56^{bright} CD25+; D: Percent CD56^{dim} CD25^{+} NK cells; E: Percent CD56^{bright} DNAM^{+} NK cells; F: Percent CD56^{dim} DNAM^{+} NK cells; G: Percent CD56^{dim} CCR7^{+} NK cells; H: Percent CD56^{dim} TRAIL^{+} NK cells; I: Percent CD56^{dim} FasL^{+} NK cells. The *P* values are shown in each graphic and the line below the *P* value connects the two groups compared. NK: Natural killer.

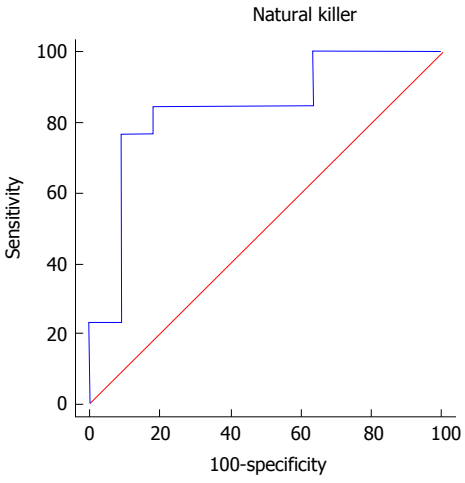

Figure 3 Area under the receiver operating characteristic curve, to evaluate the performance of natural killer cell % with liver fibrosis as the state variable.

contribute to an impaired stimulation of NK cells, and consequently a diminished anti-fibrotic activity by their action on hepatic stellate cells, favoring an accelerated liver fibrosis progression in HIV/HCV patients[17]. Yi *et al*[18] and other groups have observed that NK cells negatively regulated liver fibrosis. NK cells isolated from HCV-infected patients efficiently induced apoptosis of activated HSCs in TRAIL-, FasL-, and NKG2D-dependent manners[19]. NKp46^{high} NK cell subset potentially suppresses HCV replication and HCV-associated liver damage, leading to amelioration of liver fibrosis.

It has been described that HIV/HCV coinfection can modulate the peripheral NK phenotype[20]. In our study, we also observed differences in the NK phenotype particularly between control and HIV/HCV-coinfected patients which resemble those reported previously[21,22]. We found a lower percentage of CD56^{dim} FasL^{+} NK cells in HCV/HIV-coinfected patients compared to healthy controls. This finding could reflect a lower NK cell capacity to exert cytotoxic activity in patients with chronic HIV and HCV infection compared to non-infected individuals that could ultimately lead to a decreased capacity to regulate HSC.

Regarding HIV/HCV-coinfected individuals, no differences were observed in NK cell phenotypes according to the different degrees of liver fibrosis. Nevertheless, we could observe a statistically significant difference in the percentage of peripheral

blood NK cells in patients with high scores compared to patients with low liver fibrosis. Patients with advanced fibrosis have lower percentage of NK cells than those with low fibrosis scores. Moreover, we observed that a percentage of NK cells lower than 6.6% had 90% sensitivity and 77% specificity to predict the presence of advance fibrosis (METAVIR F3-F4). This observation could indicate, for the first time, that the evaluation of the NK cells compartment is a potential biomarker for fibrosis staging in HIV/HCV-coinfected patients.

In the era of direct antiviral agents with high efficacy for the treatment of chronic HCV, one of the main treatment access barriers for many patients is the high cost of these drugs, and where these barriers exist the assessment of liver fibrosis is mandatory to ensure treatment access. In this study, we have observed that those subjects with higher fibrosis are those with lower absolute count both of LT CD4$^+$ and lower percentage of NK cells. Although additional research is needed to confirm our findings, the evaluation of these two parameters that can be performed in a routine laboratory test may be helpful in improving the available noninvasive methods for liver fibrosis staging.

COMMENTS

Background

Different techniques to assess liver fibrosis have been developed, from liver biopsy (gold standard) to non-invasive studies (transient liver elastography; patented and non-patented biomarkers - FIB4, FibroTest, APRI, *etc*). Liver biopsy is invasive and has risk of complications. In addition, liver biopsy may be limited by the size of the specimen obtained as well as sampling, intra and inter-observer variability. On the other hand, there are many unresolved issues regarding the accuracy [especially in human immunodeficiency virus (HIV)/hepatitis C virus (HCV)-coinfected patients] of noninvasive studies, and in low-resource countries there is an important barrier to access to these methods (in particular liver elastography and patented biomarkers).

Research frontiers

The identification of non-invasive liver fibrosis biomarkers is still an open research area. In this context, the authors reasoned that the study of the phenotype of peripheral blood cells may unravel interesting clues towards the identification of such biomarkers. Some evidence indicates that the characteristics of the immune cells, including natural killer (NK) cells, observed in peripheral blood are similar to those seen in liver with relatively lower levels of magnitude. Accordingly, the aim of this study was to characterize peripheral blood NK cell phenotypes by flow cytometry as potential biomarker for liver fibrosis in patients chronically coinfected with hepatitis C and HIV.

Innovations and breakthroughs

In the era of direct antiviral agents with high efficacy for the treatment of chronic HCV, one of the main treatment access barriers for many patients is the high cost of these drugs, and where these barriers exist, the assessment of liver fibrosis is mandatory to ensure treatment access. In this study, the authors have observed that those subjects with higher fibrosis are those with lower absolute count both of LT CD4$^+$ and lower percentage of NK cells. Although additional research is needed to confirm their findings, the evaluation of these two parameters that can be performed in a routine laboratory test may be helpful in improving the available noninvasive methods for liver fibrosis staging.

Applications

The data in this study suggested that LTCD4 and NK cells could be used as potential non-invasive biomarkers of the level of liver fibrosis in HIV-HCV coinfected patients. These parameters could improve the accuracy of the available non-invasive methods to measure liver fibrosis.

Terminology

NK cells, and CD4 T lymphocytes (LTCD4) are involved in the immunological control of hepatic stellate cells that are the responsible of liver fibrosis development.

Peer-review

The identification of noninvasive liver fibrosis biomarkers is still an open research area. NK cells and LTCD4$^+$ count; are two simple parameters, that might be perform in a routine laboratory test and may serve as noninvasive biomarkers of liver fibrosis, identifying patients in need for HCV therapy in the short term.

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