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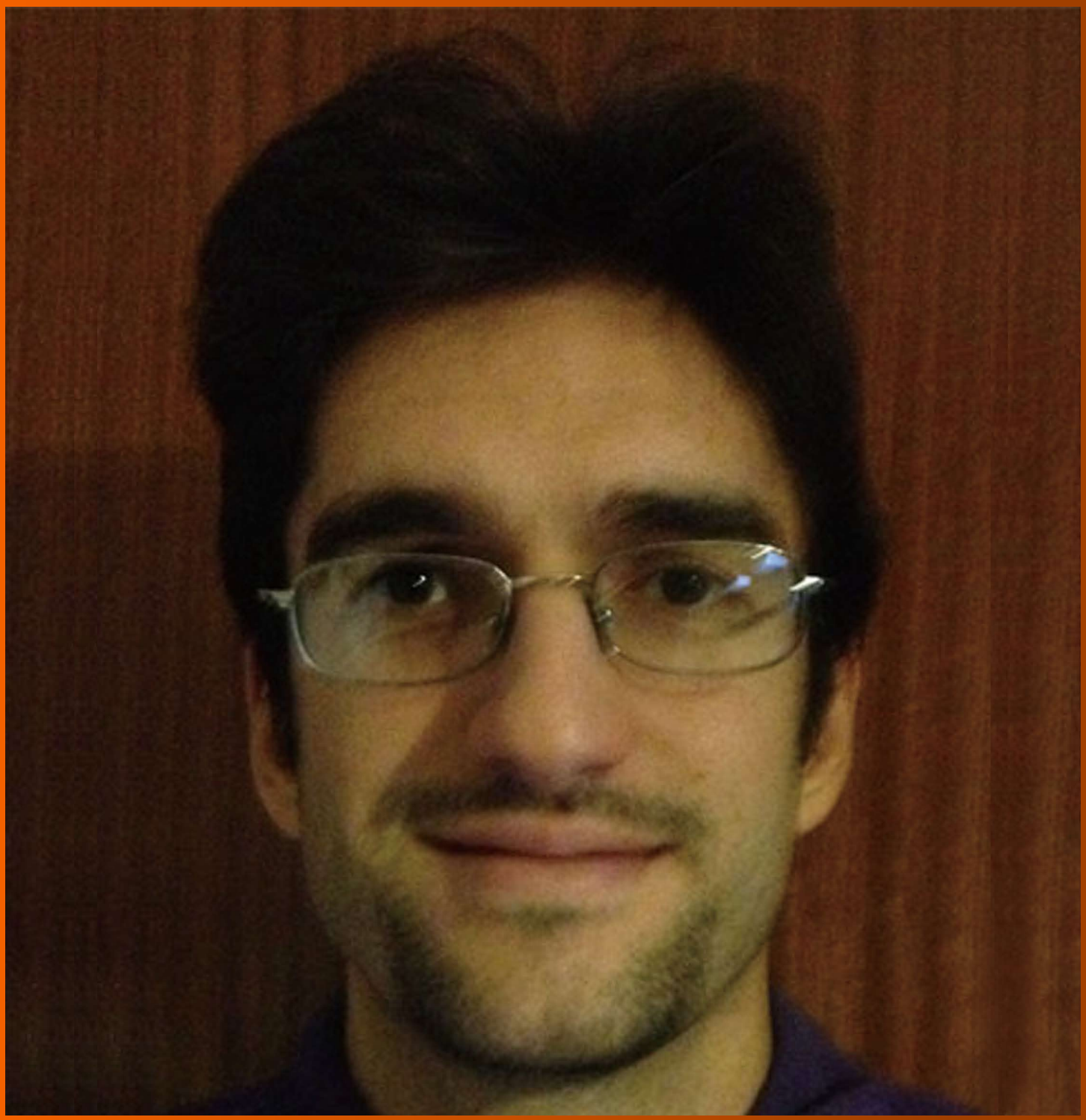

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EDITORIAL

Role natural killer group 2D-ligand interactions in hepatitis B infection

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Abstract

Hepatitis B virus (HBV) infection is the leading cause of liver disease and hepatocellular carcinoma (HCC) worldwide, in spite of prophylactic vaccination and antiviral treatment modalities. The immunopathogenesis of HBV infection has been intensively studied and is propelled by complex interactions between the virus and the host immune system. Natural killer group 2D (NKG2D) is a well-characterized activating receptor, expressed on natural killer (NK) cells, NK T cells and $CD8^+$ cytotoxic T cells. This receptor is present in both humans and mice and binds to a diverge family of ligands that resemble the MHC-class I molecules. Increasing evidence shows that NKG2D-ligand interactions are critical in the establishment of HBV persistence and the development of liver injury and HCC. The expression of NKG2D ligands depends on the presence of several polymorphisms and is also modulated post-transcriptionally by HBV. While it is known that HBV circumvents host's innate immunity *via* the NKG2D pathway but the exact mechanisms involved are still elusive. This letter discusses previous accomplishments on the role of NKG2D ligand regulation in the development of chronic HBV, liver injury and HCC.

Key words: Hepatitis B virus; Natural killer group 2D receptor; Natural killer cells; MHC class I polypeptide-related chain A; Hepatocellular carcinoma

Core tip: Hepatitis B virus (HBV) infection is the leading cause of liver disease and hepatocellular carcinoma (HCC) worldwide. HBV persistence involves complex interactions between the virus and the immune system of the host. Natural killer group 2D (NKG2D) is an activating receptor, expressed on natural killer (NK), NK T and $CD8^+$ T cells. NKG2D-ligand interactions are critical in the establishment of chronicity and the development of liver injury and HCC. However, the exact mechanisms involved are still elusive. Here previous studies are discussed on how HBV modulates the NKG2D activity to result in viral clearance, susceptibility to liver injury and tumour evasion.

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INTRODUCTION

Despite the availability of an effective vaccine and significant progress in antiviral therapy, hepatitis B virus (HBV) remains a serious global health problem. Currently about one-third of the world's population is having markers of current or past infection, far exceeding the numbers of people infected with human immunodeficiency virus and hepatitis C virus together[1]. Infection with HBV can cause a wide spectrum of clinical manifestations, ranging from asymptomatic infection to acute self-limiting or fulminant hepatitis, or chronic infection (CHB), which is presented as distinct immunological stages, including immune tolerance and immune activation phases. CHB infection may eventually progress to chronic liver injury, cirrhosis or hepatocellular carcinoma (HCC)[2]. The risk of HCC in chronic infection is 100-fold higher compared with non-carriers and is directly proportional to high levels of HBV replication[3].

Most healthy adults raise effective immune responses against HBV that clear the virus. The adaptive immune system mounts a multiprolonged immune response, but its effectiveness depends on the quality of the earlier innate immune response that begins within hours of infection. Natural killer (NK) cells are enriched markedly in the liver and play a pivotal role in self-limiting infection. In addition to the direct killing of viral-infected cells without antigen-specific priming, NK cells regulate the adaptive immune response by producing interferon (IFN)-γ, tumor necrosis factor (TNF)-α and immunoregulating cytokines. In acute HBV infection of primates and humans, NK activity has been positively correlated with viral clearance[4-6] while IFN-γ production by NK cells has been shown to contribute to the initial control of infection[5,7-9]. In CHB infection, the function and numbers of intrahepatic NK cells are reported to be higher in the immunotolerant stage than in the immuno-active stage and are accompanied with an increase of HBV-specific T cells and low viremia[10-12]. High NK cell activity early in infection and in the immunotolerant stage reflects the time for the host to mount efficient adaptive response. However, NK cells also negatively regulate specific antiviral immunity in CHB infection by contributing in the liver inflammation through TNF-related apoptosis-inducing ligand- and Fas-mediated death[13,14] and by the direct killing of HBV-specific $CD8^+$ T cells, which triggers the recruitment of inflammatory cells that sustain and amplify the hepatic damage[15,16]. NK T cells comprise about one-third of intrahepatic T cells and are known to contribute to the outcome of HBV infection. They express T cell receptors (TCR) but, unlike conventional T cells, recognize lipid-based antigens presented by the major histocompatibility complex (MHC)-like molecule CD1d. HBV infection has been shown to induce alterations in CD1d expression and NK T activation in CHB patients[17]. Moreover, NK T cells are shown to regulate NK cell activation *via* the production of cytokines IFN-γ and interleukin 4 (IL-4) in the livers of HBV-transgenic mice[18].

The effector functions of NK cells are determined by the dynamic and coordinated balance of activating and inhibitory signals through their array of receptors. A well characterized activating receptor expressed in all NK cells is the natural killer group 2D (NKG2D), a type Ⅱ transmembrane-anchored glycoprotein that plays a key role in immune mediated diseases[19,20]. In addition to NK cells, it is expressed on the surface of NK T cells, activated $CD8^+$ T lymphocytes, γ/δ T cells, and some myeloid cells[21,22]. NKG2D receptor binds to a diverge family of ligands that are distantly related homologues to MHC class Ⅰ molecules. In contrast to classical MHC molecules, NKG2D ligands (NKG2DL) do not require association with β_2 microglobulin for expression or function, and do not bind antigenic peptides[23]. In humans, these proteins are divided into two families: the MHC class-Ⅰ polypeptide-related chain (MIC) protein family that contains MICA and MICB; and the cytomegalovirus UL16-binding proteins (ULBP) family, which consists of five members, ULBP1-4 and RAET1G[24]. In mice, there are five retinoic acid early transcript 1 (RAET-1) proteins, H60 and murine ULBP-like transcript 1 (MULT-1)[24]. In addition to membrane-bound NKG2DLs, secreted forms of the ligands have been also identified in humans.

NKG2DL are expressed on diseased or stressed cells, and numerous stress pathways induce their up-regulation, including viral and bacterial infection, cellular transformation, oxidative or genotoxic stress[25]. A large body of evidence indicates that HBV infection modulates NKG2D-mediated immune responses. The engagement of NKG2D to its ligands is a sufficient stimulus to activate cytolysis and cytokine production by NK cells, to promote antitumor or antiviral immune responses and autoimmune diseases, to provide a co-stimulatory signal for the activation of $CD8^+$ T cells and probably other T cells and contributes to apoptotic cell death[18,21,22,26]. NKG2DL also participate in the cross-talk between immune cells, which can regulate innate and adaptive responses. Increased expression of NKG2DL induces NK cell-mediated cytotoxicity to eliminate over-stimulated macrophages[27], while in response to Toll-like receptor stimulation NKG2DL expressed on myeloid cells are up-regulated, contributing to T cell and NK cell activation[28].

NKG2D PATHWAY IN HBV INFECTION

There is increasing evidence that activation of the NKG2D-ligand pathway contributes to the outcome of HBV infection. Studies in a transgenic mouse model

of acute infection, demonstrated that the blockage of NKG2D receptor on only NK T cells prevents HBV infection by raising efficient acute immune responses and that the interaction between NKG2D and its RAET-1 ligand is essential in inducing HBV immunity[18]. These findings suggest that in the early stages of infection, NK T cells may be activated first in an HBV-specific and NKG2D-dependent manner that may in turn lead to the activation of NK cells. Furthermore, early in HBV infection there is an overexpression of the soluble form of MICA (sMICA) in comparison to later stages of infection, leading to the internalization and degradation of NKG2D receptor and hence to defective NK and NK T activity[26,29]. Other studies demonstrated that sMICA levels increase together with progression of liver disease[30]. Consequently, the persistence of high sMICA levels from the early to later stages may result in the establishment of CHB infection and the initiation of liver cirrhosis and HCC.

In CHB infection, NKG2D expression on intrahepatic virus-specific $CD8^+$ T cells is shown to be essential in the recognition of virus-infected hepatocytes through the up-regulation of IL-15[31]. More specifically, increased NKG2D expression provides a co-stimulatory signal in TCR-mediated $CD8^+$ T cell activation. Enhanced NKG2D and IL-15 expression may act in a way to lower the activation threshold of effector $CD8^+$ T cells, and hence to allow the efficient recognition of hepatocytes that express low levels of viral antigens[31]. In accordance with these findings, down-regulation of NKG2D and co-stimulatory receptor 2B4 on circulating NK cells has been associated with the impaired function of NK cells in CHB patients[32]. On the other hand, hepatic NK cell-mediated killing through NKG2D recognition in CHB infection induces the development of liver fibrosis and hepatic damage. NKG2D activation through the over-expression of NKG2DL on the infected hepatocytes is shown to prime them to become a target for NK cell-mediated killing and to lead to the subsequent development of liver injury[33]. In addition, in a transgenic mouse model, NK cell activation *via* the up-regulation of RAET-1 or MULT-1 on hepatocytes was shown to account for the oversensitive autoimmune hepatocyte injury, with NK T cells working as helpers necessary for NK cell activation[34].

NKG2DL EXPRESSION IS INHIBITED IN HBV-INDUCED HCC

NK and T cell activity is repressed during HCC progression and NKG2D recognition of tumor cells is implicated in the process. MIC molecules are highly expressed in transformed cells and contribute in tumor immune surveillance by promoting antitumor NK and T cell responses[35]. Tumor cells have also developed strategies to escape NKG2D immunity by the down-regulation of NKG2DL[36]. Our unpublished data and others demonstrated that HBV infection represses MICA expression in the liver of CHB patients and on hepatic tumor cell lines[37,38]. Inhibition of viral replication in HBV-expressing HCC cells in transgenic mice is reported to restore MICA expression and to induce NK-cell mediated cytolysis[38,39]. In addition, increased levels of sMICA released from the surface of tumor cells in CHB patients have been shown to sequester NKG2D in the cytoplasm and to inhibit cell-surface NKG2D expression and effector functions in malignant tumors[19,30,40]. Therefore, a tumor-specific expression pattern of MICA exists in HBV-induced HCC, while NK cells recognize hepatoma cells *via* MICA-NKG2D interaction. The reduced MICA expression by HBV weakens the immune surveillance of NK cells in chronic infection while the overexpression of sMICA by HCC cells inhibits NKG2D function leading to the impairment of NK and T cells. The persistence of high sMICA levels at the late stages of CHB infection contributes to liver cirrhosis and HCC evasion[30].

NKG2DL POLYMORPHISMS ASSOCIATE WITH HBV PERSISTENCE

NKG2DL are highly polymorphic, more than 70 alleles have been identified for the MICA gene and more than 30 alleles for the MICB gene[24]. Interestingly, allelic variants of these ligands have been shown to bind to NKG2D receptor with different affinities resulting in different degrees of activation to promote NKG2D-mediated responses. Differential distribution of MICA alleles affects the outcome of HBV infection and HCC development. Homozygous genotype *MICA-175Ser/Ser*, allele *MICA-175Ser*, haplotypes, as well as the microsatellite polymorphisms associate with CHB infection[29]. Among the MICA variants, *MICA*015* allele is characterized by high affinity to NKG2D and has been correlated with HBV persistence in a small cohort of patients[41]. A non-synonymous substitution in exon 3 (*MICA-129Met/Val*) is known to play a distinct role in NKG2D binding: *MICA-129Met* is strong binder while *MICA-129Val* is a weak binder[42]. The genotype *MICA-129Met/Met* and the allele *MICA-129Met* are reported to increase the risk of HBV-induced HCC[29]. Notably, strong binding of the *MICA-129 Met* allele to NKG2D can induce the shedding of sMICA and subsequent NKG2D inhibition, resulting in impaired NK and T cell activity and promoting tumor evasion. However, alleles that contribute to lower sMICA levels are also risk factors for HCC occurrence. This could be explained by the fact that individuals with these risk alleles would also express low levels of membrane-bound MICA leading to a poor recognition of tumor cells[43] or the shedding of sMICA may be influenced by these risk alleles during immune surveillance. Nevertheless, differential binding between NKG2D and the MICA protein as well as the MICA shedding process plays a pivotal role in tumor surveillance in HBV infection.

NKG2DLS POST-TRANSCRIPTIONAL REGULATION BY HBV

Numerous finding including our own observations show that different cells and tissues express mRNA for NKG2DL but may lack the expression of NKG2DL proteins, indicating that at least some NKG2DL are regulated post-transcriptionally. Indeed, a group of endogenous cellular microRNAs (miRNAs) have been identified that bind to the 3'-untranslated region (3'-UTR) of MICA and MICB and repress their translation[44,45]. In addition, MICA and MICB expression is upregulated upon silencing of Dicer, a key protein in the miRNA processing pathway[39]. HBV as well as HBsAg and HBx viral proteins are reported to deregulate a number of cellular miRNAs[46,47]. HBsAg was shown to repress the expression of MICA and MICB in HCC cells by inducing 142 cellular miRNAs *via* targeting 3'-UTRs of their mRNAs and therefore enhancing the resistance of HCC cells to NKG2D-mediated cytolysis[39]. This down-regulation was shown to be partially restored by inhibiting the activities of HBsAg-induced miRNAs[39]. These findings suggest that HBsAg prevents NKG2D-mediated elimination of HCC cells by inducing cellular miRNAs to inhibit MIC expression.

CONCLUDING REMARKS

The NKG2D receptor plays an important role in the outcome of HBV infection but the exact mechanisms involved are still elusive. We know that the NKG2D-induced elimination of infected hepatocytes *via* NKG2DL upregulation can be both protective by inducing viral clearance *via* NK activation early in infection and harmful by leading to susceptibility to liver injury later in infection. A tumor-specific pattern of MICA expression exists in CHB infection. MICA proteins are expressed both as membrane-bound MICA and in soluble form and the shedding of MICA is crucial in NKG2D-mediated responses to tumor. HBV can induce tumor invasion by down-regulating MICA expression on the cell surface of tumor cells and hence weakening NK surveillance while the persistence of high sMICA levels from the early to later stages of chronic infection can result to the initiation of liver cirrhosis and HCC. The NKG2D-MICA interaction may serve as an efficient innate pathway of immune surveillance against HCC and tumor evasion in CHB infection.

NKG2DLs ligands are highly polymorphic. MICA allelic variants bind with variable affinity to NKG2D resulting in differential sMICA shedding and activation of NKG2D-mediated responses. A number of MICA polymorphisms have been identified to be risk factors in HBV-induced HCC occurrence. HBV and its viral products are shown to control the expression of NKG2DLs in HCC development by regulating cellular miRNAs. Since HBV replicates less in tumor tissues than in adjacent non-tumor tissue, is possible that decreased MIC expression in HCC tissues is the consequence of down-regulation of HBV-encoded proteins, such as HBsAg, which can control the expression of the cellular miRNAs. Post-transcriptional regulation of miRNAs has the advantage that NKG2DL are already transcribed and thus, upon infection, they can be rapidly expressed. In human cytomegalovirus (HCMV) infection, immediate-early proteins are able to display histone deacetylases, which induce the transcription of MICA and MICB mRNA[48]. HBV is known to compromise host's epigenetic processes and particularly to induce the recruitment of histone deacetylases onto HBV covalently closed circular DNA minichromosome[49]. Similar to HCMV, is possible that HBV can influence NKG2DL expression post-transcriptionally by modulating the accumulation of histone deacetylases. A detailed characterization of the molecular players that link the HBV stimuli to the transcription of NKG2DL will be critical to advance our knowledge on how HBV circumvents the host's immunity. The manipulation of ligand expression shows many promises therapeutically. Understanding the mechanisms of NKG2D pathways will provide new insight on chronic HBV immunopathogenesis and HCC development and can lead to possibilities of developing effective treatment strategies.

REFERENCES

1 **Cassidy A**, Mossman S, Olivieri A, De Ridder M, Leroux-Roels G. Hepatitis B vaccine effectiveness in the face of global HBV genotype diversity. *Expert Rev Vaccines* 2011; **10**: 1709-1715 [PMID: 22085174 DOI: 10.1586/erv.11.151]

2 **Lupberger J**, Hildt E. Hepatitis B virus-induced oncogenesis. *World J Gastroenterol* 2007; **13**: 74-81 [PMID: 17206756 DOI: 10.3748/wjg.v13.i1.74]

3 **Forner A**, Llovet JM, Bruix J. Hepatocellular carcinoma. *Lancet* 2012; **379**: 1245-1255 [PMID: 22353262 DOI: 10.1016/S0140-6736(11)61347-0]

4 **Fisicaro P**, Valdatta C, Boni C, Massari M, Mori C, Zerbini A, Orlandini A, Sacchelli L, Missale G, Ferrari C. Early kinetics of innate and adaptive immune responses during hepatitis B virus infection. *Gut* 2009; **58**: 974-982 [PMID: 19201769]

5 **Webster GJ**, Reignat S, Maini MK, Whalley SA, Ogg GS, King A, Brown D, Amlot PL, Williams R, Vergani D, Dusheiko GM, Bertoletti A. Incubation phase of acute hepatitis B in man: dynamic of cellular immune mechanisms. *Hepatology* 2000; **32**: 1117-1124 [PMID: 11050064]

6 **Zhao J**, Li Y, Jin L, Zhang S, Fan R, Sun Y, Zhou C, Shang Q, Li W, Zhang Z, Wang FS. Natural killer cells are characterized by the concomitantly increased interferon-γ and cytotoxicity in acute resolved hepatitis B patients. *PLoS One* 2012; **7**: e49135 [PMID: 23133672 DOI: 10.1371/journal.pone.0049135]

7 **Bertoletti A**, Ferrari C. Kinetics of the immune response during HBV and HCV infection. *Hepatology* 2003; **38**: 4-13 [PMID: 12829979 DOI: 10.1053/jhep.2003.50310]

8 **Guidotti LG**, Rochford R, Chung J, Shapiro M, Purcell R, Chisari FV. Viral clearance without destruction of infected cells during acute HBV infection. *Science* 1999; **284**: 825-829 [PMID: 10221919 DOI: 10.1126/science.284.5415.825]

9 **Kajino K**, Jilbert AR, Saputelli J, Aldrich CE, Cullen J, Mason WS. Woodchuck hepatitis virus infections: very rapid recovery after a prolonged viremia and infection of virtually every hepatocyte. *J Virol* 1994; **68**: 5792-5803 [PMID: 7914548]

10 **Li Y**, Wang JJ, Gao S, Liu Q, Bai J, Zhao XQ, Hao YH, Ding HH, Zhu F, Yang DL, Zhao XP. Decreased peripheral natural killer cells activity in the immune activated stage of chronic hepatitis B. *PLoS*

One 2014; **9**: e86927 [PMID: 24520324 DOI: 10.1371/journal.pone.0086927]
11 **Oliviero B**, Varchetta S, Paudice E, Michelone G, Zaramella M, Mavilio D, De Filippi F, Bruno S, Mondelli MU. Natural killer cell functional dichotomy in chronic hepatitis B and chronic hepatitis C virus infections. *Gastroenterology* 2009; **137**: 1151-1160, 1160.e1-7 [PMID: 19470388 DOI: 10.1053/j.gastro.2009.05.047]
12 **Sprengers D**, van der Molen RG, Kusters JG, Hansen B, Niesters HG, Schalm SW, Janssen HL. Different composition of intrahepatic lymphocytes in the immune-tolerance and immune-clearance phase of chronic hepatitis B. *J Med Virol* 2006; **78**: 561-568 [PMID: 16555293 DOI: 10.1002/jmv.20576]
13 **Dunn C**, Brunetto M, Reynolds G, Christophides T, Kennedy PT, Lampertico P, Das A, Lopes AR, Borrow P, Williams K, Humphreys E, Afford S, Adams DH, Bertoletti A, Maini MK. Cytokines induced during chronic hepatitis B virus infection promote a pathway for NK cell-mediated liver damage. *J Exp Med* 2007; **204**: 667-680 [PMID: 17353365 DOI: 10.1084/jem.20061287]
14 **Zou Z**, Xu D, Li B, Xin S, Zhang Z, Huang L, Fu J, Yang Y, Jin L, Zhao JM, Shi M, Zhou G, Sun Y, Wang FS. Compartmentalization and its implication for peripheral immunologically-competent cells to the liver in patients with HBV-related acute-on-chronic liver failure. *Hepatol Res* 2009; **39**: 1198-1207 [PMID: 19788691 DOI: 10.1111/j.1872-034X.2009.00571.x]
15 **Bertoletti A**, Maini MK. Protection or damage: a dual role for the virus-specific cytotoxic T lymphocyte response in hepatitis B and C infection? *Curr Opin Microbiol* 2000; **3**: 387-392 [PMID: 10972499 DOI: 10.1016/S0952-7915(00)00108-4]
16 **Peppa D**, Gill US, Reynolds G, Easom NJ, Pallett LJ, Schurich A, Micco L, Nebbia G, Singh HD, Adams DH, Kennedy PT, Maini MK. Up-regulation of a death receptor renders antiviral T cells susceptible to NK cell-mediated deletion. *J Exp Med* 2013; **210**: 99-114 [PMID: 23254287 DOI: 10.1084/jem.20121172]
17 **Baron JL**, Gardiner L, Nishimura S, Shinkai K, Locksley R, Ganem D. Activation of a nonclassical NKT cell subset in a transgenic mouse model of hepatitis B virus infection. *Immunity* 2002; **16**: 583-594 [PMID: 11970881 DOI: 10.1016/S1074-7613(02)00305-9]
18 **Vilarinho S**, Ogasawara K, Nishimura S, Lanier LL, Baron JL. Blockade of NKG2D on NKT cells prevents hepatitis and the acute immune response to hepatitis B virus. *Proc Natl Acad Sci USA* 2007; **104**: 18187-18192 [PMID: 17991774 DOI: 10.1073/pnas.0708968104]
19 **Groh V**, Bruhl A, El-Gabalawy H, Nelson JL, Spies T. Stimulation of T cell autoreactivity by anomalous expression of NKG2D and its MIC ligands in rheumatoid arthritis. *Proc Natl Acad Sci USA* 2003; **100**: 9452-9457 [PMID: 12878725 DOI: 10.1073/pnas.1632807100]
20 **Ogasawara K**, Hamerman JA, Ehrlich LR, Bour-Jordan H, Santamaria P, Bluestone JA, Lanier LL. NKG2D blockade prevents autoimmune diabetes in NOD mice. *Immunity* 2004; **20**: 757-767 [PMID: 15189740 DOI: 10.1016/j.immuni.2004.05.008]
21 **Cerwenka A**, Bakker AB, McClanahan T, Wagner J, Wu J, Phillips JH, Lanier LL. Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. *Immunity* 2000; **12**: 721-727 [PMID: 10894171 DOI: 10.1016/S1074-7613(00)80222-8]
22 **Wu J**, Groh V, Spies T. T cell antigen receptor engagement and specificity in the recognition of stress-inducible MHC class I-related chains by human epithelial gamma delta T cells. *J Immunol* 2002; **169**: 1236-1240 [PMID: 12133944]
23 **Lanier LL**. NK cell recognition. *Annu Rev Immunol* 2005; **23**: 225-274 [PMID: 15771571]
24 **Stern-Ginossar N**, Mandelboim O. An integrated view of the regulation of NKG2D ligands. *Immunology* 2009; **128**: 1-6 [PMID: 19689730 DOI: 10.1111/j.1365-2567.2009.03147.x]
25 **Gasser S**, Orsulic S, Brown EJ, Raulet DH. The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. *Nature* 2005; **436**: 1186-1190 [PMID: 15995699 DOI: 10.1038/nature03884]
26 **Kahraman A**, Fingas CD, Syn WK, Gerken G, Canbay A. Role of stress-induced NKG2D ligands in liver diseases. *Liver Int* 2012; **32**: 370-382 [PMID: 22097967 DOI: 10.1111/j.1478-3231.2011.02608.x]
27 **Nedvetzki S**, Sowinski S, Eagle RA, Harris J, Vély F, Pende D, Trowsdale J, Vivier E, Gordon S, Davis DM. Reciprocal regulation of human natural killer cells and macrophages associated with distinct immune synapses. *Blood* 2007; **109**: 3776-3785 [PMID: 17218381 DOI: 10.1182/blood-2006-10-052977]
28 **Ebihara T**, Masuda H, Akazawa T, Shingai M, Kikuta H, Ariga T, Matsumoto M, Seya T. Induction of NKG2D ligands on human dendritic cells by TLR ligand stimulation and RNA virus infection. *Int Immunol* 2007; **19**: 1145-1155 [PMID: 17878262 DOI: 10.1093/intimm/dxm073]
29 **Tong HV**, Toan NL, Song LH, Bock CT, Kremsner PG, Velavan TP. Hepatitis B virus-induced hepatocellular carcinoma: functional roles of MICA variants. *J Viral Hepat* 2013; **20**: 687-698 [PMID: 24010643 DOI: 10.1111/jvh.12089]
30 **Jinushi M**, Takehara T, Tatsumi T, Hiramatsu N, Sakamori R, Yamaguchi S, Hayashi N. Impairment of natural killer cell and dendritic cell functions by the soluble form of MHC class I-related chain A in advanced human hepatocellular carcinomas. *J Hepatol* 2005; **43**: 1013-1020 [PMID: 16168521 DOI: 10.1016/j.jhep.2005.05.026]
31 **Kennedy PT**, Gehring AJ, Nowbath A, Selden C, Quaglia A, Dhillon A, Dusheiko G, Bertoletti A. The expression and function of NKG2D molecule on intrahepatic CD8+ T cells in chronic viral hepatitis. *J Viral Hepat* 2008; **15**: 901-909 [PMID: 19087227 DOI: 10.1111/j.1365-2893.2008.01049.x]
32 **Sun C**, Fu B, Gao Y, Liao X, Sun R, Tian Z, Wei H. TGF-β1 down-regulation of NKG2D/DAP10 and 2B4/SAP expression on human NK cells contributes to HBV persistence. *PLoS Pathog* 2012; **8**: e1002594 [PMID: 22438812 DOI: 10.1371/journal.ppat.1002594]
33 **Radaeva S**, Sun R, Jaruga B, Nguyen VT, Tian Z, Gao B. Natural killer cells ameliorate liver fibrosis by killing activated stellate cells in NKG2D-dependent and tumor necrosis factor-related apoptosis-inducing ligand-dependent manners. *Gastroenterology* 2006; **130**: 435-452 [PMID: 16472598 DOI: 10.1053/j.gastro.2005.10.055]
34 **Chen Y**, Wei H, Sun R, Dong Z, Zhang J, Tian Z. Increased susceptibility to liver injury in hepatitis B virus transgenic mice involves NKG2D-ligand interaction and natural killer cells. *Hepatology* 2007; **46**: 706-715 [PMID: 17626270 DOI: 10.1002/hep.21872]
35 **Groh V**, Rhinehart R, Secrist H, Bauer S, Grabstein KH, Spies T. Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. *Proc Natl Acad Sci USA* 1999; **96**: 6879-6884 [PMID: 10359807 DOI: 10.1073/pnas.96.12.6879]
36 **Sène D**, Levasseur F, Abel M, Lambert M, Camous X, Hernandez C, Pène V, Rosenberg AR, Jouvin-Marche E, Marche PN, Cacoub P, Caillat-Zucman S. Hepatitis C virus (HCV) evades NKG2D-dependent NK cell responses through NS5A-mediated imbalance of inflammatory cytokines. *PLoS Pathog* 2010; **6**: e1001184 [PMID: 21085608 DOI: 10.1371/journal.ppat.1001184]
37 **Chen CJ**, Yang HI, Su J, Jen CL, You SL, Lu SN, Huang GT, Iloeje UH. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA* 2006; **295**: 65-73 [PMID: 16391218 DOI: 10.1001/jama.295.1.65]
38 **Tang KF**, Chen M, Xie J, Song GB, Shi YS, Liu Q, Mei ZC, Steinle A, Ren H. Inhibition of hepatitis B virus replication by small interference RNA induces expression of MICA in HepG2.2.15 cells. *Med Microbiol Immunol* 2009; **198**: 27-32 [PMID: 18685862 DOI: 10.1007/s00430-008-0101-6]
39 **Wu J**, Zhang XJ, Shi KQ, Chen YP, Ren YF, Song YJ, Li G, Xue YF, Fang YX, Deng ZJ, Xu X, Gao J, Tang KF. Hepatitis B surface antigen inhibits MICA and MICB expression via induction of cellular miRNAs in hepatocellular carcinoma cells. *Carcinogenesis* 2014; **35**: 155-163 [PMID: 23917076 DOI: 10.1093/carcin/bgt268]
40 **Doubrovina ES**, Doubrovin MM, Vider E, Sisson RB, O'Reilly RJ, Dupont B, Vyas YM. Evasion from NK cell immunity by MHC class I chain-related molecules expressing colon adenocarcinoma. *J Immunol* 2003; **171**: 6891-6899 [PMID: 14662896]
41 **Karacki PS**, Gao X, Thio CL, Thomas DL, Goedert JJ, Vlahov D,

Kaslow RA, Strathdee S, Hilgartner MW, O'Brien SJ, Carrington M. MICA and recovery from hepatitis C virus and hepatitis B virus infections. *Genes Immun* 2004; **5**: 261-266 [PMID: 15029237 DOI: 10.1038/sj.gene.6364065]

42 **Steinle A**, Li P, Morris DL, Groh V, Lanier LL, Strong RK, Spies T. Interactions of human NKG2D with its ligands MICA, MICB, and homologs of the mouse RAE-1 protein family. *Immunogenetics* 2001; **53**: 279-287 [PMID: 11491531 DOI: 10.1007/s002510100325]

43 **Kumar V**, Kato N, Urabe Y, Takahashi A, Muroyama R, Hosono N, Otsuka M, Tateishi R, Omata M, Nakagawa H, Koike K, Kamatani N, Kubo M, Nakamura Y, Matsuda K. Genome-wide association study identifies a susceptibility locus for HCV-induced hepatocellular carcinoma. *Nat Genet* 2011; **43**: 455-458 [PMID: 21499248 DOI: 10.1038/ng.809]

44 **Stern-Ginossar N**, Gur C, Biton M, Horwitz E, Elboim M, Stanietsky N, Mandelboim M, Mandelboim O. Human microRNAs regulate stress-induced immune responses mediated by the receptor NKG2D. *Nat Immunol* 2008; **9**: 1065-1073 [PMID: 18677316 DOI: 10.1038/ni.1642]

45 **Yadav D**, Ngolab J, Lim RS, Krishnamurthy S, Bui JD. Cutting edge: down-regulation of MHC class I-related chain A on tumor cells by IFN-gamma-induced microRNA. *J Immunol* 2009; **182**: 39-43 [PMID: 19109132]

46 **Kong G**, Zhang J, Zhang S, Shan C, Ye L, Zhang X. Upregulated microRNA-29a by hepatitis B virus X protein enhances hepatoma cell migration by targeting PTEN in cell culture model. *PLoS One* 2011; **6**: e19518 [PMID: 21573166]

47 **Ura S**, Honda M, Yamashita T, Ueda T, Takatori H, Nishino R, Sunakozaka H, Sakai Y, Horimoto K, Kaneko S. Differential microRNA expression between hepatitis B and hepatitis C leading disease progression to hepatocellular carcinoma. *Hepatology* 2009; **49**: 1098-1112 [PMID: 19173277]

48 **van Zonneveld M**, Zondervan PE, Cakaloglu Y, Simon C, Akarca US, So TM, Flink HJ, de Man RA, Schalm SW, Janssen HL. Peg-interferon improves liver histology in patients with HBeAg-positive chronic hepatitis B: no additional benefit of combination with lamivudine. *Liver Int* 2006; **26**: 399-405 [PMID: 16629642]

49 **Pollicino T**, Belloni L, Raffa G, Pediconi N, Squadrito G, Raimondo G, Levrero M. Hepatitis B virus replication is regulated by the acetylation status of hepatitis B virus cccDNA-bound H3 and H4 histones. *Gastroenterology* 2006; **130**: 823-837 [PMID: 16530522]

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EDITORIAL

Hepatitis B virus reactivation during immunosuppressive therapy: Appropriate risk stratification

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Abstract

Our understanding of hepatitis B virus (HBV) reactivation during immunosuppresive therapy has increased remarkably during recent years. HBV reactivation in hepatitis B surface antigen (HBsAg)-positive individuals has been well-described in certain immunosuppressive regimens, including therapies containing corticosteroids, anthracyclines, rituximab, antibody to tumor necrosis factor (anti-TNF) and hematopoietic stem cell transplantation (HSCT). HBV reactivation could also occur in HBsAg-negative, antibody to hepatitis B core antigen (anti-HBc) positive individuals during therapies containing rituximab, anti-TNF or HSCT.For HBsAg-positive patients, prophylactic antiviral therapy is proven to the effective in preventing HBV reactivation. Recent evidence also demonstrated entecavir to be more effective than lamivudine in this aspect. For HBsAg-negative, anti-HBc positive individuals, the risk of reactivations differs with the type of immunosuppression. For rituximab, a prospective study demonstrated the 2-year cumulative risk of reactivation to be 41.5%, but prospective data is still lacking for other immunosupressive regimes. The optimal management in preventing HBV reactivation would involve appropriate risk stratification for different immunosuppressive regimes in both HBsAg-positive and HBsAg-negative, anti-HBc positive individuals.

Key words: Hepatitis B virus; Antibody to hepatitis B core antigen; Hepatitis B surface antigen; Rituximab; Antigen CD20; Hematopoietic stem cell transplantation; Antibody to tumor necrosis factor; Occult

Core tip: Hepatitis B virus (HBV) reactivation not only occurs in hepatitis B surface antigen (HBsAg)-positive, but also in HBsAg-negative, antibody to hepatitis B core antigen positive individuals. Immunosuppressive therapies with increased risk of HBV reactivation include corticosteroids, anthracyclines, rituximab, antibody to tumor necrosis factor and hematopoietic stem cell transplantation. The decision between prophylactic antiviral therapy *vs* routine clinical monitoring would involve appropriate risk stratification for individual types of immunosuppressives regimes.

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INTRODUCTION

The introduction of nucleoside analogue therapy has revolutionized the management of chronic hepatitis B (CHB). The current first-line therapies of entecavir and tenofovir, if taken long-term, can bring about potent virologic suppression[1], improve liver histology[2,3], and reduce cirrhotic complications[4,5], with low risk of resistance development[6,7]. Nonetheless, the efficacy of nucleoside analogue therapy remains suboptimal in one distinct clinical entity: hepatitis B virus (HBV)-related acute-on-chronic liver failure[8], in which the 3-mo survival rates were only 40%-57%[9,10]. Reactivation of HBV during immunosuppressive therapy, if caught unaware, could present as acute-on-chronic liver failure, signifying the importance of management strategies directed towards preventing HBV reactivation.

The dangers of HBV reactivation are not only limited to hepatitis B surface antigen (HBsAg)-positive patients, but could also involve HBsAg-negative, antibody to hepatitis B core antigen (anti-HBc) positive individuals. Unfortunately, despite the accumulating evidence in this field, the global oncology community remains divided on the need of routine screening of HBV serology prior to immunosuppressive therapy[11,12]. This editorial aims to provide a literature update as well as management recommendations for preventing and controlling HBV reactivation during immunosuppressive therapy.

IMMUNOSUPPRESSIVE THERAPIES WITH INCREASED RISK OF HBV REACTIVATION

Not all immunosuppressive therapies have been proven to be associated with HBV reactivation - the association is in fact limited to a selected few regimens. Corticosteroids is a well-known risk factor, in which the presence of prednisolone in chemotherapy regimens for HBsAg-positive lymphoma patients would increase the risk of HBV reactivation by 36%[13]. HBV reactivation is also possible in patients treated with steroids for non-malignant conditions, especially when the therapy duration is at least 3 mo or when the steroid dose is equivalent to 20 mg of prednisolone per day[14]. The monoclonal antibodies against B cell surface antigen CD20 (anti-CD20), rituximab and ofatumumab, could also enhance the chances of HBV reactivation, with rituximab resulting in more than five-fold increase[15]. More importantly, HBV reactivation could occur up to one year or more after cessation of rituximab[16,17].

Other biologics, including monoclonal antibodies against tumor necrosis factor (anti-TNF), *e.g.*, infliximab, adalimumab and etanercept have been demonstrated to have a 35% HBV reactivation rate in HBsAg-positive patients with rheumatoid arthritis, inflammatory bowel disease and other non-malignant conditions[18]. Other agents proven to increase the risk for HBV reactivation include transarterial chemo-embolization (TACE) for hepatocellular carcinoma[19], hematopoietic stem cell transplantation (HSCT)[20], methotrexate[21], anthracyclines[22], and other biologic agents including tyrosine kinase inhibitors[23] and ustekinumab[24] (Table 1).

HBSAG-NEGATIVE, ANTI-HBC POSITIVE HBV REACTIVATION

HBV reactivation is also possible in HBsAg-negative individuals who have occult HBV infection - defined as HBsAg-negativity but with detectable HBV DNA in serum or liver[25]. Such individuals could had CHB, achieved HBsAg seroclearance, but with intrahepatic HBV DNA remaining[26]. They may or may not possess serum antibody to the hepatitis B surface antibody (anti-HBs), with the only positive serologic marker being anti-HBc, indicating past HBV exposure.

HBV reactivation in HBsAg-negative, anti-HBc positive patients has been extensively reported in rituximab-containing chemotherapy (Table 2). Previous retrospective studies reported a reactivation rate of 8.9% to 23.8%[17,27,28]. This large variability could be due to the lack of regular serologic and virologic monitoring, with only HBV reactivation noted when biochemical hepatitis (a late event) occurred. Two recent prospective studies described the risk of HBV reactivation in better detail. The first, when using multiple virologic endpoints, found the rate of reactivation to vary from 11.3% to 18.9%[29]. The second, when defining detectable HBV DNA (> 10 IU/mL) as HBV reactivation, found the cumulative 2-year reactivation rate to be 41.5%. This second study also found patients with negative anti-HBs to have a higher cumulative rate of reactivation than those with positive anti-HBs (68% *vs* 34% at 2 years respectively)[16]. Patients with detectable HBV DNA all responded well to entecavir, with no cases of hepatitic flares.

HBV reactivation has also been reported in HBsAg-negative anti-HBc-positive patients undergoing HSCT. Retrospective studies again found variable rates of reactivation (8.9% to 19.7%)[30-32], again limited by the lack of routine clinical monitoring. Nonetheless, HBV reactivation could occur many months (up to 47 mo) after HSCT, indicating prolonged clinical monitoring would be needed post-HSCT to ensure early detection of HBV reactivation. The preliminary results of an ongoing prospective study found HBsAg-negative, anti-HBc positive HSCT recipients developing graft-*vs*-host disease to have an increased chance of HBV reactivation[33] - these results would need further validation.

Anti-TNF therapy could also increase the risk of

Table 1 Immunosuppressive regimens known to increase risk of hepatitis B virus reactivation

HBsAg-positive	HBsAg-negative Anti-HBc positive
Corticosteroids	Anti-CD20 (*e.g.*, rituximab)
Anti-CD20 (*e.g.*, rituximab)	HSCT
HSCT	Anti-TNF
Anti-TNF	TACE for hepatocellular carcinoma
Anthracyclines	Methotrexate
TACE for hepatocellular carcinoma	
Methotrexate	
Ustekinumab	
Tyrosine kinase inhibitors	

HBsAg: Hepatitis B surface antigen; Anti-HBc: Antibody to hepatitis B core antigen; Anti-CD20: Antibody against CD20; Anti-TNF: Antibody against tumor necrosis factor; TACE: Transarterial chemo-embolization; HSCT: Hematopoietic stem cell transplantation.

HBV reactivation in HBsAg-negative, anti-HBc positive patients, although when compared to HBsAg-positive patients, reactivation rates were much lower (1.7% to 5%)[18,34]. Other regimens known to be associated with HBV reactivation among HBsAg-negative, anti-HBc positive patients include TACE for hepatocellular carcinoma[35] and methotrexate[21] (Table 1).

RECOMMENDED STRATEGY TO MANAGE HBV REACTIVATION

Screening for HBsAg and anti-HBc prior to immunosuppressive therapy

Despite the lack of consensus among the global oncology community[11,12], current guidelines from international liver societies[36,37] recommend mandatory screening for serum HBsAg and anti-HBc prior to all forms of immunosuppressive therapy. Screening would be particularly important in HBV-endemic regions, and is cost-effective[38].

Prophylactic nucleoside analogue therapy for HBsAg-positive individuals

The provision of concomitant nucleoside analogue therapy at the commencement of immunosuppression has been demonstrated to be effective in reducing the risk of HBV reactivation for both hematological malignancies and solid-organ tumors[39]. For most immunosuppressive regimes, nucleoside analogue therapy should be kept until at least 6 mo after the last dose of immunosuppressive therapy. The exception is rituximab, with nucleoside analogue therapy continued until at least 12 mo after completion of rituximab-containing chemotherapy[15].

In terms of the choice of nucleoside analogue for prophylactic therapy, lamivudine has been used most extensively, achieving a 79% risk reduction on HBV reactivation[40]. The disadvantage of lamivudine is its low genetic barrier to resistance[4], such that it is no longer a recommended first-line treatment for CHB. Hence, the two first-line therapies, *i.e.*, entecavir or tenofovir, both with a high genetic barrier to resistance should be used instead[6,7]. This is supported by a recent randomized controlled trial demonstrating entecavir to be superior to lamivudine in the prevention of HBV reactivation among HBsAg-positive individuals undergoing rituximab-containing chemotherapy[41].

Prophylactic nucleoside analogue therapy with a finite therapy duration should be only for CHB patients with quiescent disease, as indicated by baseline HBV DNA < 2000 IU/mL. For CHB patients with baseline HBV DNA ⩾ 2000 IU/mL, long-term nucleoside analogue therapy should be considered to reduce the risk of liver-related complications associated with high viral loads[42,43].

Monitoring HBsAg-negative, anti-HBc positive individuals

Defining the optimal management strategy for HBsAg-negative, anti-HBc positive individuals is more difficult. A randomized controlled trial did demonstrate the efficacy of prophylactic nucleoside analogue therapy during rituximab-containing chemotherapy[44]. Nonetheless, within the HBsAg-negative anti-HBc positive population, HBV reactivation seemed to occur only among specific immunosuppressive regimes (Table 1). The risk of HBV reactivation among certain therapies is also low (*e.g.*, < 5% during anti-TNF therapy). Another factor to consider is the seroprevalence of anti-HBc, which could be > 40% among HBV-endemic regions in East Asia[45,46]. Hence, the universal provision of prophylactic nucleoside analogue therapy for all immunosuppressive regimens might not be cost-effective.

Currently, the regular monitoring of serum HBV DNA would probably be the preferred strategy. The optimal interval of monitoring is uncertain - a suggestion would be for every 1-3 mo[36], although there is no high-quality data to support this. Prophylactic nucleoside analogue therapy can still be considered for specific population groups, *e.g.*, anti-HBs negative patients undergoing rituximab-containing chemotherapy[16].

FUTURE DIRECTIONS

More studies would be needed for risk stratification. Can the current data in HBsAg-positive patients be extrapolated to all forms of immunosuppressive therapy? There is a paucity of data concerning HBV reactivation among traditional immunomodulators, *e.g.*, azathioprine, thalidomide or methotrexate. Other immunosuppressive agents lacking HBV reactivation data include non-steroid or anthracycline-containing chemotherapeutic regimens, monoclonal antibodies other than anti-CD20 or anti-TNF, epidermal growth factor receptor inhibitors and proteasome inhibitors. If current guidelines continue to emphasize prophylactic HBV therapy for all forms of immunosuppression, then cost-effective studies would be

Table 2 Rates of hepatitis B virus reactivation during rituximab-containing chemotherapy in hepatitis B surface antigen-negative, antibody to hepatitis B core antigen positive individuals as described by various studies

Study region	Study nature	No. of patients	HBV reactivation rate	Definition of HBV reactivation
Hong Kong[17]	Retrospective	23	23.8%	HBsAg seroreversion
Japan[27]	Retrospective	56	8.9%	HBsAg seroreversion
Asia-Pacific[28]	Retrospective	178	9.6%	HBsAg seroreversion
Taiwan[29]	Prospective	150	11.3%-18.9%	Multiple virologic endpoints
Hong Kong[16]	Prospective	63	41.5%	Detectable HBV DNA

HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus.

needed to justify their usage.

For HBsAg-negative, anti-HBc positive individuals undergoing rituximab-containing chemotherapy, the discrepancies in reactivation rates from previous studies (Table 2) could indicate not all cases of HBV reactivation, when defined as detectable serum HBV DNA, would end up being clinically relevant. In addition, prospective data is still needed to clearly define the risk of HBV reactivation among HSCT and anti-TNF therapy, as well as to identify additional risk factors besides anti-HBs status.

Hopefully, future studies in these directions would help in stratifying the risk of HBV reactivation among different immunosuppressive regimes and improve disease outcomes of HBV-infected individuals undergoing immunosuppressive therapy.

REFERENCES

1 **Yuen MF**, Seto WK, Fung J, Wong DK, Yuen JC, Lai CL. Three years of continuous entecavir therapy in treatment-naïve chronic hepatitis B patients: VIRAL suppression, viral resistance, and clinical safety. *Am J Gastroenterol* 2011; **106**: 1264-1271 [PMID: 21364549 DOI: 10.1038/ajg.2011.45]

2 **Marcellin P**, Gane E, Buti M, Afdhal N, Sievert W, Jacobson IM, Washington MK, Germanidis G, Flaherty JF, Schall RA, Bornstein JD, Kitrinos KM, Subramanian GM, McHutchison JG, Heathcote EJ. Regression of cirrhosis during treatment with tenofovir disoproxil fumarate for chronic hepatitis B: a 5-year open-label follow-up study. *Lancet* 2013; **381**: 468-475 [PMID: 23234725 DOI: 10.1016/S0140-6736(12)61425-1]

3 **Chang TT**, Liaw YF, Wu SS, Schiff E, Han KH, Lai CL, Safadi R, Lee SS, Halota W, Goodman Z, Chi YC, Zhang H, Hindes R, Iloeje U, Beebe S, Kreter B. Long-term entecavir therapy results in the reversal of fibrosis/cirrhosis and continued histological improvement in patients with chronic hepatitis B. *Hepatology* 2010; **52**: 886-893 [PMID: 20683932 DOI: 10.1002/hep.23785]

4 **Yuen MF**, Seto WK, Chow DH, Tsui K, Wong DK, Ngai VW, Wong BC, Fung J, Yuen JC, Lai CL. Long-term lamivudine therapy reduces the risk of long-term complications of chronic hepatitis B infection even in patients without advanced disease. *Antivir Ther* 2007; **12**: 1295-1303 [PMID: 18240869]

5 **Wong GL**, Chan HL, Mak CW, Lee SK, Ip ZM, Lam AT, Iu HW, Leung JM, Lai JW, Lo AO, Chan HY, Wong VW. Entecavir treatment reduces hepatic events and deaths in chronic hepatitis B patients with liver cirrhosis. *Hepatology* 2013; **58**: 1537-1547 [PMID: 23389810 DOI: 10.1002/hep.26301]

6 **Tenney DJ**, Rose RE, Baldick CJ, Pokornowski KA, Eggers BJ, Fang J, Wichroski MJ, Xu D, Yang J, Wilber RB, Colonno RJ. Long-term monitoring shows hepatitis B virus resistance to entecavir in nucleoside-naïve patients is rare through 5 years of therapy. *Hepatology* 2009; **49**: 1503-1514 [PMID: 19280622 DOI: 10.1002/hep.22841]

7 **Kitrinos KM**, Corsa A, Liu Y, Flaherty J, Snow-Lampart A, Marcellin P, Borroto-Esoda K, Miller MD. No detectable resistance to tenofovir disoproxil fumarate after 6 years of therapy in patients with chronic hepatitis B. *Hepatology* 2014; **59**: 434-442 [PMID: 23939953 DOI: 10.1002/hep.26686]

8 **Seto WK**, Lai CL, Yuen MF. Acute-on-chronic liver failure in chronic hepatitis B. *J Gastroenterol Hepatol* 2012; **27**: 662-669 [PMID: 22098452 DOI: 10.1111/j.1440-1746.2011.06971.x]

9 **Cui YL**, Yan F, Wang YB, Song XQ, Liu L, Lei XZ, Zheng MH, Tang H, Feng P. Nucleoside analogue can improve the long-term prognosis of patients with hepatitis B virus infection-associated acute on chronic liver failure. *Dig Dis Sci* 2010; **55**: 2373-2380 [PMID: 20512414 DOI: 10.1007/s10620-010-1257-7]

10 **Garg H**, Sarin SK, Kumar M, Garg V, Sharma BC, Kumar A. Tenofovir improves the outcome in patients with spontaneous reactivation of hepatitis B presenting as acute-on-chronic liver failure. *Hepatology* 2011; **53**: 774-780 [PMID: 21294143 DOI: 10.1002/hep.24109]

11 **Tan D**, Tan SY, Lim ST, Kim SJ, Kim WS, Advani R, Kwong YL. Management of B-cell non-Hodgkin lymphoma in Asia: resource-stratified guidelines. *Lancet Oncol* 2013; **14**: e548-e561 [PMID: 24176573]

12 **Artz AS**, Somerfield MR, Feld JJ, Giusti AF, Kramer BS, Sabichi AL, Zon RT, Wong SL. American Society of Clinical Oncology provisional clinical opinion: chronic hepatitis B virus infection screening in patients receiving cytotoxic chemotherapy for treatment of malignant diseases. *J Clin Oncol* 2010; **28**: 3199-3202 [PMID: 20516452 DOI: 10.1200/JCO.2010.30.0673]

13 **Cheng AL**, Hsiung CA, Su IJ, Chen PJ, Chang MC, Tsao CJ, Kao WY, Uen WC, Hsu CH, Tien HF, Chao TY, Chen LT, Whang-Peng J. Steroid-free chemotherapy decreases risk of hepatitis B virus (HBV) reactivation in HBV-carriers with lymphoma. *Hepatology* 2003; **37**: 1320-1328 [PMID: 12774010 DOI: 10.1053/jhep.2003.50220]

14 **Lai CL**, Yuen MF. Systemic corticosteroid and reactivation of chronic hepatitis B. *Respirology* 2010; **15**: 1017-1018 [PMID: 20874743 DOI: 10.1111/j.1440-1843.2010.01822.x]

15 **Evens AM**, Jovanovic BD, Su YC, Raisch DW, Ganger D, Belknap SM, Dai MS, Chiu BC, Fintel B, Cheng Y, Chuang SS, Lee MY, Chen TY, Lin SF, Kuo CY. Rituximab-associated hepatitis B virus (HBV) reactivation in lymphoproliferative diseases: meta-analysis and examination of FDA safety reports. *Ann Oncol* 2011; **22**: 1170-1180 [PMID: 21115603 DOI: 10.1093/annonc/mdq583]

16 **Seto WK**, Chan TS, Hwang YY, Wong DK, Fung J, Liu KS, Gill H, Lam YF, Lie AK, Lai CL, Kwong YL, Yuen MF. Hepatitis B reactivation in patients with previous hepatitis B virus exposure undergoing rituximab-containing chemotherapy for lymphoma: a prospective study. *J Clin Oncol* 2014; **32**: 3736-3743 [PMID: 25287829 DOI: 10.1200/JCO.2014.56.7081]

17 **Yeo W**, Chan TC, Leung NW, Lam WY, Mo FK, Chu MT, Chan HL, Hui EP, Lei KI, Mok TS, Chan PK. Hepatitis B virus reactivation in lymphoma patients with prior resolved hepatitis B undergoing anticancer therapy with or without rituximab. *J Clin Oncol* 2009; **27**: 605-611 [PMID: 19075267 DOI: 10.1200/JCO.2008.18.0182]

18 **Pérez-Alvarez R**, Díaz-Lagares C, García-Hernández F, Lopez-Roses L, Brito-Zerón P, Pérez-de-Lis M, Retamozo S, Bové A, Bosch X, Sanchez-Tapias JM, Forns X, Ramos-Casals M. Hepatitis B virus (HBV) reactivation in patients receiving tumor necrosis factor (TNF)-targeted therapy: analysis of 257 cases. *Medicine* (Baltimore) 2011; **90**: 359-371 [PMID: 22033451 DOI: 10.1097/MD.0b013e3182380a76]

19 **Jang JW**, Choi JY, Bae SH, Kim CW, Yoon SK, Cho SH, Yang JM, Ahn BM, Lee CD, Lee YS, Chung KW, Sun HS. Transarterial chemo-lipiodolization can reactivate hepatitis B virus replication in patients with hepatocellular carcinoma. *J Hepatol* 2004; **41**: 427-435 [PMID: 15336446 DOI: 10.1016/j.jhep.2004.05.014]

20 **Locasciulli A**, Bruno B, Alessandrino EP, Meloni G, Arcese W, Bandini G, Cassibba V, Rotoli B, Morra E, Majolino I, Alberti A, Bacigalupo A. Hepatitis reactivation and liver failure in haemopoietic stem cell transplants for hepatitis B virus (HBV)/hepatitis C virus (HCV) positive recipients: a retrospective study by the Italian group for blood and marrow transplantation. *Bone Marrow Transplant* 2003; **31**: 295-300 [PMID: 12621466 DOI: 10.1038/sj.bmt.1703826]

21 **Tamori A**, Koike T, Goto H, Wakitani S, Tada M, Morikawa H, Enomoto M, Inaba M, Nakatani T, Hino M, Kawada N. Prospective study of reactivation of hepatitis B virus in patients with rheumatoid arthritis who received immunosuppressive therapy: evaluation of both HBsAg-positive and HBsAg-negative cohorts. *J Gastroenterol* 2011; **46**: 556-564 [PMID: 21246383 DOI: 10.1007/s00535-010-0367-5]

22 **Ling WH**, Soe PP, Pang AS, Lee SC. Hepatitis B virus reactivation risk varies with different chemotherapy regimens commonly used in solid tumours. *Br J Cancer* 2013; **108**: 1931-1935 [PMID: 23652302 DOI: 10.1038/bjc.2013.225]

23 **Lai GM**, Yan SL, Chang CS, Tsai CY. Hepatitis B reactivation in chronic myeloid leukemia patients receiving tyrosine kinase inhibitor. *World J Gastroenterol* 2013; **19**: 1318-1321 [PMID: 23483799 DOI: 10.3748/wjg.v19.i8.1318]

24 **Navarro R**, Vilarrasa E, Herranz P, Puig L, Bordas X, Carrascosa JM, Taberner R, Ferrán M, García-Bustinduy M, Romero-Maté A, Pedragosa R, García-Diez A, Daudén E. Safety and effectiveness of ustekinumab and antitumour necrosis factor therapy in patients with psoriasis and chronic viral hepatitis B or C: a retrospective, multicentre study in a clinical setting. *Br J Dermatol* 2013; **168**: 609-616 [PMID: 22985451 DOI: 10.1111/bjd.12045]

25 **Raimondo G**, Pollicino T, Cacciola I, Squadrito G. Occult hepatitis B virus infection. *J Hepatol* 2007; **46**: 160-170 [PMID: 17112622 DOI: 10.1016/j.jhep.2006.10.007]

26 **Yuen MF**, Wong DK, Fung J, Ip P, But D, Hung I, Lau K, Yuen JC, Lai CL. HBsAg Seroclearance in chronic hepatitis B in Asian patients: replicative level and risk of hepatocellular carcinoma. *Gastroenterology* 2008; **135**: 1192-1199 [PMID: 18722377 DOI: 10.1053/j.gastro.2008.07.008]

27 **Matsue K**, Kimura S, Takanashi Y, Iwama K, Fujiwara H, Yamakura M, Takeuchi M. Reactivation of hepatitis B virus after rituximab-containing treatment in patients with CD20-positive B-cell lymphoma. *Cancer* 2010; **116**: 4769-4776 [PMID: 20597091 DOI: 10.1002/cncr.25253]

28 **Kim SJ**, Hsu C, Song YQ, Tay K, Hong XN, Cao J, Kim JS, Eom HS, Lee JH, Zhu J, Chang KM, Reksodiputro AH, Tan D, Goh YT, Lee J, Intragumtornchai T, Chng WJ, Cheng AL, Lim ST, Suh C, Kwong YL, Kim WS. Hepatitis B virus reactivation in B-cell lymphoma patients treated with rituximab: analysis from the Asia Lymphoma Study Group. *Eur J Cancer* 2013; **49**: 3486-3496 [PMID: 23910494 DOI: 10.1016/j.ejca.2013.07.006]

29 **Hsu C**, Tsou HH, Lin SJ, Wang MC, Yao M, Hwang WL, Kao WY, Chiu CF, Lin SF, Lin J, Chang CS, Tien HF, Liu TW, Chen PJ, Cheng AL. Chemotherapy-induced hepatitis B reactivation in lymphoma patients with resolved HBV infection: a prospective study. *Hepatology* 2014; **59**: 2092-2100 [PMID: 24002804 DOI: 10.1002/hep.26718]

30 **Hammond SP**, Borchelt AM, Ukomadu C, Ho VT, Baden LR, Marty FM. Hepatitis B virus reactivation following allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* 2009; **15**: 1049-1059 [PMID: 19660717 DOI: 10.1016/j.bbmt.2009.05.001]

31 **Ceneli O**, Ozkurt ZN, Acar K, Rota S, Aki SZ, Yeğin ZA, Yağci M, Ozenirler S, Sucak GT. Hepatitis B-related events in autologous hematopoietic stem cell transplantation recipients. *World J Gastroenterol* 2010; **16**: 1765-1771 [PMID: 20380010 DOI: 10.3748/wjg.v16.i14.1765]

32 **Viganò M**, Vener C, Lampertico P, Annaloro C, Pichoud C, Zoulim F, Facchetti F, Poli F, Scalamogna M, Deliliers GL, Colombo M. Risk of hepatitis B surface antigen seroreversion after allogeneic hematopoietic SCT. *Bone Marrow Transplant* 2011; **46**: 125-131 [PMID: 20383209 DOI: 10.1038/bmt.2010.70]

33 **Seto WK**, Chan TS, Hwang YY, Choi O, Wong DK, Fung J, Lie AK, Lai CL, Kwong YL, Yuen MF. Interim analysis of hepatitis B reactivation in patients with prior HBV exposure undergoing hematopoietic stem cell transplant: a prospective study (abstract). *Hepatology* 2013; **58** (S1): 650A [DOI: 10.1002/hep.26727]

34 **Lee YH**, Bae SC, Song GG. Hepatitis B virus (HBV) reactivation in rheumatic patients with hepatitis core antigen (HBV occult carriers) undergoing anti-tumor necrosis factor therapy. *Clin Exp Rheumatol* 2013; **31**: 118-121 [PMID: 23111095]

35 **Peng JW**, Lin GN, Xiao JJ, Jiang XM. Hepatitis B virus reactivation in hepatocellular carcinoma patients undergoing transcatheter arterial chemoembolization therapy. *Asia Pac J Clin Oncol* 2012; **8**: 356-361 [PMID: 22897940 DOI: 10.1111/j.1743-7563.2012.01534.x]

36 **European Association For The Study Of The Liver**. EASL clinical practice guidelines: Management of chronic hepatitis B virus infection. *J Hepatol* 2012; **57**: 167-185 [PMID: 22436845]

37 **Lok AS**, McMahon BJ. Chronic hepatitis B: update 2009. *Hepatology* 2009; **50**: 661-662 [PMID: 19714720 DOI: 10.1002/hep.23190]

38 **Zurawska U**, Hicks LK, Woo G, Bell CM, Krahn M, Chan KK, Feld JJ. Hepatitis B virus screening before chemotherapy for lymphoma: a cost-effectiveness analysis. *J Clin Oncol* 2012; **30**: 3167-3173 [PMID: 22711851 DOI: 10.1200/JCO.2011.40.7510]

39 **Yeo W**, Chan PK, Ho WM, Zee B, Lam KC, Lei KI, Chan AT, Mok TS, Lee JJ, Leung TW, Zhong S, Johnson PJ. Lamivudine for the prevention of hepatitis B virus reactivation in hepatitis B s-antigen seropositive cancer patients undergoing cytotoxic chemotherapy. *J Clin Oncol* 2004; **22**: 927-934 [PMID: 14990649 DOI: 10.1200/JCO.2004.05.161]

40 **Loomba R**, Rowley A, Wesley R, Liang TJ, Hoofnagle JH, Pucino F, Csako G. Systematic review: the effect of preventive lamivudine on hepatitis B reactivation during chemotherapy. *Ann Intern Med* 2008; **148**: 519-528 [PMID: 18378948 DOI: 10.7326/0003-4819-148-7-200804010-00008]

41 **Huang H**, Li X, Zhu J, Ye S, Zhang H, Wang W, Wu X, Peng J, Xu B, Lin Y, Cao Y, Li H, Lin S, Liu Q, Lin T. Entecavir vs lamivudine for prevention of hepatitis B virus reactivation among patients with untreated diffuse large B-cell lymphoma receiving R-CHOP chemotherapy: a randomized clinical trial. *JAMA* 2014; **312**: 2521-2530 [PMID: 25514302 DOI: 10.1001/jama.2014.15704]

42 **Chen CJ**, Yang HI, Su J, Jen CL, You SL, Lu SN, Huang GT, Iloeje UH. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA* 2006; **295**: 65-73 [PMID: 16391218 DOI: 10.1001/jama.295.1.65]

43 **Iloeje UH**, Yang HI, Su J, Jen CL, You SL, Chen CJ. Predicting cirrhosis risk based on the level of circulating hepatitis B viral load. *Gastroenterology* 2006; **130**: 678-686 [PMID: 16530509 DOI: 10.1053/j.gastro.2005.11.016]

44 **Huang YH**, Hsiao LT, Hong YC, Chiou TJ, Yu YB, Gau JP, Liu CY, Yang MH, Tzeng CH, Lee PC, Lin HC, Lee SD. Randomized controlled trial of entecavir prophylaxis for rituximab-associated hepatitis B virus reactivation in patients with lymphoma and resolved hepatitis B. *J Clin Oncol* 2013; **31**: 2765-2772 [PMID: 23775967 DOI: 10.1200/JCO.2012.48.5938]

45 **Shen LP**, Zhang Y, Wang F, Zhang S, Yang JY, Fang KX, Yu T, Wang XY, Zhang WY, Bi SL. Epidemiological changes in hepatitis B prevalence in an entire population after 20 years of the universal HBV vaccination programme. *Epidemiol Infect* 2011; **139**:

1159-1165 [PMID: 21156099 DOI: 10.1017/S0950268810002827]
46 **Zhang H**, Li Q, Sun J, Wang C, Gu Q, Feng X, Du B, Wang W, Shi X, Zhang S, Li W, Jiang Y, Feng J, He S, Niu J. Seroprevalence and risk factors for hepatitis B infection in an adult population in Northeast China. *Int J Med Sci* 2011; **8**: 321-331 [PMID: 21611114 DOI: 10.7150/ijms.8.321]

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REVIEW

Hepatitis C virus genetic variability and evolution

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Abstract

Hepatitis C virus (HCV) has infected over 170 million people worldwide and creates a huge disease burden due to chronic, progressive liver disease. HCV is a single-stranded, positive sense, RNA virus, member of the *Flaviviridae* family. The high error rate of RNA-dependent RNA polymerase and the pressure exerted by the host immune system, has driven the evolution of HCV into 7 different genotypes and more than 67 subtypes. HCV evolves by means of different mechanisms of genetic variation. On the one hand, its high mutation rates generate the production of a large number of different but closely related viral variants during infection, usually referred to as a quasispecies. The great quasispecies variability of HCV has also therapeutic implications since the continuous generation and selection of resistant or fitter variants within the quasispecies spectrum might allow viruses to escape control by antiviral drugs. On the other hand HCV exploits recombination to ensure its survival. This enormous viral diversity together with some host factors has made it difficult to control viral dispersal. Current treatment options involve pegylated interferon-α and ribavirin as dual therapy or in combination with a direct-acting antiviral drug, depending on the country. Despite all the efforts put into antiviral therapy studies, eradication of the virus or the development of a preventive vaccine has been unsuccessful so far. This review focuses on current available data reported to date on the genetic mechanisms driving the molecular evolution of HCV populations and its relation with the antiviral therapies designed to control HCV infection.

Key words: Recombination; Quasispecies; Hepatitis C virus; RNA; Evolution; Antiviral therapy

Core tip: Hepatitis C virus (HCV) is the major causative agent of parenterally-acquired hepatitis. To date there is

no preventive vaccine, and though antiviral therapy has been improved in the past few years, not all patients eradicate the virus as a result of it. The main reason lies in the intrinsic genetic variability that characterises RNA viruses, such as HCV, whose RNA polymerase lacks proof-reading activity, leading to a high mutation rate and the generation of a wide range of genome variants better known as a quasispecies. Therefore this review summarises current data on HCV quasispecies dynamics, antiviral therapy and recombination events.

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INTRODUCTION

Hepatitis C virus (HCV) has infected over 170 million people worldwide and therefore creates a huge disease burden due to chronic, progressive liver disease[1]. Infections with HCV have become a major cause of liver cancer and one of the most common indications for liver transplantation[2-4]. The fact that chronic infection with HCV can lead to cirrhosis and hepatocellular carcinoma creates the need to develop drugs that effectively eradicate the infection[5] and a prophylactic vaccine that prevents its dissemination. Unfortunately, to date there is no effective vaccine available[6]. Currently, the standard of care (SOC) therapy involves pegylated interferon α (INF-α-peg) and ribavirin (RBV)[7]. In addition, the new SOC (NSOC) therapy of protease inhibitors boceprevir or telaprevir in combination with INF-α-peg and RBV have been approved for the eradication of HCV genotype 1 in the United States, Europe and Japan[8-11]. Unfortunately, interferon is not widely available globally, not always well tolerated and some genotypes of HCV respond better than others causing that not all patients achieve a sustained virological response (SVR)[12]. Other adverse events such as rash have also been associated with the NSOC[13].

The main route of transmission is direct or indirect exposure to contaminated blood, either through blood transfusions or blood products, through the consumption of intravenous drugs, use of surgical material poorly sterilized, organ transplants, accidents in health centres, vertical transmission from mother to child, *etc*.[12].

HCV is a member of the family *Flaviviridae*, although it differs from other members of this family in many details of its genome organization[1]. HCV is a single-stranded, positive sense, RNA virus with a genome of approximately 9600 nucleotides in length. Most of the genome carries a single open reading frame that encodes three structural (core, E1, E2) and seven non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins (Figure 1)[14,15]. In addition, alternative translation products (F protein) have been detected from a reading frame overlapping the core gene (core + 1/ARFP)[16,17]. Possible roles in regulation of gene expression, cell signalling and apoptosis have been suggested[18-20]. Short untranslated regions at each end of the genome (5′-NCR and 3′-NCR) are required for its translation and replication[21,22]. The mechanism of translation initiation is dependent on an internal ribosomal entry site in the 5′-NCR, which interacts directly with the 40S ribosomal subunit[23].

The high error rate of RNA-dependent RNA polymerase and the pressure exerted by the host immune system, has driven the evolution of HCV towards the development of a global diversity that revealed the existence of seven genetic lineages (genotypes 1 to 7) (Figure 2)[24]. On average, the complete genome differs in 31%-33% of nucleotide sites[4]. Genotypes 1 to 6 of HCV contain a series of more closely related sub-types (67 accepted subtypes and many more to be confirmed) that typically differ from each other by at least 15% in nucleotide positions within the coding region[24]. Subtypes 1a, 1b and 3a are widely distributed and account for the vast majority of infections in Western countries[1].

HCV genetic variability is not evenly distributed across the viral genome. The regions of the genome that correspond to essential viral functions (such as those involved in translation and replication) or those with major structural domains (5′-NCR and 3′-NCR) are the most conserved. The 5′-NCR region is the most conserved region of the genome with 90% sequence identity between distant strains[25,26]. The region encoding the viral capsid is also highly conserved with 81%-88% sequence identity between different isolates. The most variable region of the genome is the one that codes for the membrane glycoproteins E1 and E2[27]. The sequences belonging to the hypervariable regions 1 and 2 (*HVR1* and *HVR2*) of E2 gene are the ones that show less sequence homology with only 50% identity between different isolates[28]. Factors that may contribute to high genetic variability of these viruses include large population sizes, short generation times and high replication rates[29].

An important breakthrough in the treatment of chronic HCV infection was undoubtedly the introduction of alpha interferon (IFN-α) plus RBV as combination therapy[27]. However, the rate of sustained virological response is still unsatisfactory[30,31], particularly in patients infected with genotype 1[32], the most prevalent in many geographic regions of the world[4,33]. Although IFN-α is effective in reducing the viral load, complete eradication of the virus is achieved in less than 20% of patients treated with IFN-α alone[32]. In those patients who initially respond to IFN-α, ribavirin helps increasing the frequency of virus eradication, yet its effect on non-responder patients is still limited[7]. Although viral genotype and viral load, as well as serum HCV RNA clearance during therapy are definitely related to response, further insight into viral factors involved in

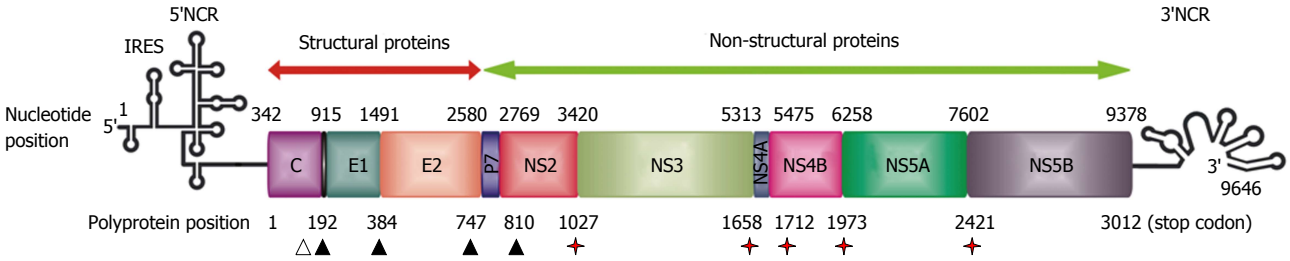

Figure 1 Organisation of hepatitis C virus genome and hepatitis C virus polyprotein processing. Schematic representation of the 9.6 kb positive-stranded RNA genome. Simplified RNA secondary structures in the 5' and 3' non-coding regions (NCRs) are shown. Internal ribosome entry site (IRES)-mediated translation produces a polyprotein precursor that is processed into the mature structural and non-structural proteins. Nucleotide positions are shown by numbers on the upper part of the scheme. Amino acid positions are shown by numbers in the lower part of the scheme. The coding region is depicted by rectangles showing the corresponding encoded proteins. Solid arrowheads denote cleavages by the endoplasmic reticulum signal peptidase. The open arrowhead indicates further C-terminal processing of the core protein by signal peptide peptidase. Red stars indicate cleavages by the hepatitis C virus NS2 and NS3-4A proteases.

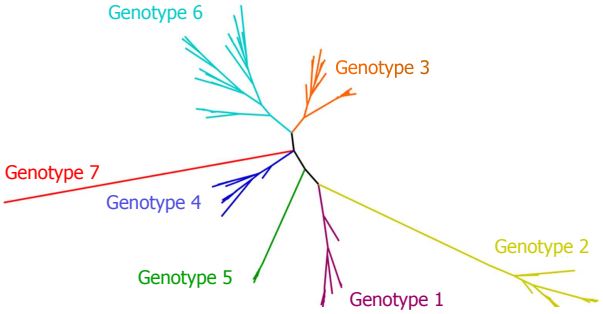

Figure 2 Evolutionary tree of the seven genotypes and all known subtypes of hepatitis C virus. The tree was constructed using the maximum likelihood method using GTR + I + G (general time-reversible substitution model considering invariable sites and gamma distribution) as the nucleotide substitution model that best fitted the data using a 307-nucleotide sequence from the NS5B-coding region. Sequences used for the construction of this phylogenetic tree were extracted from Yusim *et al*[155].

therapeutic responsiveness is still necessary[27]. The different genotypes and subtypes vary in their responses to treatment with INF-α or INF-α/RBV. As mentioned above, only 10%-20% of individuals chronically infected with HCV genotype 1 treated with IFN-α monotherapy and 40%-50% of those treated with combination therapy (INF-α/RBV) exhibit a complete and permanent disappearance of the virus. These percentages are lower than rates of 50 and 70%-80%, respectively, observed in the treatment of patients infected with HCV genotypes 2 or 3[3,34]. Despite these facts, the use of INF-α-peg has been associated with a significant increase in these rates[35].

Two inhibitors of the NS3/4A serine protease, boceprevir (BOC) and telaprevir (TVR) have demonstrated potent inhibition of HCV genotype 1 replication and markedly improved SVR rates in treatment-naïve and treatment-experienced patients[36]. Nowadays the NSOC therapy for genotype 1, chronic HCV infection is the use of BOC or TVR in combination with INF-α-peg and RBV[37-40]. Additionally, two other direct antiviral drugs (DAAs), simeprevir (protease-inhibitor)[41] and sofosbuvir (nucleotide analogue of the NS5B RNA-polymerase)[42] have been recently approved for triple therapy in the United States and Europe. Despite these new advances in what respects to triple therapy, it is worth to note that a wide range of different DAAs are currently under clinical trials aiming at all-oral IFN-free regimens[43-47].

MUTATIONS AND QUASISPECIES DYNAMICS

HCV evolution is a highly dynamic process[48]. Like most RNA viruses, HCV exploits all possible mechanisms of genetic variation to ensure its survival. Mutation at the nucleotide level seems to be the main cause of genetic variation in RNA viruses, such as HCV. These mutations are primarily generated by an error-prone, non-proofreading RNA-dependent RNA-polymerase which directs the replication of the virus genetic material[1,49]. The mutation rate of HCV, estimated at 10^{-4} substitutions per site and round of replication[50], is among the highest for RNA viruses including retroviruses[51], and would seem to be high enough to generate all the genetic variation found in this virus. Due to this feature and to the high replication rate of HCV, a large number of different but closely related viral variants are continuously produced during infection. These circulate *in vivo* as a complex population commonly referred to as a quasispecies[52-58].

The idea of quasispecies was first used by Eigen *et al*[59,60] to refer to the first self-replicating structures. Originally conceived as a mathematical framework formulated to explain the evolution of life in the "pre-cellular RNA world"[61], quasispecies theory is based on classical population genetics, but seeks to explore the consequences of error-prone replication and near-infinite population sizes for genome evolution. More recently, quasispecies theory has been used to describe the evolutionary dynamics of RNA viruses[62]. These structured populations also possess a high mutation rate which would lead to the existence of a complex mixture of different but related genomes that would behave as a selection unit (Figure 3). At a particular point of infection, the HCV quasispecies viral distribution reflects the balance between the continued generation of new variants, the need to preserve essential viral functions and the positive selective

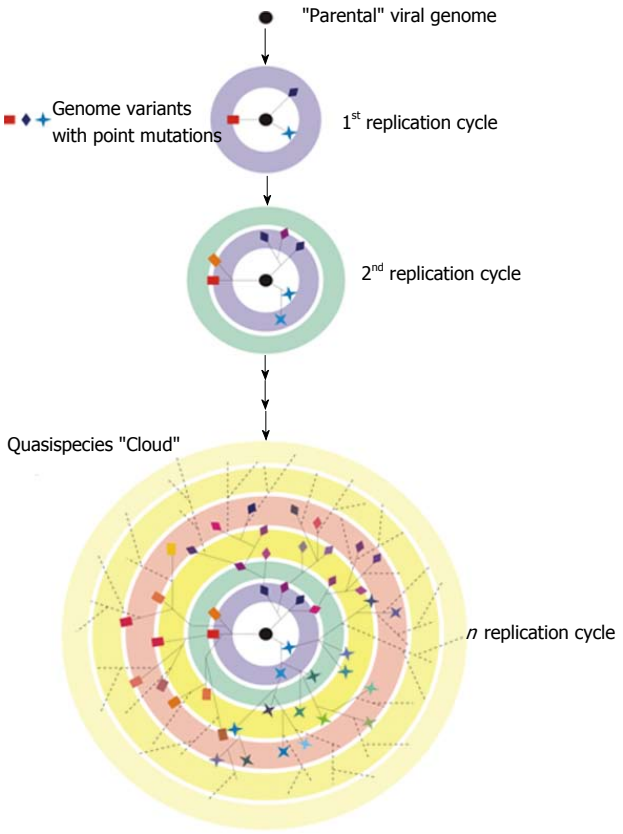

Figure 3 Viral quasispecies. A virus replicating with a high mutation rate will generate a diverse mutant repertoire over the course of a few generations. In this schematic representation, a "parental" viral genome (black filled circle) gives rise to different variants (coloured squares, prisms and stars), each linked to another one by a point mutation. The concentric circles represent replication cycles. The resulting distribution is often referred to as quasispecies "Cloud".

pressure exerted by the environment[63]. It is important to highlight that multiple viral quasispecies co-exist in infected individuals at different replicative sites and in consecutive times, which offers a rich environment for intra and inter quasispecies interactions.

The theory of quasispecies predicts that a particular mutant surrounded by a more favourable mutant spectrum (more related) can dominate another one with better fitness. This phenomenon has been called suppressive effect. This suppressive or interfering effect of the mutant spectrum rises along with the mutation rates in viral quasispecies, as has been suggested by the strong suppressive effect in infectivity that viral populations have near to extinction[64]. It has been proposed that interference generated by defective genomes contributes to viral extinction due to an increased mutagenesis rate. We can also see the opposite effect: genome complementation between different components of the mutant spectrum, which demonstrates that the behaviour of quasispecies cannot be understood solely as the sum of individual behaviours[65]. Because of this, quasispecies is defined as a dynamic distribution of genomes subject to variation, competition and selection, and that act as a selection unit. This means that the quasispecies as a whole, instead of a particular viral variant, is the target of the selection process[62,64,66].

As mentioned before, low fitness variants can be preserved at higher than expected frequencies just because they are coupled to a well represented higher fitness genotype in sequence space. One of the defining characteristics of a quasispecies is the phenomenon of mutational coupling, as it places individual mutants within a functional network of variants[62]. The elevated mutation rates in RNA viruses mean that a fast replicator will generate genetically diverse progeny, many of which will be significantly less fit than the parent. As a result, quasispecies theory predicts that slower replicators will be favoured if they give rise to a fitter progeny[67].

One of the consequences of quasispecies dynamics is the existence of a threshold error to the preservation of genetic information[64]. When the error rate exceeds a tolerable limit (related to genome size, fitness and population size of the quasispecies) distribution collapses and the nucleotide sequence loses its information. This transition is known as the entrance to catastrophic error and its application to viral extinction through mutagenesis is called lethal mutagenesis[64]. There are experimental evidences that show that RNA viruses replicate very closely to this threshold error and that an increase in mutation rates can have a negative impact on the viability of viral populations.

The theory of evolution predicts that, in a dynamic environment, high mutation rates are favoured, and therefore viral error rates may have been optimized by natural selection[68,69]. For RNA viruses, low replicative fidelity generates a diverse population of variants. Even though many of these variants are generally less fit, they may take over if an unexpected change in environment occurs, such as immune pressure, shifting the corresponding fitness landscape. On the contrary, a homogeneous population, generated by high replicative fidelity, would lack this flexibility and might be less successful in the dynamic host environment. Experimental support for this hypothesis has been provided by two different groups[70,71]. They isolated a poliovirus variant, resistant to ribavirin, which had a single amino acid substitution in the viral polymerase. This mutant exhibited a moderate resistance to lethal mutagenesis and assays for selectable markers indicated that this population had a lower mutation rate and it consequently displayed less genetic diversity. More recently, Vignuzzi *et al*[72] (2008) proposed that this attenuated high fidelity variant could be employed for vaccine development.

High mutation rates and quasispecies dynamics confer great adaptability to RNA viruses and represent one of the major obstacles for the control and prevention of RNA viral diseases[15,73]. The great quasispecies variability of HCV has also therapeutic implications since, by means of generating and selecting fitter variants within the quasispecies cloud, viruses might escape control by antiviral drugs[74]. The way HCV quasispecies evolves is highly dynamic, and for this reason, the complexity of the genetic information gathered from quasispecies populations cannot be accurately analysed

by a unique analytical tool[75]. The three parameters most commonly used to determine the complexity of the quasispecies mutant spectrum are: mutation frequency (defined as the proportion of mutated nucleotides within a genome distribution relative to the consensus sequence), Shannon entropy (defined as the proportion of different genomes within a mutant distribution) and Hamming distance (defined as the number of mutations that differentiate two sequences within the mutant spectrum)[27,64]. The average of all the comparisons between possible pairs reflects the genetic complexity of quasispecies[62]. As stated previously, a fundamental feature of viral quasispecies, predicted from quasispecies theory, is that the target of selection is the mutant distribution as a whole rather than an individual genome. Selective transmission of predominant and minor HCV quasispecies has been shown in humans[76-80] and in experimentally infected chimpanzees[81,82]. Further understanding of quasispecies dynamics in infected individuals is necessary to gain knowledge on how to apply virus-specific drugs and to identify key parameters that are critical for the development of effective antiviral strategies[55]. The genetic variability within quasispecies level has been frequently used as a predictor of the response to antiviral therapy[83]. Several studies have reported that genetic diversity within different regions of HCV genome before combined therapy with INF/RBV was higher in non-responders than in responders[53,83-86]. Nevertheless, Cristina *et al*[15] (2007) showed that the response to antiviral therapy is independent from genetic variability within quasispecies populations at the beginning of therapy. Ueda *et al*[86] (2004) reported that during combined therapy with INF/RBV, HCV quasispecies significantly decreased in non-responders. However, another study showed that populations fluctuate along therapy, in treated patients that achieved a sustained virological response as well as in those non-responders. This would indicate that these fluctuations are intrinsic to each patient and that HCV follows different evolution paths in different patients[15]. The variations in the results obtained by the different research groups might be caused by different methodological approaches used to study the real variability within circulating quasispecies populations[87]. Another explanation to this might be that in viral populations with a high degree of genetic variability, the probability of finding variants that would replicate effectively in the presence of antiviral drugs is high. Despite this, those populations with lower variability degree might also present these types of resistant variants, though in lower probability. Likewise, given the stochastic nature of this phenomenon, the opposite situation might as well occur in these two same cases[88]. These correlations can probably mask the expected one between genetic variability and response to treatment, thus providing no conclusive results on these matters.

In addition to the multiplicity of viral genetic factors reported so far, host factors have also shown to be involved in the development of HCV infection. Since 2009, several single nucleotide polymorphisms (SNP) have been reported near the interleukin 28B gene (*IL-28B*) that influence response to dual antiviral therapy[89-94]. However, it was not until 2011 that the relationship between HCV quasispecies diversity and the host IL-28B genotype was investigated[95]. Their results evidenced a clear association between IL-28B risk allele (SNP rs8099917 - G allele) and a lower NS3 protease amino acid quasispecies diversity in infected patients, hence suggesting that IL-28B risk allele carriers exert less positive selection pressure on the NS3/4 protease[95]. However, it would be interesting to address whether the lower amino acid quasispecies diversification in patients with an IL-28B risk allele is restricted to the NS3 coding region or if it affects other viral genomic regions. Interestingly, also unfavourable rs8099917 genotypes were found linked to time-dependent changes in the core coding region, specifically the shift to residue 70Q associated with hepatocellular carcinoma[96]. These results might indicate that the IL-28B genotype influences viral evolution and disease outcome in addition to the behaviour of the innate immune system. More recently, Yuan *et al*[94] (2012) found that, despite exhibiting similar baseline viral loads, more chronically-infected patients with the rs12979860-CC polymorphism had amino acid substitutions in NS5A compared to non-CC patients. This result suggests that patients with the CC genotype undergo early viral evolution probably as a consequence of the selective pressure exerted by the use of interferon at the beginning of treatment. These studies raise the question of whether host genetics shapes viral evolution in response to immunity, and if the differences observed in the evolution of HCV quasispecies are implicated in the mechanism by which the IL-28B genotype influences the outcome of acute HCV infection and treatment response[97].

As previously mentioned, RNA virus populations exist as a cloud of sequence variants (continuously being generated by mutation) strongly related, therefore a better understanding of these populations within infected individuals is needed in order to apply antiviral strategies and to define critical parameters to the development of new and more effective antiviral therapies[62]. Nevertheless, to date, our ability to know in depth the quasispecies cloud from the study of isolated clones is limited. The recent techniques of deep sequencing allow us, for the first time to overcome these obstacles and observe quasispecies as a whole. These techniques are already being used on clinical samples to study different viral models[98-100].

NEXT GENERATION SEQUENCING IN HCV QUASISPECIES ANALYSIS

Since 2005, the development of high throughput, or so-called next generation sequencing technologies (NGS), has allowed a huge increase in capacity to sequence genomes at a reasonably low cost and in a short time frame[101]. NGS comprises a set of high-throughput

Table 1 Representative next generation sequencing platforms and their characteristics

Platform	Run time (h)	Read length (bp)	Throughput per run (Mb)	Typical errors	Main biological applications	Company URL
Roche 454 FLX +	23	700, up to 1000	700	Insertions/deletions (indels) at homopolymer regions	Microbial genome sequencing, human genome sequencing, transcriptomics, metagenomics	http://www.my454.com/
Illumina HySeq 1000 MySeq	8	2 × 100	400000	Indels, especially end of reads	Microbial genome sequencing, human genome sequencing, transcriptomics, metagenomics	http://www.illumina.com/systems.ilmn
2000 V3	10	2 × 150	< 600000			
SOLiD 4	12	50 × 35	71000	End of read substitution errors	Microbial genome sequencing, human genome sequencing, transcriptomics, metagenomics	http://www.appliedbiosystems.com/absite/us/en/home.html
Ion torrent PGM 318 Chip	3	200	1000	Indels at homopolymer regions	Microbial genome sequencing, human genome sequencing, transcriptomics, metagenomics	http://www.iontorrent.com/
Pacific Biosciences				Random indel errors	Full-length transcriptomics, discovering large structural variants and haplotypes	http://www.pacificbiosciences.com/

sequencing technologies, which make it possible to sequence several genomes from individual templates in a parallel fashion[102]. The current NGS technologies are known as second generation technologies (Table 1), to distinguish them from the first generation (Sanger sequencing), and the third generation (based on single molecule sequencing)[103].

As it is mentioned before, RNA viruses have very high mutation frequencies. The error rate of viral RNA-dependent RNA polymerases is estimated to be between 10^{-3} and 10^{-6} per nucleotide copied compared to 10^{-8}-10^{-11} for DNA polymerases[72]. Consequently, an RNA virus population consists not of a single genotype but of an ensemble of closely related genotypes, termed as a quasispecies, centred on a master sequence. This genetic diversity creates a cloud of potentially beneficial mutations, which is thought to allow rapid adaptation to a constantly changing environment[52]. Quasispecies theory makes a number of predictions about the behaviour of viral populations and the consequences of altering diversity.

NGS technologies have redefined the modus operandi in virus genetics research, allowing the unprecedented generation of very large sequencing datasets on a short time scale and at affordable costs[101]. A significant technical challenge to address how we can measure viral diversity could be NGS technology. Ultra-deep sequencing has the sensitivity and quantitative nature required for this kind of investigations into viral genetic drift, natural selection and response to antiviral drugs. The analysis of viral quasispecies has been greatly enhanced by the recent emergence of these powerful technologies that allow the simultaneous sequencing of 400000-10000000 individual target sequences[104].

As previously reported, drug-resistant variants may already exist previous to a particular antiviral treatment embedded in a predominantly wild-type virus quasispecies population. Consequently, as they may be present at different low-level frequencies, this may lead to varying degrees of viral response and therefore the mutant genome will become enriched upon treatment with HCV inhibitors. Hence, determining the natural levels of low frequency resistant variants before starting a treatment might be relevant to better predict viral response to HCV inhibitors[105].

The classic method for detecting these drug-resistant variants in infected patients is population-based DNA sequencing, in other words: bacterial cloning. This method provides a good idea of the major sequences present, but unfortunately it cannot detect minor variants that are present at a frequency below 20%-25%[106]. With the development of deep sequencing technologies, detection of drug-resistant variants became more sensitive allowing the identification of variants present at very low frequencies (about 0.1%-1%)[107-109]. For HCV, the deep sequencing method was first used to detect emergence of NS3 mutants. This way it was clearly demonstrated that *de novo* telaprevir-resistant NS3 mutants arose in mice injected with wild-type HCV only 2 wk after the beginning of treatment[108]. Deep sequencing was also used to confirm results of naturally occurring drug-resistant *HCV* mutants detected by a novel mismatch amplification mutation assay polymerase chain reaction[107].

As can be noted, deep sequencing technology provided a comprehensive view of the viral population dynamics during monotherapy with NS3 protease inhibitors. It allowed the estimation of pre-treatment levels of NS3 drug resistant mutants, suggesting therefore a limitation of HCV viral suppression with NS3 protease inhibitor monotherapy caused by the pre-existence of drug resistant mutants. This finding strongly evidences the need for a combined therapy to durably treat HCV infection[105].

In addition, NGS technology has already been implemented in order to study the transmission event of HCV among injection drug users. In this case NGS was used to determine intra-host viral genetic variation by deep sequencing the HCV hypervariable region, which allowed a detailed analysis of the structure of the viral quasispecies in the patients' population under study[110].

NGS approaches are powerful methods that allow a rather comprehensive analysis of the intra-host viral genetic variation[107]. Moreover, these technologies are becoming rapidly accessible all around the world which will likely revolutionise the field of molecular epidemiology[110].

It is worth mentioning that these technologies offer, as already discussed, several advantages over conventional methods, such as consensus sequencing, bacterial cloning, and endpoint limiting dilution[107]. Furthermore, as the development of a variety of software and algorithms capable of handling the massive amount of data generated by NGS platforms is increasing in parallel with the advances in these technologies, it will likely expedite the implementation of such approaches in a variety of settings in the near future[110]. Taking this into consideration, the use of NGS in HCV outbreak investigations will presumably improve molecular epidemiology studies as well as provide a vast amount of information that will need to be handled appropriately both for the benefit of infected patients and the management of public health systems.

RECOMBINATION

In addition to mutations, it is widely accepted that recombination plays an important role in the evolution of RNA viruses by creating genetic variation through the exchange of nucleotide sequences between different genomic RNA molecules[111]. Therefore it is considered as a key mechanism for the production of new genomes with selective growth advantage[112]. Homologous recombination occurs, in some cases of RNA virus recombination, when the donor sequence neatly replaces a homologous region of the acceptor sequence leaving its structure unchanged. In these cases, not only the parental RNAs are homologous, but also crossovers occur at homologous sites[112,113]. Nevertheless, hybrid sequences may originate as a result of aberrant homologous recombination (when similar viruses exchange sequence without maintaining strict alignment) and non-homologous recombination (recombination between unrelated RNA sequences)[112,113]. RNA recombination involves replication of genomic RNA as a necessary component of the process. If in the middle of the replication process, the viral RNA-dependent RNA polymerase complex switches from one RNA parental strand to another, hybrid complementary RNA strands will be formed. Yet, if the replicase continues to copy the new strand at the same site where it left the parental one, this constitutes a homologous recombination event. On the other hand, aberrant or non-homologous recombination will occur if the copying process is not as precise[112]. This template strand exchange mechanism of recombination is known as "copy choice". The exact mechanism of exchange of strand is not known, but could be promoted by the pause of the polymerase during chain elongation. Thus far, nearly all studies on the mechanisms of recombination in RNA viruses support a copy-choice model, originally proposed for poliovirus[114]. It is of note, that this template-switching mechanism greatly differs from the enzyme-driven breakage-rejoining mechanism of homologous recombination in DNA, mainly because it resorts to replication as an essential step of the process[112].

Recombination in RNA virus was first discovered in poliovirus infected cells in which the frequent recovery of poliovirus that results from recombination has the potential to produce "escape mutants" in nature as well as in experiments[115]. Subsequently, recombination was found to occur in other RNA viruses positive and negative-stranded and more recently, recombination between unrelated groups of RNA and DNA viruses was discovered in a novel virus genome isolated from an extreme environment[116]. The presence of recombination in several members of the family *Flaviviridae* such as Pestivirus, Flavivirus and Hepacivirus has been demonstrated[117-122].

Regarding HCV, recombination has been reported both inter and intragenotypic in populations in different geographic locations (Table 2). Some earlier reports described some HCV strains from Honduras in which the study of partial sequences from different regions of the viral genome resulted in HCV discordant genotype, providing first evidence for the possible existence of HCV recombination[123]. However it was not until 2002 that the first convincing report of an intergenotypic HCV recombinant strain was published by Kalinina *et al*[124] (2002) in Saint Petersburg (Russia)[49,124]. These authors described six different natural HCV strains that belonged to different subtypes, 2k and 1b. They found that the 5' untranslated region and the core coding region belonged to subtype 2k, whereas the NS5B region corresponded to subtype 1b[124]. Sequencing the E2-p7-NS2 region, they were able to map the crossover point within the NS2 region, estimating it most likely between positions 3175 and 3176 (according to the numbering system for strain pj6CF). The reported recombinant was cautiously designated RF1_2k/1b, in agreement with the nomenclature used for human immunodeficiency virus (HIV) recombinants[124]. This same recombinant strain has since then been isolated in other countries, like Ireland[125], Uzbekistan[126], Cyprus[127], France[128] and Estonia[129] which would suggest that, although its generation might not be favoured by natural selection, it would also not be selected against[49]. Additionally, at least ten other different intergenotypic recombinant forms (RFs) of HCV have been described and are totally or partially characterised (Table 1). Within this group of recombinants, we can observe the presence of recombinant forms between genotypes 2 and 6

Table 2 Main features of intergenotype, intersubtype and intrapatient recombination in hepatitis C virus published cases

Strain	Genotype	Country	Recombination breakpoint(s)	Ref.
Intergenotype				
RF1_2k/1b	2k/1b	Russia, Ireland, Uzbekistan, Georgia, France, Cyprus, Estonia	NS2, positions 3175-3176	[124-129]
D3	2i/6p	Viet Nam	NS2/NS3 junction, between positions 3405 and 3464	[130]
SE-03-07-1689	RF3_2b/1b	Philippines	NS3, positions 3466-3467	[137]
HC10-0804	2b/1b	Japan	NS2/NS3 junction, positions 3443-3444	[135]
B5808, M2123, M4430, M4416, M4414, M4431, M2777, M8774	2b/1b	Japan	NS2, putative position 3301	[136]
R1	2/5	France	NS2/NS3 junction, between residues 3420 and 3440	[132]
D177	RF_2b/6w	Taiwan	NS2/NS3 junction, position 3429	[131,133]
	RF_3a/1b	Taiwan, China	Undetermined	
	RF_2a/1a	Taiwan	Undetermined	
JF779679	2b/1a	United States	NS2/NS3 junction, positions 3405-3416	[134]
Intersubtype	Subtypes			
PE22	RF2_1b/1a	Peru	NS5B, position 8321	[141]
H23	1b/1a	Uruguay	Core, at position 387	[111]
HC-J1	1a/1c	Japan	2 sites in E1-E2, at positions 1407 and 2050	[142]
Khajal	1a/1c	India	5 sites, from core to NS3, at positions 801 1261, 2181, 3041 and 3781	[143]
R49	4a/4d	Portugal	Undetermined	[140]
EU246930	6a/6o	Viet Nam	NS5B, between positions 8345-9073	[144]
EU246932	6e/6o	Viet Nam	NS5B, between positions 8358-8977	[144]
EU246937	6n/6o	Thailand	NS5B, between positions 8372-9033	[144]
EU246931	6e/6h	Viet Nam	NS5B, between positions 8356-9019	[144]
Intrapatient	Subtype			
	1b	Spain	NS5B, at residue 286	[145]
	1a, 1b, 3a	Spain	1 or 2 sites within E1-E2 or NS5A	[146]
	4a	Ireland	E2 glycoprotein, HVR1 region	[147]

Modified from ref. [49].

described in Vietnam[130] and Taiwan[131], between genotypes 2 and 5 described in France[132], between genotypes 3 and 1 in Taiwan[131] and China[133], and between genotypes 2 and 1 reported in Japan, the United States and the Philippines[134-137]. It is interesting to note that recombinant forms found so far have a wide geographic distribution. Besides, all HCV genotypes except for genotype 4 and 7 have been found in them. Other interesting feature is that all these recombinants but one (RF_3a/1b) originated by the combination of a 5'-end of genotype 2 and a 3'-end of a different genotype. The 3'-end of subtype 1b seems to be the only one appearing in more than one recombinant form[49]. Oddly enough, genotype 2 is present in the majority of the recombinants found to date, which might suggest a critical role in order for the process to take place[138] or even for the stability and functionality of the resulting recombinant genome. The fact that some recombinants involving genotype 2 and subtype 1b have been frequently found in older patients and in cases not usually related to the epidemic spread associated to a higher use of intravenous drugs, makes it difficult to assess whether this pattern derives from adaptive selection or is simply due to chance[1,49]. As we can observe in Table 2, another characteristic feature of intergenotypic recombination in HCV seems to be that the crossover points appear to be located within either gene *NS2* or *NS3*[49]. An apparent hotspot has been identified between amino acids 1022 and 1042 (corresponding to the vicinity of the NS2/NS3 junction). Considering how short this region seems to be, and despite the existence of only a few reports inquiring into RNA secondary structures involved in the recombination process[130,139], all seems to indicate that a copy choice mechanism might be responsible for the generation of these recombinant forms[49].

With respects to intragenotypic recombination, nine recombinant forms have been described. As we mentioned before, each of the six major genotypes of HCV (except for genotype 7 for which there is only one complete-genome sequence available) can be subdivided into closely related sub-types that differ from each other by at least 15% in nucleotide sequences[24]. Therefore, the same methodological procedures based on phylogenetic incongruence used to detect intergenotypic recombinants are also applicable to detect intragenotype/intersubtype RFs. Only one of the intragenotype recombinants described involved genotype 4[140], four involved different subtypes of genotype 1 and the remaining four involved different subtypes of genotype 6. Examples of recombinants (1a/1b) have been identified in Peru as well as in Uruguay[111,141] and (1a/1c) in Japan and in India[142,143]. Interestingly, although the recombinants reported in

Uruguay and Peru are (1a/1b), their recombination breakpoints were found in different regions of the genome as shown in Table 2. The same happens in the case of the recombinant forms described in India and in Japan. Therefore, unlike what it is observed in intergenotype recombination, where all shared a common genome region in which recombination occurs, in this case these points are highly variable in their location. Only two of the intragenotype 1 RFs have been fully sequenced[142,143] and both involve the same subtypes (1a and 1c). Interestingly, they revealed the existence of more than one cross-over point, resulting in mosaic recombinant forms. In addition, the sequences showed a very dissimilar size, exhibiting relatively short segments of one subtype embedded within a genome of the other subtype[49]. The most recent description of intersubtype recombinant forms involves genotype 6, and these were identified thanks to full-genome sequence analysis[144]. The remaining three cases of intersubtypic RFs reported have only been partially characterised at the genome level[49]. One of the cases was detected by discordant phylogenetic analysis of the regions coding for E1 and NS5B isolated from an intravenous drug user from Portugal[140]. This case should be considered as a putative example since no recombination breakpoint has been mapped for this RF. The two remaining cases are different as they have been described only by sequencing one single portion of the HCV genome. Contrary to what occurred with the other example, the corresponding breakpoints for these two were identified within the core and NS5B genes each[111,141], genes that are relatively conserved and are therefore suitable for phylogenetic typing and subtyping of HCV isolates[49].

When we talk about recombination in HCV we can not forget to mention the existence of intrapatient or also called intra quasispecies recombination. With respect to this matter, three reports have been published thus far. Two of them involve intrapatient recombination in individual patients undergoing therapy[145,146]. The most recent one involves quasispecies evolution from a chronically infected, treatment naïve individual[147]. In the first report of intra-quasispecies recombination, analysed sequences were obtained from the *NS5A* gene of HCV quasispecies populations from six patients being treated with IFN + RBV combined therapy[145]. Only one recombinant strain was detected in all patient quasispecies populations studied and its recombination crossover point was found within the protein kinase R (PKR)-binding region of NS5A[145]. This region has a particular importance since previous work by Enomoto *et al*[148] (1995) suggested that the genetic heterogeneity of the interferon sensitivity determining region domain of HCV NS5A (IFN sensitivity-determining region), linked to response to therapy in Japanese patients[148]. Although there seems not to be a consensus on this issue[85], the published information supports the hypothesis that an association indeed exists between NS5A and response to therapy[149-151]. Some reports suggest that HCV NS5A protein can act *in vivo* repressing PKR function, and presumably allowing HCV to escape the antiviral effects of interferon[1,3,152,153]. Analysing NS5A protein sequences of both the recombinant and putative parental-like virus provided evidence in favour of the possibility that the recombinant isolate might have acquired amino acids already known for being present in HCV strains resistant to interferon treatment[145]. The results of these studies support the fact that recombination cannot be denied as an evolutionary mechanism for generating diversity in HCV *in vivo* in patients undergoing antiviral therapy[145]. In spite of this fact, recombination does not seem to play a major role in the evolution of HCV quasispecies populations; at least this is what can be extrapolated from the study of *NS5A* genes, since only one recombinant isolate was found among all HCV quasispecies populations studied. Contrary to this finding, Sentandreu *et al*[146] (2008) identified a high frequency of intra-patient recombination events (18.01% of the 111 analysed patients) analysing a large data set of HCV sequences (around 17700) from intra-patient viral population[146]. They retrospectively studied NS5A and E1-E2 coding regions from samples isolated from two different sets of groups: HCV mono-infected patients, both naïve and non-responders to antiviral treatment; and HCV/HIV co-infected patients, both treatment-naïve and under HAART[146]. These authors found recombination within the E1-E2 region (9.1%), and within the NS5A region (9.6%), with specific areas being proposed as the crossover points. Although no structural analyses were performed in this study, these results are consistent with the implication of RNA secondary structure in favouring the hotspots or zones where recombination can occur within the HCV genome[49]. As per these results, where recombination intra-patient was found in 18% of the HCV infected patients studied, intra-quasispecies recombination events seem relatively frequent. Moreover, this might be an underestimation of the real frequency of HCV recombination due to the difficulty in detecting recombination events if they occur between genetically very similar variants as is the case of variants within a quasispecies[49,146]. More recently, Palmer *et al*[147] (2012) detected putative intra-subtype recombinants, as well as the likely ancestral parental donors. By retrospective clonal analysis they explored quasispecies evolution evaluated at the HVR1 region from serum samples isolated from the same chronically infected, treatment naïve individual which were collected over 9.6 years[147]. Their detailed analysis clearly documents the emergence, maintenance and final removal of HCV variants, which in this case has been demonstrated in a patient who did not undergo antiviral therapy highlighting the importance of HCV quasispecies dynamics even in absence of a clear selective pressure.

Even though the regions apparently involved in the crossover events seem to be different when comparing intergenotype *vs* intraquasispecies recombination, they seem to be quite conserved within each of the different categories. This might represent another indication

of the importance of RNA secondary structure for recombination events to take place and might as well hint at a possible factor determining their occurrence.

Having detected so many recombinant strains (Table 2) proves that HCV is capable of successfully completing all the steps leading to this event: simultaneous infection of the same cell by different viral strains, simultaneous replication of both viral genomes, strand shift by the viral RNA polymerase without disturbing the correct reading frame, and encapsidation and release of the recombinant genomes as viable viral particles[49]. The resulting products will then be subjected to the same population processes governing the maintenance, growth or disappearance of new variants in a heterogeneous viral population[49,146].

If we analyse in depth the recombination events, we can consider that recombination in HCV may be underestimated. Why? Three different factors might account for this: Firstly, in recombination events between subtype viral strains, there is a trade-off between the capability of homologous recombination event to occur, and the intra-patient viral diversity, since homologous recombination requires a minimum length of sequence identity. Secondly, another trade-off occurs between the intra-patient viral diversity and the discrimination power to detect recombination with the different methods available. Finally, despite recombination events between different genotypes/subtypes co-infecting the same patient are probably easier to detect, they are less likely to occur, since the strains of different subtypes differ more between them than between those from the same subtype; this would imply a lower probability of template switching and moreover, if a recombination event does indeed happen, it will likely generate recombinant sequences less viable than the parental ones. Some or even all these factors acting in concert might explain why the frequency of recombinant HCV sequences reported to date is so low[49,111,124-137,140-147].

How relevant recombination for HCV long term evolution and its incidence in HCV infection is, has not been thoroughly investigated yet, but these findings support a potentially significant role for recombination by creating genetic variation through the reshuffling of independent variants[146]. Recombination may serve two opposite purposes: simply to explore a new genomic combination or to rescue viable genomes from debilitated parental ones[29,154]. Considering that recombination may influence vaccine development, virus control programs, patient management as well as antiviral therapies, it is clearly important to determine the extent to which this mechanism plays a role in HCV evolution[111].

CONCLUSION

RNA viruses exist as complex mutant distributions commonly known as viral quasispecies. This is the result of high mutation rates due to the lack of proofreading activities in their RNA-dependent RNA polymerases[87]. The evolution of the HCV quasispecies is a highly dynamic process that has therapeutic implications due to the continuous generation and selection of fitter variants within the quasispecies spectrum which might allow viruses to escape control by antiviral drugs and treatment[15,74]. Further studies on HCV quasispecies is needed in order to develop appropriate strategies for effective antiviral control[55]. HCV utilizes all known genetic mechanisms, including mutation and recombination. NGS technologies may represent an important improvement to identify key parameters in our understanding of HCV evolution in relation to current and new therapies against HCV.

REFERENCES

1 **Simmonds P**. Genetic diversity and evolution of hepatitis C virus--15 years on. *J Gen Virol* 2004; **85**: 3173-3188 [PMID: 15483230 DOI: 10.1099/vir.0.80401-0]

2 **Hoofnagle JH**. Course and outcome of hepatitis C. *Hepatology* 2002; **36**: S21-S29 [PMID: 12407573 DOI: 10.1053/jhep.2002.36227]

3 **Pawlotsky JM**. The nature of interferon-alpha resistance in hepatitis C virus infection. *Curr Opin Infect Dis* 2003; **16**: 587-592 [PMID: 14624110 DOI: 10.1097/01.qco.0000104300.87920.03]

4 **Simmonds P**, Bukh J, Combet C, Deléage G, Enomoto N, Feinstone S, Halfon P, Inchauspé G, Kuiken C, Maertens G, Mizokami M, Murphy DG, Okamoto H, Pawlotsky JM, Penin F, Sablon E, Shin-I T, Stuyver LJ, Thiel HJ, Viazov S, Weiner AJ, Widell A. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology* 2005; **42**: 962-973 [PMID: 16149085 DOI: 10.1002/hep.20819]

5 **Rosen HR**, Gretch DR. Hepatitis C virus: current understanding and prospects for future therapies. *Mol Med Today* 1999; **5**: 393-399 [PMID: 10462751]

6 **Keyvani H**, Fazlalipour M, Monavari SH, Mollaie HR. Hepatitis C virus--proteins, diagnosis, treatment and new approaches for vaccine development. *Asian Pac J Cancer Prev* 2012; **13**: 5931-5949 [PMID: 23464383]

7 **Hu KQ**, Vierling JM, Redeker AG. Viral, host and interferon-related factors modulating the effect of interferon therapy for hepatitis C virus infection. *J Viral Hepat* 2001; **8**: 1-18 [PMID: 11155147]

8 **Ghany MG**, Strader DB, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C: an update. *Hepatology* 2009; **49**: 1335-1374 [PMID: 19330875 DOI: 10.1002/hep.22759]

9 **Kanda T**, Imazeki F, Yokosuka O. New antiviral therapies for chronic hepatitis C. *Hepatol Int* 2010; **4**: 548-561 [PMID: 21063477 DOI: 10.1007/s12072-010-9193-3]

10 **European Association for the Study of the Liver**. EASL Clinical Practice Guidelines: management of hepatitis C virus infection. *J Hepatol* 2011; **55**: 245-264 [PMID: 21371579 DOI: 10.1016/j.jhep.2011.02.023]

11 **Editors of the Drafting Committee for Hepatitis Management Guidelines: The Japan Society of Hepatology**. Guidelines for the Management of Hepatitis C Virus Infection: First edition, May 2012, The Japan Society of Hepatology. *Hepatol Res* 2013; **43**: 1-34 [PMID: 23332085 DOI: 10.1111/hepr.12020]

12 Available from: URL: http: //www.who.int

13 **Lawitz EJ**. Diagnosis and management of telaprevir-associated rash. *Gastroenterol Hepatol* (N Y) 2011; **7**: 469-471 [PMID: 22298982]

14 **Bartenschlager R**, Penin F, Lohmann V, André P. Assembly of infectious hepatitis C virus particles. *Trends Microbiol* 2011; **19**: 95-103 [PMID: 21146993 DOI: 10.1016/j.tim.2010.11.005]

15 **Cristina J**, del Pilar Moreno M, Moratorio G. Hepatitis C virus genetic variability in patients undergoing antiviral therapy. *Virus Res* 2007; **127**: 185-194 [PMID: 17449128 DOI: 10.1016/

j.virusres.2007.02.023]
16 **Varaklioti A**, Vassilaki N, Georgopoulou U, Mavromara P. Alternate translation occurs within the core coding region of the hepatitis C viral genome. *J Biol Chem* 2002; **277**: 17713-17721 [PMID: 11884417 DOI: 10.1074/jbc.M201722200]
17 **Xu Z**, Choi J, Lu W, Ou JH. Hepatitis C virus f protein is a short-lived protein associated with the endoplasmic reticulum. *J Virol* 2003; **77**: 1578-1583 [PMID: 12502871]
18 **Ma HC**, Lin TW, Li H, Iguchi-Ariga SM, Ariga H, Chuang YL, Ou JH, Lo SY. Hepatitis C virus ARFP/F protein interacts with cellular MM-1 protein and enhances the gene trans-activation activity of c-Myc. *J Biomed Sci* 2008; **15**: 417-425 [PMID: 18398700 DOI: 10.1007/s11373-008-9248-9]
19 **Shao SW**, Wu WB, Bian ZQ, Yu JG, Zhao P, Zhao LJ, Zhu SY, Qi ZT. Hepatitis C virus F protein inhibits cell apoptosis by activation of intracellular NF-kappaB pathway. *Hepatol Res* 2009; **39**: 282-289 [PMID: 19054148 DOI: 10.1111/j.1872-034X.2008.00452.x]
20 **Wu WB**, Shao SW, Zhao LJ, Luan J, Cao J, Gao J, Zhu SY, Qi ZT. Hepatitis C virus F protein up-regulates c-myc and down-regulates p53 in human hepatoma HepG2 cells. *Intervirology* 2007; **50**: 341-346 [PMID: 17700029 DOI: 10.1159/000107271]
21 **Friebe P**, Bartenschlager R. Genetic analysis of sequences in the 3' nontranslated region of hepatitis C virus that are important for RNA replication. *J Virol* 2002; **76**: 5326-5338 [PMID: 11991961]
22 **Kieft JS**, Zhou K, Jubin R, Doudna JA. Mechanism of ribosome recruitment by hepatitis C IRES RNA. *RNA* 2001; **7**: 194-206 [PMID: 11233977]
23 **Pestova TV**, Shatsky IN, Fletcher SP, Jackson RJ, Hellen CU. A prokaryotic-like mode of cytoplasmic eukaryotic ribosome binding to the initiation codon during internal translation initiation of hepatitis C and classical swine fever virus RNAs. *Genes Dev* 1998; **12**: 67-83 [PMID: 9420332]
24 **Smith DB**, Bukh J, Kuiken C, Muerhoff AS, Rice CM, Stapleton JT, Simmonds P. Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: updated criteria and genotype assignment web resource. *Hepatology* 2014; **59**: 318-327 [PMID: 24115039 DOI: 10.1002/hep.26744]
25 **Bukh J**, Purcell RH, Miller RH. Sequence analysis of the 5' noncoding region of hepatitis C virus. *Proc Natl Acad Sci USA* 1992; **89**: 4942-4946 [PMID: 1317578]
26 **Piñeiro D**, Martinez-Salas E. RNA structural elements of hepatitis C virus controlling viral RNA translation and the implications for viral pathogenesis. *Viruses* 2012; **4**: 2233-2250 [PMID: 23202462 DOI: 10.3390/v4102233]
27 **Argentini C**, Genovese D, Dettori S, Rapicetta M. HCV genetic variability: from quasispecies evolution to genotype classification. *Future Microbiol* 2009; **4**: 359-373 [PMID: 19327119 DOI: 10.2217/fmb.09.8]
28 **Le Guillou-Guillemette H**, Vallet S, Gaudy-Graffin C, Payan C, Pivert A, Goudeau A, Lunel-Fabiani F. Genetic diversity of the hepatitis C virus: impact and issues in the antiviral therapy. *World J Gastroenterol* 2007; **13**: 2416-2426 [PMID: 17552024]
29 **Domingo E**, Holland JJ. RNA virus mutations and fitness for survival. *Annu Rev Microbiol* 1997; **51**: 151-178 [PMID: 9343347 DOI: 10.1146/annurev.micro.51.1.151]
30 **Davis GL**, Esteban-Mur R, Rustgi V, Hoefs J, Gordon SC, Trepo C, Shiffman ML, Zeuzem S, Craxi A, Ling MH, Albrecht J. Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; **339**: 1493-1499 [PMID: 9819447 DOI: 10.1056/NEJM199811193392102]
31 **McHutchison JG**, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, Goodman ZD, Ling MH, Cort S, Albrecht JK. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; **339**: 1485-1492 [PMID: 9819446 DOI: 10.1056/NEJM199811193392101]
32 **Poynard T**, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G, Bain V, Heathcote J, Zeuzem S, Trepo C, Albrecht J. Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT) *Lancet* 1998; **352**: 1426-1432 [PMID: 9807989]
33 **Ogata N**, Alter HJ, Miller RH, Purcell RH. Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. *Proc Natl Acad Sci USA* 1991; **88**: 3392-3396 [PMID: 1849654]
34 **Zeuzem S**. Heterogeneous virologic response rates to interferon-based therapy in patients with chronic hepatitis C: who responds less well? *Ann Intern Med* 2004; **140**: 370-381 [PMID: 14996679]
35 **Fried MW**, Hadziyannis SJ. Treatment of chronic hepatitis C infection with peginterferons plus ribavirin. *Semin Liver Dis* 2004; **24** Suppl 2: 47-54 [PMID: 15346246 DOI: 10.1055/s-2004-832928]
36 **Ghany MG**, Nelson DR, Strader DB, Thomas DL, Seeff LB. An update on treatment of genotype 1 chronic hepatitis C virus infection: 2011 practice guideline by the American Association for the Study of Liver Diseases. *Hepatology* 2011; **54**: 1433-1444 [PMID: 21898493 DOI: 10.1002/hep.24641]
37 **Bacon BR**, Gordon SC, Lawitz E, Marcellin P, Vierling JM, Zeuzem S, Poordad F, Goodman ZD, Sings HL, Boparai N, Burroughs M, Brass CA, Albrecht JK, Esteban R. Boceprevir for previously treated chronic HCV genotype 1 infection. *N Engl J Med* 2011; **364**: 1207-1217 [PMID: 21449784 DOI: 10.1056/NEJMoa1009482]
38 **Jacobson IM**, McHutchison JG, Dusheiko G, Di Bisceglie AM, Reddy KR, Bzowej NH, Marcellin P, Muir AJ, Ferenci P, Flisiak R, George J, Rizzetto M, Shouval D, Sola R, Terg RA, Yoshida EM, Adda N, Bengtsson L, Sankoh AJ, Kieffer TL, George S, Kauffman RS, Zeuzem S. Telaprevir for previously untreated chronic hepatitis C virus infection. *N Engl J Med* 2011; **364**: 2405-2416 [PMID: 21696307 DOI: 10.1056/NEJMoa1012912]
39 **Poordad F**, McCone J, Bacon BR, Bruno S, Manns MP, Sulkowski MS, Jacobson IM, Reddy KR, Goodman ZD, Boparai N, DiNubile MJ, Sniukiene V, Brass CA, Albrecht JK, Bronowicki JP. Boceprevir for untreated chronic HCV genotype 1 infection. *N Engl J Med* 2011; **364**: 1195-1206 [PMID: 21449783 DOI: 10.1056/NEJMoa1010494]
40 **Zeuzem S**, Andreone P, Pol S, Lawitz E, Diago M, Roberts S, Focaccia R, Younossi Z, Foster GR, Horban A, Ferenci P, Nevens F, Müllhaupt B, Pockros P, Terg R, Shouval D, van Hoek B, Weiland O, Van Heeswijk R, De Meyer S, Luo D, Boogaerts G, Polo R, Picchio G, Beumont M. Telaprevir for retreatment of HCV infection. *N Engl J Med* 2011; **364**: 2417-2428 [PMID: 21696308 DOI: 10.1056/NEJMoa1013086]
41 **Rosenquist Å**, Samuelsson B, Johansson PO, Cummings MD, Lenz O, Raboisson P, Simmen K, Vendeville S, de Kock H, Nilsson M, Horvath A, Kalmeijer R, de la Rosa G, Beumont-Mauviel M. Discovery and development of simeprevir (TMC435), a HCV NS3/4A protease inhibitor. *J Med Chem* 2014; **57**: 1673-1693 [PMID: 24446688 DOI: 10.1021/jm401507s]
42 **Sofia MJ**, Bao D, Chang W, Du J, Nagarathnam D, Rachakonda S, Reddy PG, Ross BS, Wang P, Zhang HR, Bansal S, Espiritu C, Keilman M, Lam AM, Steuer HM, Niu C, Otto MJ, Furman PA. Discovery of a β-d-2'-deoxy-2'-α-fluoro-2'-β-C-methyluridine nucleotide prodrug (PSI-7977) for the treatment of hepatitis C virus. *J Med Chem* 2010; **53**: 7202-7218 [PMID: 20845908 DOI: 10.1021/jm100863x]
43 **Everson GT**, Sims KD, Rodriguez-Torres M, Hézode C, Lawitz E, Bourlière M, Loustaud-Ratti V, Rustgi V, Schwartz H, Tatum H, Marcellin P, Pol S, Thuluvath PJ, Eley T, Wang X, Huang SP, McPhee F, Wind-Rotolo M, Chung E, Pasquinelli C, Grasela DM, Gardiner DF. Efficacy of an interferon- and ribavirin-free regimen of daclatasvir, asunaprevir, and BMS-791325 in treatment-naive patients with HCV genotype 1 infection. *Gastroenterology* 2014; **146**: 420-429 [PMID: 24184132 DOI: 10.1053/j.gastro.2013.10.057]
44 **Fontana RJ**, Hughes EA, Bifano M, Appelman H, Dimitrova D, Hindes R, Symonds WT. Sofosbuvir and daclatasvir combination therapy in a liver transplant recipient with severe recurrent cholestatic hepatitis C. *Am J Transplant* 2013; **13**: 1601-1605

[PMID: 23593993 DOI: 10.1111/ajt.12209]
45 **Jacobson IM**, Gordon SC, Kowdley KV, Yoshida EM, Rodriguez-Torres M, Sulkowski MS, Shiffman ML, Lawitz E, Everson G, Bennett M, Schiff E, Al-Assi MT, Subramanian GM, An D, Lin M, McNally J, Brainard D, Symonds WT, McHutchison JG, Patel K, Feld J, Pianko S, Nelson DR. Sofosbuvir for hepatitis C genotype 2 or 3 in patients without treatment options. *N Engl J Med* 2013; **368**: 1867-1877 [PMID: 23607593 DOI: 10.1056/NEJMoa1214854]
46 **Lawitz E**, Mangia A, Wyles D, Rodriguez-Torres M, Hassanein T, Gordon SC, Schultz M, Davis MN, Kayali Z, Reddy KR, Jacobson IM, Kowdley KV, Nyberg L, Subramanian GM, Hyland RH, Arterburn S, Jiang D, McNally J, Brainard D, Symonds WT, McHutchison JG, Sheikh AM, Younossi Z, Gane EJ. Sofosbuvir for previously untreated chronic hepatitis C infection. *N Engl J Med* 2013; **368**: 1878-1887 [PMID: 23607594 DOI: 10.1056/NEJMoa1214853]
47 **Sulkowski MS**, Gardiner DF, Rodriguez-Torres M, Reddy KR, Hassanein T, Jacobson I, Lawitz E, Lok AS, Hinestrosa F, Thuluvath PJ, Schwartz H, Nelson DR, Everson GT, Eley T, Wind-Rotolo M, Huang SP, Gao M, Hernandez D, McPhee F, Sherman D, Hindes R, Symonds W, Pasquinelli C, Grasela DM. Daclatasvir plus sofosbuvir for previously treated or untreated chronic HCV infection. *N Engl J Med* 2014; **370**: 211-221 [PMID: 24428467 DOI: 10.1056/NEJMoa1306218]
48 **Cristina J**. Genetic diversity and evolution of hepatitis C virus in the Latin American region. *J Clin Virol* 2005; **34** Suppl 2: S1-S7 [PMID: 16461234]
49 **González-Candelas F**, López-Labrador FX, Bracho MA. Recombination in hepatitis C virus. *Viruses* 2011; **3**: 2006-2024 [PMID: 22069526 DOI: 10.3390/v3102006]
50 **Bartenschlager R**, Lohmann V. Replication of hepatitis C virus. *J Gen Virol* 2000; **81**: 1631-1648 [PMID: 10859368]
51 **Kim T**, Mudry RA, Rexrode CA, Pathak VK. Retroviral mutation rates and A-to-G hypermutations during different stages of retroviral replication. *J Virol* 1996; **70**: 7594-7602 [PMID: 8892879]
52 **Biebricher CK**, Eigen M. The error threshold. *Virus Res* 2005; **107**: 117-127 [PMID: 15649558 DOI: 10.1016/j.virusres.2004.11.002]
53 **Chambers TJ**, Fan X, Droll DA, Hembrador E, Slater T, Nickells MW, Dustin LB, Dibisceglie AM. Quasispecies heterogeneity within the E1/E2 region as a pretreatment variable during pegylated interferon therapy of chronic hepatitis C virus infection. *J Virol* 2005; **79**: 3071-3083 [PMID: 15709027 DOI: 10.1128/JVI.79.5.3071-3083.2005]
54 **Domingo E**, Baranowski E, Ruiz-Jarabo CM, Martín-Hernández AM, Sáiz JC, Escarmís C. Quasispecies structure and persistence of RNA viruses. *Emerg Infect Dis* 1998; **4**: 521-527 [PMID: 9866728 DOI: 10.3201/eid0404.980402]
55 **Domingo E**, Escarmís C, Lázaro E, Manrubia SC. Quasispecies dynamics and RNA virus extinction. *Virus Res* 2005; **107**: 129-139 [PMID: 15649559 DOI: 10.1016/j.virusres.2004.11.003]
56 **Feliu A**, Gay E, García-Retortillo M, Saiz JC, Forns X. Evolution of hepatitis C virus quasispecies immediately following liver transplantation. *Liver Transpl* 2004; **10**: 1131-1139 [PMID: 15350003 DOI: 10.1002/lt.20206]
57 **Laskus T**, Wilkinson J, Gallegos-Orozco JF, Radkowski M, Adair DM, Nowicki M, Operskalski E, Buskell Z, Seeff LB, Vargas H, Rakela J. Analysis of hepatitis C virus quasispecies transmission and evolution in patients infected through blood transfusion. *Gastroenterology* 2004; **127**: 764-776 [PMID: 15362033]
58 **Martell M**, Esteban JI, Quer J, Genescà J, Weiner A, Esteban R, Guardia J, Gómez J. Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution. *J Virol* 1992; **66**: 3225-3229 [PMID: 1313927]
59 **Eigen M**, Gardiner W, Schuster P, Winkler-Oswatitsch R. The origin of genetic information. *Sci Am* 1981; **244**: 88-92, 96, et passim [PMID: 6164094]
60 **Eigen M**, Schuster P. The hypercycle. A principle of natural self-organization. Part A: Emergence of the hypercycle. *Naturwissenschaften* 1977; **64**: 541-565 [PMID: 593400]
61 **Eigen M**. Selforganization of matter and the evolution of biological macromolecules. *Naturwissenschaften* 1971; **58**: 465-523 [PMID: 4942363]
62 **Domingo E**, Martin V, Perales C, Grande-Pérez A, García-Arriaza J, Arias A. Viruses as quasispecies: biological implications. *Curr Top Microbiol Immunol* 2006; **299**: 51-82 [PMID: 16568896]
63 **Pellerin M**, Lopez-Aguirre Y, Penin F, Dhumeaux D, Pawlotsky JM. Hepatitis C virus quasispecies variability modulates nonstructural protein 5A transcriptional activation, pointing to cellular compartmentalization of virus-host interactions. *J Virol* 2004; **78**: 4617-4627 [PMID: 15078944]
64 **Domingo E**, Gomez J. Quasispecies and its impact on viral hepatitis. *Virus Res* 2007; **127**: 131-150 [PMID: 17349710 DOI: 10.1016/j.virusres.2007.02.001]
65 **Martín V**, Domingo E. Influence of the mutant spectrum in viral evolution: focused selection of antigenic variants in a reconstructed viral quasispecies. *Mol Biol Evol* 2008; **25**: 1544-1554 [PMID: 18436553 DOI: 10.1093/molbev/msn099]
66 **Vignuzzi M**, Stone JK, Arnold JJ, Cameron CE, Andino R. Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population. *Nature* 2006; **439**: 344-348 [PMID: 16327776 DOI: 10.1038/nature04388]
67 **Wilke CO**. Quasispecies theory in the context of population genetics. *BMC Evol Biol* 2005; **5**: 44 [PMID: 16107214 DOI: 10.1186/1471-2148-5-44]
68 **Elena SF**, Sanjuán R. Adaptive value of high mutation rates of RNA viruses: separating causes from consequences. *J Virol* 2005; **79**: 11555-11558 [PMID: 16140732 DOI: 10.1128/JVI.79.18.11555-11558.2005]
69 **Holmes EC**. Error thresholds and the constraints to RNA virus evolution. *Trends Microbiol* 2003; **11**: 543-546 [PMID: 14659685]
70 **Arnold JJ**, Vignuzzi M, Stone JK, Andino R, Cameron CE. Remote site control of an active site fidelity checkpoint in a viral RNA-dependent RNA polymerase. *J Biol Chem* 2005; **280**: 25706-25716 [PMID: 15878882 DOI: 10.1074/jbc.M503444200]
71 **Pfeiffer JK**, Kirkegaard K. Ribavirin resistance in hepatitis C virus replicon-containing cell lines conferred by changes in the cell line or mutations in the replicon RNA. *J Virol* 2005; **79**: 2346-2355 [PMID: 15681435 DOI: 10.1128/JVI.79.4.2346-2355.2005]
72 **Vignuzzi M**, Stone JK, Andino R. Ribavirin and lethal mutagenesis of poliovirus: molecular mechanisms, resistance and biological implications. *Virus Res* 2005; **107**: 173-181 [PMID: 15649563 DOI: 10.1016/j.virusres.2004.11.007]
73 **Fint SJEL**, Racaniello VR, Skalka AM. Principles of virology. Third ed. Washington, DC, United States of America: ASM Press, 2009: 569
74 **Pawlotsky JM**. Hepatitis C virus population dynamics during infection. *Curr Top Microbiol Immunol* 2006; **299**: 261-284 [PMID: 16568902]
75 **Baccam P**, Thompson RJ, Fedrigo O, Carpenter S, Cornette JL. PAQ: Partition Analysis of Quasispecies. *Bioinformatics* 2001; **17**: 16-22 [PMID: 11222259]
76 **Cody SH**, Nainan OV, Garfein RS, Meyers H, Bell BP, Shapiro CN, Meeks EL, Pitt H, Mouzin E, Alter MJ, Margolis HS, Vugia DJ. Hepatitis C virus transmission from an anesthesiologist to a patient. *Arch Intern Med* 2002; **162**: 345-350 [PMID: 11822928]
77 **Gretch DR**, Polyak SJ, Wilson JJ, Carithers RL, Perkins JD, Corey L. Tracking hepatitis C virus quasispecies major and minor variants in symptomatic and asymptomatic liver transplant recipients. *J Virol* 1996; **70**: 7622-7631 [PMID: 8892882]
78 **Lin HJ**, Seeff LB, Barbosa L, Hollinger FB. Occurrence of identical hypervariable region 1 sequences of hepatitis C virus in transfusion recipients and their respective blood donors: divergence over time. *Hepatology* 2001; **34**: 424-429 [PMID: 11481629 DOI: 10.1053/jhep.2001.26635]
79 **Manzin A**, Solforosi L, Debiaggi M, Zara F, Tanzi E, Romanò L, Zanetti AR, Clementi M. Dominant role of host selective pressure in driving hepatitis C virus evolution in perinatal infection. *J Virol* 2000; **74**: 4327-4334 [PMID: 10756048]

80 **Weiner AJ**, Thaler MM, Crawford K, Ching K, Kansopon J, Chien DY, Hall JE, Hu F, Houghton M. A unique, predominant hepatitis C virus variant found in an infant born to a mother with multiple variants. *J Virol* 1993; **67**: 4365-4368 [PMID: 8389933]

81 **Hijikata M**, Mizuno K, Rikihisa T, Shimizu YK, Iwamoto A, Nakajima N, Yoshikura H. Selective transmission of hepatitis C virus in vivo and in vitro. *Arch Virol* 1995; **140**: 1623-1628 [PMID: 7487493]

82 **Kojima M**, Osuga T, Tsuda F, Tanaka T, Okamoto H. Influence of antibodies to the hypervariable region of E2/NS1 glycoprotein on the selective replication of hepatitis C virus in chimpanzees. *Virology* 1994; **204**: 665-672 [PMID: 7941335 DOI: 10.1006/viro.1994.1582]

83 **Cuevas JM**, Torres-Puente M, Jiménez-Hernández N, Bracho MA, García-Robles I, Carnicer F, Olmo JD, Ortega E, González-Candelas F, Moya A. Combined therapy of interferon plus ribavirin promotes multiple adaptive solutions in hepatitis C virus. *J Med Virol* 2009; **81**: 650-656 [PMID: 19235859 DOI: 10.1002/jmv.21460]

84 **Cuevas JM**, Gonzalez M, Torres-Puente M, Jiménez-Hernández N, Bracho MA, García-Robles I, González-Candelas F, Moya A. The role of positive selection in hepatitis C virus. *Infect Genet Evol* 2009; **9**: 860-866 [PMID: 19463971 DOI: 10.1016/j.meegid.2009.05.007]

85 **Puig-Basagoiti F**, Forns X, Furcić I, Ampurdanés S, Giménez-Barcons M, Franco S, Sánchez-Tapias JM, Saiz JC. Dynamics of hepatitis C virus NS5A quasispecies during interferon and ribavirin therapy in responder and non-responder patients with genotype 1b chronic hepatitis C. *J Gen Virol* 2005; **86**: 1067-1075 [PMID: 15784900 DOI: 10.1099/vir.0.80526-0]

86 **Ueda E**, Enomoto N, Sakamoto N, Hamano K, Sato C, Izumi N, Watanabe M. Changes of HCV quasispecies during combination therapy with interferon and ribavirin. *Hepatol Res* 2004; **29**: 89-96 [PMID: 15163430 DOI: 10.1016/j.hepres.2004.02.014]

87 **Ojosnegros S**, Agudo R, Sierra M, Briones C, Sierra S, González-López C, Domingo E, Cristina J. Topology of evolving, mutagenized viral populations: quasispecies expansion, compression, and operation of negative selection. *BMC Evol Biol* 2008; **8**: 207 [PMID: 18637173 DOI: 10.1186/1471-2148-8-207]

88 **Torres-Puente M**, Cuevas JM, Jiménez-Hernández N, Bracho MA, García-Robles I, Wrobel B, Carnicer F, Del Olmo J, Ortega E, Moya A, González-Candelas F. Genetic variability in hepatitis C virus and its role in antiviral treatment response. *J Viral Hepat* 2008; **15**: 188-199 [PMID: 18233992 DOI: 10.1111/j.1365-2893.2007.00929.x]

89 **Ge D**, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, Sulkowski M, McHutchison JG, Goldstein DB. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 2009; **461**: 399-401 [PMID: 19684573 DOI: 10.1038/nature08309]

90 **Rallón NI**, Naggie S, Benito JM, Medrano J, Restrepo C, Goldstein D, Shianna KV, Vispo E, Thompson A, McHutchison J, Soriano V. Association of a single nucleotide polymorphism near the interleukin-28B gene with response to hepatitis C therapy in HIV/hepatitis C virus-coinfected patients. *AIDS* 2010; **24**: F23-F29 [PMID: 20389235 DOI: 10.1097/QAD.0b013e3283391d6d]

91 **Suppiah V**, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, Riordan S, Sheridan D, Smedile A, Fragomeli V, Müller T, Bahlo M, Stewart GJ, Booth DR, George J. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet* 2009; **41**: 1100-1104 [PMID: 19749758 DOI: 10.1038/ng.447]

92 **Tanaka Y**, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009; **41**: 1105-1109 [PMID: 19749757 DOI: 10.1038/ng.449]

93 **Urban TJ**, Thompson AJ, Bradrick SS, Fellay J, Schuppan D, Cronin KD, Hong L, McKenzie A, Patel K, Shianna KV, McHutchison JG, Goldstein DB, Afdhal N. IL28B genotype is associated with differential expression of intrahepatic interferon-stimulated genes in patients with chronic hepatitis C. *Hepatology* 2010; **52**: 1888-1896 [PMID: 20931559 DOI: 10.1002/hep.23912]

94 **Yuan H**, Adams-Huet B, Petersen T, Attar N, Lee WM, Jain MK. A single nucleotide polymorphism in IL28B affects viral evolution of hepatitis C quasispecies after pegylated interferon and ribavirin therapy. *J Med Virol* 2012; **84**: 1913-1919 [PMID: 23080496 DOI: 10.1002/jmv.23407]

95 **Aparicio E**, Franco S, Parera M, Andrés C, Tural C, Clotet B, Martínez MA. Complexity and catalytic efficiency of hepatitis C virus (HCV) NS3 and NS4A protease quasispecies influence responsiveness to treatment with pegylated interferon plus ribavirin in HCV/HIV-coinfected patients. *J Virol* 2011; **85**: 5961-5969 [PMID: 21471227 DOI: 10.1128/JVI.00308-11]

96 **Miura M**, Maekawa S, Kadokura M, Sueki R, Komase K, Shindo H, Ohmori T, Kanayama A, Shindo K, Amemiya F, Nakayama Y, Kitamura T, Uetake T, Inoue T, Sakamoto M, Okada S, Enomoto N. Analysis of viral amino acids sequences and the IL28B SNP influencing the development of hepatocellular carcinoma in chronic hepatitis C. *Hepatol Int* 2011; Epub ahead of print [PMID: 22020823 DOI: 10.1007/s12072-011-9307-6]

97 **Valenti L**, Pulixi E, La Spina S. IL28B, HCV core mutations, and hepatocellular carcinoma: does host genetic make-up shape viral evolution in response to immunity? *Hepa Inter* 2012; **6**: 356-359 [DOI: 10.1007/s12072-011-9327-2]

98 **Bushman FD**, Hoffmann C, Ronen K, Malani N, Minkah N, Rose HM, Tebas P, Wang GP. Massively parallel pyrosequencing in HIV research. *AIDS* 2008; **22**: 1411-1415 [PMID: 18614863 DOI: 10.1097/QAD.0b013e3282fc972e]

99 **Eriksson N**, Pachter L, Mitsuya Y, Rhee SY, Wang C, Gharizadeh B, Ronaghi M, Shafer RW, Beerenwinkel N. Viral population estimation using pyrosequencing. *PLoS Comput Biol* 2008; **4**: e1000074 [PMID: 18437230 DOI: 10.1371/journal.pcbi.1000074]

100 **Margeridon-Thermet S**, Shulman NS, Ahmed A, Shahriar R, Liu T, Wang C, Holmes SP, Babrzadeh F, Gharizadeh B, Hanczaruk B, Simen BB, Egholm M, Shafer RW. Ultra-deep pyrosequencing of hepatitis B virus quasispecies from nucleoside and nucleotide reverse-transcriptase inhibitor (NRTI)-treated patients and NRTI-naive patients. *J Infect Dis* 2009; **199**: 1275-1285 [PMID: 19301976 DOI: 10.1086/597808]

101 **Luciani F**, Bull RA, Lloyd AR. Next generation deep sequencing and vaccine design: today and tomorrow. *Trends Biotechnol* 2012; **30**: 443-452 [PMID: 22721705 DOI: 10.1016/j.tibtech.2012.05.005]

102 **Metzker ML**. Sequencing technologies - the next generation. *Nat Rev Genet* 2010; **11**: 31-46 [PMID: 19997069 DOI: 10.1038/nrg2626]

103 **Schadt EE**, Turner S, Kasarskis A. A window into third-generation sequencing. *Hum Mol Genet* 2010; **19**: R227-R240 [PMID: 20858600 DOI: 10.1093/hmg/ddq416]

104 **Kelleher RJ**, Geigenmüller U, Hovhannisyan H, Trautman E, Pinard R, Rathmell B, Carpenter R, Margulies D. High-throughput sequencing of mGluR signaling pathway genes reveals enrichment of rare variants in autism. *PLoS One* 2012; **7**: e35003 [PMID: 22558107 DOI: 10.1371/journal.pone.0035003]

105 **Svarovskaia ES**, Martin R, McHutchison JG, Miller MD, Mo H. Abundant drug-resistant NS3 mutants detected by deep sequencing in hepatitis C virus-infected patients undergoing NS3 protease inhibitor monotherapy. *J Clin Microbiol* 2012; **50**: 3267-3274 [PMID: 22837328 DOI: 10.1128/JCM.00838-12]

106 **Leitner T**, Halapi E, Scarlatti G, Rossi P, Albert J, Fenyö EM, Uhlén M. Analysis of heterogeneous viral populations by direct DNA sequencing. *Biotechniques* 1993; **15**: 120-127 [PMID: 8363827]

107 **Fonseca-Coronado S**, Escobar-Gutiérrez A, Ruiz-Tovar K, Cruz-Rivera MY, Rivera-Osorio P, Vazquez-Pichardo M, Carpio-Pedroza JC, Ruíz-Pacheco JA, Cazares F, Vaughan G. Specific detection

of naturally occurring hepatitis C virus mutants with resistance to telaprevir and boceprevir (protease inhibitors) among treatment-naïve infected individuals. *J Clin Microbiol* 2012; **50**: 281-287 [PMID: 22116161 DOI: 10.1128/JCM.05842-11]

108 **Hiraga N**, Abe H, Imamura M, Tsuge M, Takahashi S, Hayes CN, Ochi H, Tateno C, Yoshizato K, Nakamura Y, Kamatani N, Chayama K. Impact of viral amino acid substitutions and host interleukin-28b polymorphism on replication and susceptibility to interferon of hepatitis C virus. *Hepatology* 2011; **54**: 764-771 [PMID: 21618576 DOI: 10.1002/hep.24453]

109 **Ninomiya M**, Ueno Y, Funayama R, Nagashima T, Nishida Y, Kondo Y, Inoue J, Kakazu E, Kimura O, Nakayama K, Shimosegawa T. Use of illumina deep sequencing technology to differentiate hepatitis C virus variants. *J Clin Microbiol* 2012; **50**: 857-866 [PMID: 22205816 DOI: 10.1128/JCM.05715-11]

110 **Escobar-Gutiérrez A**, Vazquez-Pichardo M, Cruz-Rivera M, Rivera-Osorio P, Carpio-Pedroza JC, Ruíz-Pacheco JA, Ruiz-Tovar K, Vaughan G. Identification of hepatitis C virus transmission using a next-generation sequencing approach. *J Clin Microbiol* 2012; **50**: 1461-1463 [PMID: 22301026 DOI: 10.1128/JCM.00005-12]

111 **Moreno P**, Alvarez M, López L, Moratorio G, Casane D, Castells M, Castro S, Cristina J, Colina R. Evidence of recombination in Hepatitis C Virus populations infecting a hemophiliac patient. *Virol J* 2009; **6**: 203 [PMID: 19922637 DOI: 10.1186/1743-422X-6-203]

112 **Worobey M**, Holmes EC. Evolutionary aspects of recombination in RNA viruses. *J Gen Virol* 1999; **80** (Pt 10): 2535-2543 [PMID: 10573145]

113 **Lai MM**. RNA recombination in animal and plant viruses. *Microbiol Rev* 1992; **56**: 61-79 [PMID: 1579113]

114 **Copper PD**, Steiner-Pryor A, Scotti PD, Delong D. On the nature of poliovirus genetic recombinants. *J Gen Virol* 1974; **23**: 41-49 [PMID: 4364875]

115 **Kew O**, Nottay B. Evolution of the oral polio vaccine strains in humans occurs by both mutation and intra-molecular recombination. In: Chanock RM LR, editor. Modern approaches to vaccines. Molecular and chemical basis of virus virulence and immunogenicity. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, 1984: 457

116 **Diemer GS**, Stedman KM. A novel virus genome discovered in an extreme environment suggests recombination between unrelated groups of RNA and DNA viruses. *Biol Direct* 2012; **7**: 13 [PMID: 22515485 DOI: 10.1186/1745-6150-7-13]

117 **Becher P**, Orlich M, Thiel HJ. RNA recombination between persisting pestivirus and a vaccine strain: generation of cytopathogenic virus and induction of lethal disease. *J Virol* 2001; **75**: 6256-6264 [PMID: 11413291 DOI: 10.1128/JVI.75.14.6256-6264.2001]

118 **Holmes EC**, Worobey M, Rambaut A. Phylogenetic evidence for recombination in dengue virus. *Mol Biol Evol* 1999; **16**: 405-409 [PMID: 10331266]

119 **Tolou HJ**, Couissinier-Paris P, Durand JP, Mercier V, de Pina JJ, de Micco P, Billoir F, Charrel RN, de Lamballerie X. Evidence for recombination in natural populations of dengue virus type 1 based on the analysis of complete genome sequences. *J Gen Virol* 2001; **82**: 1283-1290 [PMID: 11369871]

120 **Twiddy SS**, Holmes EC. The extent of homologous recombination in members of the genus Flavivirus. *J Gen Virol* 2003; **84**: 429-440 [PMID: 12560576]

121 **Uzcategui NY**, Camacho D, Comach G, Cuello de Uzcategui R, Holmes EC, Gould EA. Molecular epidemiology of dengue type 2 virus in Venezuela: evidence for in situ virus evolution and recombination. *J Gen Virol* 2001; **82**: 2945-2953 [PMID: 11714970]

122 **Worobey M**, Holmes EC. Homologous recombination in GB virus C/hepatitis G virus. *Mol Biol Evol* 2001; **18**: 254-261 [PMID: 11158384]

123 **Yun Z**, Lara C, Johansson B, Lorenzana de Rivera I, Sönnerborg A. Discrepancy of hepatitis C virus genotypes as determined by phylogenetic analysis of partial NS5 and core sequences. *J Med Virol* 1996; **49**: 155-160 [PMID: 8818959 DOI: 10.1002/(SICI)1096-9071(199607)49:3<155::AID-JMV1>3.0.CO;2-3]

124 **Kalinina O**, Norder H, Mukomolov S, Magnius LO. A natural intergenotypic recombinant of hepatitis C virus identified in St. Petersburg. *J Virol* 2002; **76**: 4034-4043 [PMID: 11907242]

125 **Moreau I**, Hegarty S, Levis J, Sheehy P, Crosbie O, Kenny-Walsh E, Fanning LJ. Serendipitous identification of natural intergenotypic recombinants of hepatitis C in Ireland. *Virol J* 2006; **3**: 95 [PMID: 17107614 DOI: 10.1186/1743-422X-3-95]

126 **Kurbanov F**, Tanaka Y, Avazova D, Khan A, Sugauchi F, Kan N, Kurbanova-Khudayberganova D, Khikmatullaeva A, Musabaev E, Mizokami M. Detection of hepatitis C virus natural recombinant RF1_2k/1b strain among intravenous drug users in Uzbekistan. *Hepatol Res* 2008; **38**: 457-464 [PMID: 18034826 DOI: 10.1111/j.1872-034X.2007.00293.x]

127 **Demetriou VL**, Kyriakou E, Kostrikis LG. Near-full genome characterisation of two natural intergenotypic 2k/1b recombinant hepatitis C virus isolates. *Adv Virol* 2011; **2011**: 710438 [PMID: 22315602 DOI: 10.1155/2011/710438]

128 **Morel V**, Descamps V, François C, Fournier C, Brochot E, Capron D, Duverlie G, Castelain S. Emergence of a genomic variant of the recombinant 2k/1b strain during a mixed Hepatitis C infection: a case report. *J Clin Virol* 2010; **47**: 382-386 [PMID: 20153975 DOI: 10.1016/j.jcv.2010.01.011]

129 **Tallo T**, Norder H, Tefanova V, Krispin T, Schmidt J, Ilmoja M, Orgulas K, Pruunsild K, Priimägi L, Magnius LO. Genetic characterization of hepatitis C virus strains in Estonia: fluctuations in the predominating subtype with time. *J Med Virol* 2007; **79**: 374-382 [PMID: 17311333 DOI: 10.1002/jmv.20828]

130 **Nopporpanth S**, Lien TX, Poovorawan Y, Smits SL, Osterhaus AD, Haagmans BL. Identification of a naturally occurring recombinant genotype 2/6 hepatitis C virus. *J Virol* 2006; **80**: 7569-7577 [PMID: 16840336 DOI: 10.1128/JVI.00312-06]

131 **Lee YM**, Lin HJ, Chen YJ, Lee CM, Wang SF, Chang KY, Chen TL, Liu HF, Chen YM. Molecular epidemiology of HCV genotypes among injection drug users in Taiwan: Full-length sequences of two new subtype 6w strains and a recombinant form_2b6w. *J Med Virol* 2010; **82**: 57-68 [PMID: 19950240 DOI: 10.1002/jmv.21658]

132 **Legrand-Abravanel F**, Claudinon J, Nicot F, Dubois M, Chapuy-Regaud S, Sandres-Saune K, Pasquier C, Izopet J. New natural intergenotypic (2/5) recombinant of hepatitis C virus. *J Virol* 2007; **81**: 4357-4362 [PMID: 17267503 DOI: 10.1128/JVI.02639-06]

133 **Du H**, Qi Y, Hao F, Huang Y, Mao L, Ji S, Huang M, Qin C, Yan R, Zhu X, Zhang C. Complex patterns of HCV epidemic in Suzhou: evidence for dual infection and HCV recombination in East China. *J Clin Virol* 2012; **54**: 207-212 [PMID: 22595306 DOI: 10.1016/j.jcv.2012.04.017]

134 **Bhattacharya D**, Accola MA, Ansari IH, Striker R, Rehrauer WM. Naturally occurring genotype 2b/1a hepatitis C virus in the United States. *Virol J* 2011; **8**: 458 [PMID: 21967740 DOI: 10.1186/1743-422X-8-458]

135 **Yokoyama K**, Takahashi M, Nishizawa T, Nagashima S, Jirintai S, Yotsumoto S, Okamoto H, Momoi MY. Identification and characterization of a natural inter-genotypic (2b/1b) recombinant hepatitis C virus in Japan. *Arch Virol* 2011; **156**: 1591-1601 [PMID: 21643787 DOI: 10.1007/s00705-011-1038-4]

136 **Hoshino H**, Hino K, Miyakawa H, Takahashi K, Akbar SM, Mishiro S. Inter-genotypic recombinant hepatitis C virus strains in Japan noted by discrepancies between immunoassay and sequencing. *J Med Virol* 2012; **84**: 1018-1024 [PMID: 22585717 DOI: 10.1002/jmv.23300]

137 **Kageyama S**, Agdamag DM, Alesna ET, Leaño PS, Heredia AM, Abellanosa-Tac-An IP, Jereza LD, Tanimoto T, Yamamura J, Ichimura H. A natural inter-genotypic (2b/1b) recombinant of hepatitis C virus in the Philippines. *J Med Virol* 2006; **78**: 1423-1428 [PMID: 16998890 DOI: 10.1002/jmv.20714]

138 **Morel V**, Fournier C, François C, Brochot E, Helle F, Duverlie G, Castelain S. Genetic recombination of the hepatitis C virus: clinical implications. *J Viral Hepat* 2011; **18**: 77-83 [PMID: 21235686 DOI: 10.1111/j.1365-2893.2010.01367.x]

139 **Kalinina O**, Norder H, Magnius LO. Full-length open reading frame of a recombinant hepatitis C virus strain from St Petersburg:

proposed mechanism for its formation. *J Gen Virol* 2004; **85**: 1853-1857 [PMID: 15218169 DOI: 10.1099/vir.0.79984-0]

140 **Calado RA**, Rocha MR, Parreira R, Piedade J, Venenno T, Esteves A. Hepatitis C virus subtypes circulating among intravenous drug users in Lisbon, Portugal. *J Med Virol* 2011; **83**: 608-615 [PMID: 21328374 DOI: 10.1002/jmv.21955]

141 **Colina R**, Casane D, Vasquez S, García-Aguirre L, Chunga A, Romero H, Khan B, Cristina J. Evidence of intratypic recombination in natural populations of hepatitis C virus. *J Gen Virol* 2004; **85**: 31-37 [PMID: 14718617]

142 **Cristina J**, Colina R. Evidence of structural genomic region recombination in Hepatitis C virus. *Virol J* 2006; **3**: 53 [PMID: 16813646 DOI: 10.1186/1743-422X-3-53]

143 **Ross RS**, Verbeeck J, Viazov S, Lemey P, Van Ranst M, Roggendorf M. Evidence for a complex mosaic genome pattern in a full-length hepatitis C virus sequence. *Evol Bioinform Online* 2008; **4**: 249-254 [PMID: 19204822]

144 **Shi W**, Freitas IT, Zhu C, Zheng W, Hall WW, Higgins DG. Recombination in hepatitis C virus: identification of four novel naturally occurring inter-subtype recombinants. *PLoS One* 2012; **7**: e41997 [PMID: 22911872 DOI: 10.1371/journal.pone.0041997]

145 **Moreno MP**, Casane D, López L, Cristina J. Evidence of recombination in quasispecies populations of a Hepatitis C Virus patient undergoing anti-viral therapy. *Virol J* 2006; **3**: 87 [PMID: 17062150 DOI: 10.1186/1743-422X-3-87]

146 **Sentandreu V**, Jiménez-Hernández N, Torres-Puente M, Bracho MA, Valero A, Gosalbes MJ, Ortega E, Moya A, González-Candelas F. Evidence of recombination in intrapatient populations of hepatitis C virus. *PLoS One* 2008; **3**: e3239 [PMID: 18800167 DOI: 10.1371/journal.pone.0003239]

147 **Palmer BA**, Moreau I, Levis J, Harty C, Crosbie O, Kenny-Walsh E, Fanning LJ. Insertion and recombination events at hypervariable region 1 over 9.6 years of hepatitis C virus chronic infection. *J Gen Virol* 2012; **93**: 2614-2624 [PMID: 22971825 DOI: 10.1099/vir.0.045344-0]

148 **Enomoto N**, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Izumi N, Marumo F, Sato C. Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. *J Clin Invest* 1995; **96**: 224-230 [PMID: 7542279 DOI: 10.1172/JCI118025]

149 **Gale MJ**, Korth MJ, Tang NM, Tan SL, Hopkins DA, Dever TE, Polyak SJ, Gretch DR, Katze MG. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. *Virology* 1997; **230**: 217-227 [PMID: 9143277 DOI: 10.1006/viro.1997.8493]

150 **Giménez-Barcons M**, Franco S, Suárez Y, Forns X, Ampurdanès S, Puig-Basagoiti F, Sánchez-Fueyo A, Barrera JM, Llovet JM, Bruix J, Sánchez-Tapias JM, Rodés J, Saiz JC. High amino acid variability within the NS5A of hepatitis C virus (HCV) is associated with hepatocellular carcinoma in patients with HCV-1b-related cirrhosis. *Hepatology* 2001; **34**: 158-167 [PMID: 11431747 DOI: 10.1053/jhep.2001.25512]

151 **Witherell GW**, Beineke P. Statistical analysis of combined substitutions in nonstructural 5A region of hepatitis C virus and interferon response. *J Med Virol* 2001; **63**: 8-16 [PMID: 11130893]

152 **Gale M**, Blakely CM, Kwieciszewski B, Tan SL, Dossett M, Tang NM, Korth MJ, Polyak SJ, Gretch DR, Katze MG. Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: molecular mechanisms of kinase regulation. *Mol Cell Biol* 1998; **18**: 5208-5218 [PMID: 9710605]

153 **Pawlotsky JM**, Germanidis G, Neumann AU, Pellerin M, Frainais PO, Dhumeaux D. Interferon resistance of hepatitis C virus genotype 1b: relationship to nonstructural 5A gene quasispecies mutations. *J Virol* 1998; **72**: 2795-2805 [PMID: 9525599]

154 **Costa-Mattioli M**, Ferré V, Casane D, Perez-Bercoff R, Coste-Burel M, Imbert-Marcille BM, Andre EC, Bressollette-Bodin C, Billaudel S, Cristina J. Evidence of recombination in natural populations of hepatitis A virus. *Virology* 2003; **311**: 51-59 [PMID: 12832202]

155 **Yusim K**, Richardson R, Tao N, Dalwani A, Agrawal A, Szinger J, Funkhouser R, Korber B, Kuiken C. Los alamos hepatitis C immunology database. *Appl Bioinformatics* 2005; **4**: 217-225 [PMID: 16309340]

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REVIEW

Non-alcoholic fatty liver disease: The diagnosis and management

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is now the most frequent chronic liver disease that occurs across all age groups and is recognized to occur in 14%-30% of the general population, representing a serious and growing clinical problem due to the growing prevalence of obesity and overweight. Histologically, it resembles alcoholic liver injury but occurs in patients who deny significant alcohol consumption. NAFLD encompasses a spectrum of conditions, ranging from benign hepatocellular steatosis to inflammatory nonalcoholic steatohepatitis, fibrosis, and cirrhosis. The majority of hepatocellular lipids are stored as triglycerides, but other lipid metabolites, such as free fatty acids, cholesterol, and phospholipids, may also be present and play a role in disease progression. NAFLD is associated with obesity and insulin resistance and is considered the hepatic manifestation of the metabolic syndrome, a combination of medical conditions including type 2 diabetes mellitus, hypertension, hyperlipidemia, and visceral adiposity. Confirmation of the diagnosis of NAFLD can usually be achieved by imaging studies; however, staging the disease requires a liver biopsy. Current treatment relies on weight loss and exercise, although various insulin-sensitizing agents, antioxidants and medications appear promising. The aim of this review is to highlight the current information regarding epidemiology, diagnosis, and management of NAFLD as well as new information about pathogenesis, diagnosis and management of this disease.

Key words: Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Liver disease

Core tip: Non-alcoholic fatty liver disease (NAFLD) is a serious and growing clinical problem due to the growing prevalence of obesity and overweight. Histologically, it resembles alcoholic liver injury but occurs in patients who deny significant alcohol consumption. NAFLD encompasses a spectrum of conditions, ranging from benign hepatocellular steatosis to inflammatory nonalcoholic steatohepatitis, fibrosis, and cirrhosis. The majority of hepatocellular lipids are stored as triglycerides, but other lipid metabolites, such as free fatty acids, cholesterol, and phospholipids, may also be present and play a role in disease progression. NAFLD is associated with obesity and insulin resistance and is considered the hepatic manifestation of the metabolic syndrome, a combination of medical conditions including type 2 diabetes mellitus,

hypertension, hyperlipidemia, and visceral adiposity. Confirmation of the diagnosis of NAFLD can usually be achieved by imaging studies; however, staging the disease requires a liver biopsy. Current treatment relies on weight loss and exercise, although various insulin-sensitizing agents, antioxidants and medications appear promising. The aim of this review is to highlight the current information regarding epidemiology, diagnosis, and management of NAFLD as well as new information about pathogenesis, diagnosis and management of this disease.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a common clinicopathological condition characterized by significant lipid deposition in the hepatocytes of the liver parenchyma and persistent abnormalities in liver enzyme. The rising prevalence of NAFLD is related to the epidemic of obesity[1]. Although the histologic picture resembles that of alcohol-induced liver injury, NAFLD occurs in patients who do not abuse alcohol[2]. NAFLD comprises a wide spectrum of liver damage, ranging from simple macrovesicular steatosis to steatohepatitis, advanced fibrosis, and cirrhosis[3]. NAFLD is now increasingly being recognized as a cause of end-stage liver disease and is associated with increased rates of hepatocellular carcinoma (HCC), liver transplantation, and death[4-6]. Significant research endeavors are being directed toward understanding the pathogenesis of NAFLD and designing therapeutic strategies. This article provides a clinical overview of NAFLD, focusing on its epidemiology, etiology, pathogenesis, diagnosis, natural history and treatment.

PREVALENCE AND EPIDEMIOLGY

NAFLD is defined as either excessive fat accumulation in the liver with more than 5% of hepatocytes containing visible intracellular triglycerides or steatosis affecting at least 5% of the liver volume or weight in patients consuming less than 30 g (three units) of alcohol per day for men and less than 20 g (two units) of alcohol per day for women. One unit of alcohol (10 g) is defined as one glass of beer (25 cL), one glass of wine (20 cL) or one glass of whisky (3 cL)[7,8].

Globally, NAFLD is the most common form of chronic liver disease among adults and children[9,10]. However, the prevalence of non-alcoholic steatohepatitis (NASH) in the general population is not known. Depending on the cutoff values used to define the upper limit of normal for aminotransferase levels, the estimated prevalence of NAFLD in the general United States population ranges from 5.4% to 24%, but these values may be underestimations because aminotransferase levels have limited sensitivity for steatosis[11,12]. Histologic estimates of NAFLD prevalence *via* preoperative or intraoperative liver biopsy, mainly obtained from individuals evaluated as donors for living-donor liver transplantation, are 33% to 88%[13-15]. In children, NAFLD prevalence has been estimated to be 9.6%; of great concern, 2% to 8% of children with NAFLD progress to cirrhosis[16,17].

Obesity is the most important risk factor for NAFLD; the prevalence of NAFLD is 4.6 times greater in the obese population, and up to 74% of obese individuals have fatty livers[18]. Among morbidly obese patients undergoing bariatric surgery for weight loss, 84% to 96% have NAFLD and 2% to 12% have severe fibrosis or cirrhosis[19-22]. NAFLD is also strongly associated with hepatic and adipose tissue insulin resistance and metabolic syndrome[23]. Although NAFLD is clearly linked to obesity and metabolic syndrome, it may occur in up to 29% of lean patients lacking associative risk factors[24]. The prevalence of NAFLD is estimated to be at least twice as common among individuals who meet criteria for metabolic syndrome[25]. Among individuals with NAFLD, it is estimated that over 90% have some features of metabolic syndrome[26]. Diabetes is reported in 33% to 50% of patients with NAFLD, whereas insulin resistance may occur in as many as 75%[27].

Other factors that influence the development of NAFLD include age, sex, race, and ethnicity[28-30]. The prevalence of NAFLD increases with age in both adults and children[31]. NAFLD is more common among men than women younger than the age of 50; however, higher prevalence rates are seen in women older than the age of 50, perhaps related to hormonal changes occurring after menopause[11]. The prevalence of NAFLD across the globe varies but in some populations, half of all people may be affected. Among the Asian population, the prevalence of NAFLD diagnosed by ultrasound varies between 5% and 40%[32].

ETIOLGY

NAFLD encompasses a group of conditions, ranging from benign hepatocellular steatosis to inflammatory NASH, fibrosis, and cirrhosis[9]. The causes may be divided into two main categories: (1) acquired or congenital metabolic abnormalities; and (2) toxins and drugs[26]. Potential causes of NAFLD are listed in Table 1.

Obesity is often associated with NAFLD as the degree of steatosis was found to be correlated with body mass index (BMI)[33,34]; however, there is a significant correlation between the degree of steatosis, waist-to-hip ratio and the risk of metabolic syndrome[35-37]. Also, there is a strong correlation between glucose intolerance, type 2 diabetes mellitus (T2DM) and NAFLD[38]. Moreover, Diabetes mellitus may be an independent predictor of advanced liver cirrhosis, HCC and NAFLD[24,39-41].

Table 1 Causes of nonalcoholic fatty liver disease[69]

Acquired metabolic disorders
Diabetes mellitus
Dyslipidemia
Kwashiorkor and marasmus
Obesity
Starvation
Cytotoxic and cytostatic drugs
L-asparaginase
Azacitidine
Azaserine
Bleomycin
Methotrexate
Puromycin
Tetracycline
Other drugs and toxins
Amiodarone
4,4'-diethylaminoethoxyhexestrol
Dichlorethylene
Ethionine
Ethyl bromide
Estrogens
Glucocorticoids
Highly active antiretroviral therapy
Hydrazine
Hypoglycin
Orotate
Perhexilene maleate
Safrole
Tamoxifen
Metals
Antimony
Barium salts
Chromates
Phosphorus
Rare earths of low atomic number
Thallium compounds
Uranium compounds
Inborn errors of metabolism
Abetalipoproteinemia
Familial hepatosteatosis
Galactosemia
Glycogen storage disease
Hereditary fructose intolerance
Homocystinuria
Systemic carnitine deficiency
Tyrosinemia
Weber-Christian syndrome
Wilson disease
Surgical procedures
Biliopancreatic diversion
Extensive small bowel resection
Gastric bypass
Jejunoileal bypass
Miscellaneous conditions
Industrial exposure to petrochemicals
Inflammatory bowel disease
Partial lipodystrophy
Jejunal diverticulosis with bacterial overgrowth
Severe anemia
Total parenteral nutrition

PATHOGENESIS

The pathogenesis of NAFLD is fully understood, however, no single pathogenic mechanism has been identified[42]. Currently, the development of NASH is considered to be through a "two hit" process[43]. The first "hit" includes accumulation of fat in liver cells, which is usually associated with insulin resistance, central obesity along with triglyceride accumulation inside the liver, and fatty acid metabolism dysregulation that leads to steatosis. The second "hit" causes hepatocyte inflammation and necrosis, which lead to cirrhosis and fibrosis in some patients with NAFLD[43].

ROLE OF OXIDATIVE STRESS

Oxidative stress has a principal role in the pathogenesis of NAFLD as levels of lipid peroxide are increased in both NASH and hepatic steatosis[44].

Oxidative stress plays a key role in the second "hit", which also involves lipid peroxidation in steatotic hepatocytes. Induction of hepatic CYP2E1 promotes oxidative stress and lipid peroxidation[45] and mitochondrial dysfunction leads to reactive oxygen species formation[46]. Moreover, immune responses to lipid peroxidation products may share in NAFLD progression[47,48].

IMMUNE AND INFLAMMATORY PATHWAYS IN NASH

Adaptive and innate immune dysfunction along with inflammatory pathways is involved in the development of NAFLD[49]. Neutrophils, kupffer cells (KCs), natural killer (NK) cells and dendritic cells play an important role in the pathogenesis of NASH.

KCs are activated in acute or chronic liver disease, and this activation increases the pro-inflammatory cytokines, *e.g.*, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and IL-1b, which activate T cells and induce hepatocytes apoptosis[50]. Also, activation of neutrophils increases the release of pro-inflammatory cytokines and leads to oxidative damage to hepatic cells[51,52]. Moreover, NK cells are abundant in liver tissue and have an anti-fibrotic effect in the liver[53], and reduction of NK cell activity and levels may increase susceptibility to liver cirrhosis among obese subjects. Therefore, NK cells have a role in the development of liver injury and fibrosis and contribute to NASH and NAFLD development[54].

NATURAL HISTORY

NAFLD is the most common liver disorder worldwide, affecting 20%-40% of population in Western countries and 5%-40% of the general population across the Asia-Pacific region[55,56]. The prevalence of NAFLD, including NASH, is rising in parallel with the obesity, T2DM, and metabolic syndrome[57]. A certain proportion of NASH patients progress to cirrhosis and HCC[58]. Previous studies showed that 10%-29% of NASH patients may have liver cirrhosis within 10 years, and 4%-27% of these patients may have HCC (Figure 1)[59]. Therefore, NAFLD/NASH will gradually become the major etiology

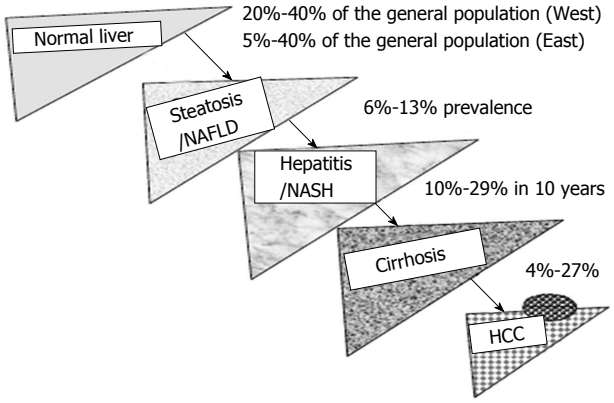

Figure 1 Natural history of nonalcoholic fatty liver disease. Data adapted from Hsu *et al*[59]. NAFLD: Non-alcoholic fatty liver disease; HCC: Hepatocellular carcinoma; NASH: Non-alcoholic steatohepatitis.

of chronic liver disease worldwide[60].

CLINICAL FEATURES OF NAFLD

Most subjects with NAFLD are clinically silent and asymptomatic, but can manifest with non-specific symptoms such as right upper quadrant discomfort or fatigue. Liver enzymes are usually minimally perturbed with mostly increased levels of alanine aminotransferase (ALT) and gamma-glutamyl transpeptidase. The diagnosis is often made incidentally in these individuals because of either abnormal liver enzyme levels or radiological features of a fatty liver. In others, NAFLD may be diagnosed either as a result of an unusual appearance of the liver during abdominal surgery or because of persistent hepatomegaly. It is important to recognize that only a minority of subjects has NAFLD been diagnosed and that it currently remains undiagnosed in the great majority of afflicted individuals[61]. NAFLD is a diagnosis of exclusion, so its workup needs to exclude other causes such as significant alcohol consumption (defined as > 30 g/d of ethanol for men and 20 g/d of ethanol for women), hepatitis B and/or C infection, drug abuse, autoimmune liver disease, haemochromatosis or Wilson's disease[62].

The principal risk factors for developing NAFLD are obesity and insulin resistance. More generally, any elements constituting the metabolic syndrome such as type 2 diabetes, dyslipidaemia and hypertension are linked to the development of NAFLD, and approximately 85% of patients with NAFLD have at least one such constituent. The metabolic syndrome itself is present in 30% of patients with NAFLD[62]. The association of NAFLD with obesity, diabetes, hypertriglyceridemia, and hypertension is well known. However, other associations include cardiovascular morbidity and mortality[63-65], sleep abnormalities[66], psychiatric illness[62], chronic fatigue and pain syndrome[67] and abnormalities of the coagulation cascade[68].

LABORATORY FEATURES OF NAFLD

Suspicion for NAFLD is triggered by abnormalities of liver chemistry tests that are usually performed for non-liver-related reasons. About 50% of patients with simple steatosis have higher liver biochemical test levels, which occur in 80% of patients with advanced NAFLD. Also, serum aspartate aminotransferase or ALT level, or both is usually increased up to 1.5- to 4-fold and levels rarely exceed 10 times the upper limit of normal. However, the gamma glutamyl transpeptidase and alkaline phosphatase levels may be elevated, but the serum prothrombin time, bilirubin level and serum albumin level are normal, except in patients with NAFLD-associated cirrhosis[69]. Moreover, about one fourth of NAFLD patients may have antinuclear antibodies (ANA) in low titers (less than 1:320)[70]. Serum ferritin level may be higher in 20% to 50% of NAFLD patients and can be considered a marker for advanced disease[24,71]. Hyperglycemia and dyslipidemia may be detected in 30% to 50% of NAFLD subjects[40]. Laboratory and clinical findings do not correlate with NAFLD histologic severity[68].

IMAGING FEATURES OF NAFLD

The radiologic features of fatty liver disease stem from the increased fat content of the liver parenchyma. The spatial pattern may be diffuse and homogeneous or heterogeneous, with focal fat deposition in an otherwise normal liver or areas of focal fat sparing in a diffusely fatty liver. The homogeneous form is the most common; the heterogeneous and focal forms may simulate perfusion abnormalities, diffusely infiltrative disease, nodular lesions, or masses[72,73]. Therefore, it is not only important to recognize fatty liver on imaging but also to discriminate it from other pathologic processes. The most important modalities used in the assessment of hepatic steatosis are ultrasonography, computed tomography (CT), and magnetic resonance (MR) imaging and MR spectroscopy. However, plain radiography has no significant role in the assessment of NAFLD[74].

CT has been widely used in evaluation of NAFLD in adults. Use of ionizing radiation precludes its use as a research tool in children, although fatty liver may be observed in children on scans done for clinical purposes[75]. Deposition of fat in the liver is characterized by a reduction in the attenuation of the hepatic parenchyma. On unenhanced CT, normal liver parenchyma has slightly greater attenuation than the spleen or blood. However, with increasing hepatic steatosis, liver attenuation decreases and the liver may become less dense than the intrahepatic vessels, simulating the appearance on a contrast-enhanced scan (Figure 2)[76]. Liver attenuation may be affected by a variety of factors other than liver fat, such as iron, copper, glycogen, fibrosis, edema, or amiodarone use. Assessment of liver fat by CT attenuation may be unreliable, and CT methods are

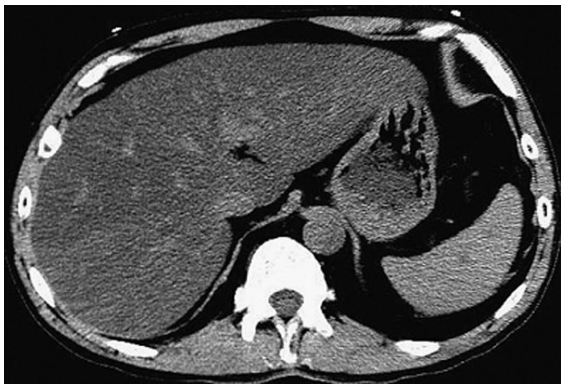

Figure 2 Axial unenhanced computed tomography scan reveals diffuse liver steatosis. Data adapted from Charatcharoenwitthaya *et al*[76].

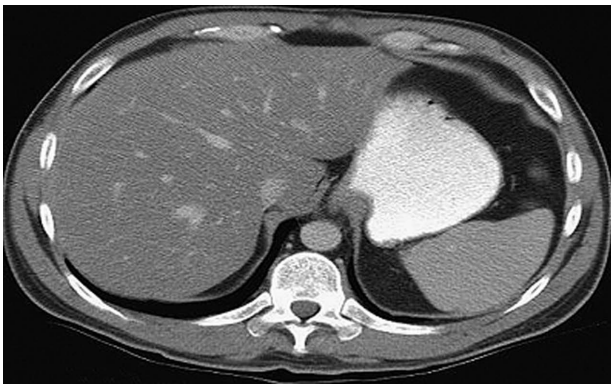

Figure 3 Axial enhanced computed tomography scan reveals diffuse liver steatosis. Data adapted from Rofsky *et al*[75].

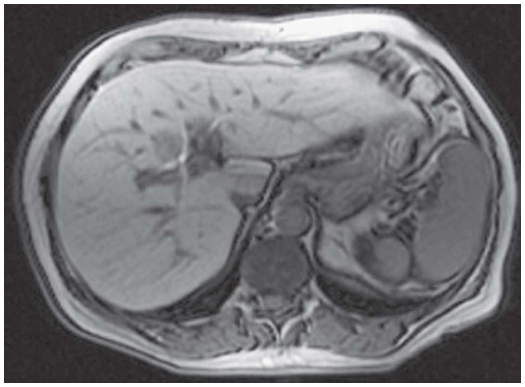

Figure 4 T1-weighted magnetic resonance imaging of fatty liver, demonstrating a bright liver. Data adapted from Mazhar *et al*[74].

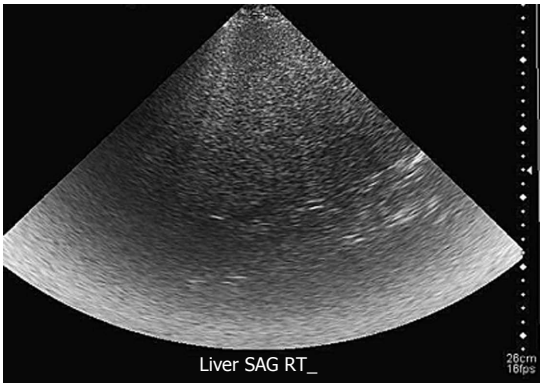

Figure 5 Sagittal ultrasound image of the liver shows diffuse liver steatosis. Data adapted from Yokoo *et al*[79].

insensitive to mild steatosis. The reported sensitivity and specificity of unenhanced CT for detection of moderate/severe steatosis (> 30% on histology) range from 73% to 100% and 95% to 100%, respectively[75].

At enhanced CT, the presence of iodine contrast interferes with attenuation, adding a new confounding factor. Perfusion alterations, timing of acquisitions, and contrast type, dosage, and injection rate all may influence hepatic and splenic attenuation. Nevertheless, criteria have been proposed to detect hepatic steatosis at enhanced CT, including a liver-spleen attenuation difference of at least 20 HU between 80 to 100 s, or at least 18.5 HU between 100 to 120 s, after intravenous contrast injection (Figure 3). Sensitivity and specificity of these attenuation differences range from 54% to 93% and 87% to 93%, respectively. Ultimately, however, the quantitative criteria for diagnosing fatty liver at enhanced CT are protocol specific and have significant overlap of liver-spleen attenuation values between normal and fatty liver, thereby limiting its clinical role[75].

MR imaging is more sensitive than CT for hepatic steatosis assessment. Recently, MR imaging provides a highly validated and reproducible hepatic triglyceride content measurement[77]. MR imaging is generally considered the most definitive radiologic modality for the qualitative and quantitative assessment of fatty liver disease but is relatively costly (Figure 4)[74]. However, proton MR spectroscopy is evolving to detect not only the full spectrum of steatosis but also other features such as the degree of fibrosis[13,78].

Transabdominal ultrasonography is the most common imaging technique to diagnose hepatic steatosis due to its widespread availability, noninvasiveness and low cost[76]. At ultrasonography, diffuse fatty liver is characterized by hyperechogenicity of the liver parenchyma relative to the adjacent right kidney or spleen (the so-called bright liver). Focal fat deposition appears as a hyperechoic area in an otherwise normal liver, whereas focal fat sparing is represented by a hypoechoic area within diffusely hyperechoic liver parenchyma[73]. Other frequently described ultrasound features of fatty liver include decreased visualization of vascular margins, attenuation of the ultrasound beam, loss of definition of the diaphragm, and hepatomegaly (Figure 5)[79].

Ultrasonography has several limitations in the detection of both diffuse and focal hepatic steatosis. It is highly operator dependent, nonreproducible, and limited by abdominal gas and patient body habitus. The last inadequacy is highlighted in this patient population because the majority of cases of fatty liver disease occur in overweight or obese individuals. Similar to CT, however, ultrasonography is not a quantitative method

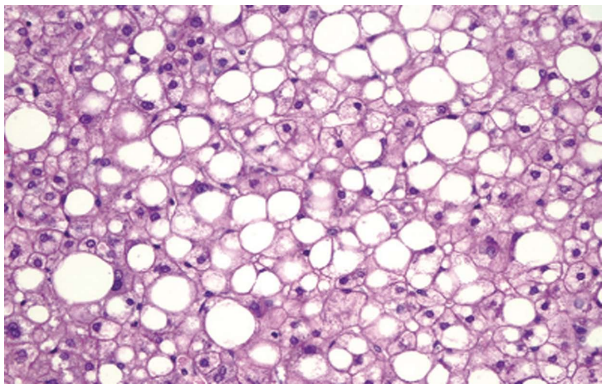

Figure 6 Histologic features of simple steatosis (fatty liver). Data adapted from Feldman *et al*[69].

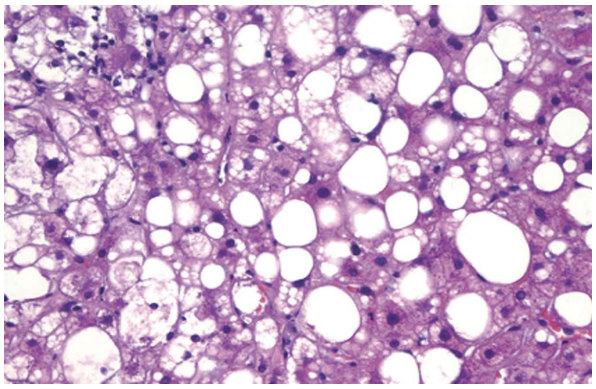

Figure 7 Histologic features of nonalcoholic steatohepatitis. Data adapted from Cortez-Pinto *et al*[83].

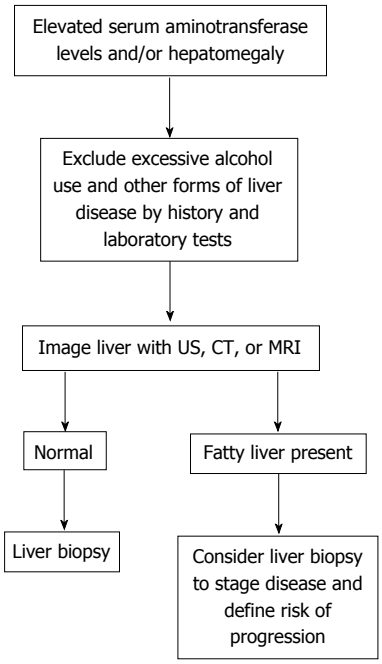

Figure 8 Diagnostic approach to patients with suspected nonalcoholic fatty liver disease. Data adapted from Feldman *et al*[69]. CT: Computed tomography; MRI: Magnetic resonance imaging; US: Ultrasound.

and may be unable to distinguish simple steatosis from advanced fibrosis or early cirrhosis. Ultrasonography has low sensitivity and specificity for detecting small amounts of fat in the liver[75].

Transient elastography, a recently developed technique based on ultrasound monitoring of the passage of a low frequency pressure wave through tissues, has been found to be a promising non-invasive technique for the detection of advanced fibrosis caused by chronic viral hepatitis and NASH[80], although abdominal obesity may compromise its utility in the NASH patient population[81].

Scintigraphy with xenon-133 (133Xe) as a nuclear medicine imaging technique was used to detect hepatic steatosis in the 1980s and 1990s but is now no longer incorporated in diagnostic algorithms. 133Xe is a highly fat-soluble gas that, after being inhaled or injected, remains in the fatty tissue after blood pool clearing. The 133Xe hepatic retention ratio is increased in patients with fatty liver[82].

HISTOLOGICAL FEATURES OF NAFLD

The main histologic features of NAFLD are similar to those of alcohol-induced liver disease and include steatohepatitis (fatty liver plus parenchymal inflammation with or without accompanying focal necrosis), steatosis (fatty liver) and varying degrees of fibrosis, including cirrhosis. Steatosis is predominantly macrovesicular and usually is distributed diffusely throughout the liver lobule, although prominent microvesicular steatosis and zone 3 (perivenular) steatosis have been reported occasionally (Figure 6). Mild neutrophilic, lymphocytic, or mixed inflammatory infiltrates also may be observed, and glycogenated nuclei are common[69]. NASH, which is an advanced form of NAFLD, is indistinguishable histologically from alcoholic hepatitis (Figure 7)[83].

Literature reviews indicate that similar factors and markers of inflammation are present in paediatric NAFLD as in adults[84-86]. However, many differences are noted in comparison to adult histology and include: (1) greater severity of steatosis; (2) less or no ballooning or Mallory-Denk bodies; (3) less lobular inflammation; (4) few or no polymorphonuclear leukocytes; and (5) increased portal tract inflammation and fibrosis[87]. Moreover, cirrhosis in children is rare but is reported[88].

DIAGNOISIS

Establishing a definitive diagnosis of NAFLD requires both clinical and histologic data (Figure 8). Most patients with NAFLD are evaluated because of chronically elevated liver biochemical test levels, with or without hepatomegaly. The combination of the patient's history, clinical examination, radiologic findings and blood test results is essential for accurate diagnosis of NAFLD[89]. Anti-smooth muscle antibodies and ANA are common in patients with NASH and most frequently represent a nonspecific antibody response that is not associated

Table 2 Brunt schema for grading nonalcoholic steatohepatitis[99]

Severe (grade 3)	Moderate (grade 2)	Mild (grade 1)	
Typically > 66% (panacinar); commonly mixed steatosis	Any degree and usually mixed macrovesicular and microvesicular	Predominantly macrovesicular; involves < 33%-66% of the lobules	Steatosis
Predominantly zone 3; marked	Obvious and present in zone 3	Occasionally observed; zone 3 hepatocytes	Ballooning
Scattered acute and chronic inflammation; polymorphs may appear concentrated in zone 3 areas of ballooning and perisinusoidal fibrosis	Polymorphs may be noted associated with ballooned hepatocytes, pericellular fibrosis; mild chronic inflammation may be seen	Scattered and mild acute (polymorphs) inflammation and occasional chronic inflammation (mononuclear cells)	Lobular inflammation
Mild or moderate	Mild to moderate	None or mild	Portal inflammation

with the pattern or severity of injury on liver biopsy[90]. However, alcoholic liver disease must be excluded in order to establish NAFLD diagnosis.

DIFFERENTIAL DIAGNOSIS

The diagnosis of fatty liver is confirmed by imaging studies and the clinician is challenged with establishing the etiology of hepatic steatosis. Among patients with elevated serum aminotransferase values, the etiology is usually established through a careful evaluation of their history (medication use, risk factors for viral hepatitis, history of alcohol and drug use, and review of comorbidities), a series of screening blood tests for causes of chronic liver disease (viral serologic studies, iron studies, autoimmune markers, ceruloplasmin, and α1-antitrypsin), supportive imaging studies (initial evaluation usually by ultrasonography), and, sometimes, liver biopsy[91,92].

Alcoholic liver disease includes a spectrum of conditions provoked by alcohol ingestion, including alcoholic hepatitis, fatty liver disease and cirrhosis. It has been estimated that almost all patients with heavy alcohol consumption develop fatty liver, although only 10% to 35% develop alcoholic hepatitis and 8% to 20% progress to alcoholic cirrhosis[93]. In individuals who admit to moderate alcohol intake, the differentiation between NAFLD and alcoholic fatty liver disease is difficult because laboratory, imaging, and histologic findings are similar. Unfortunately, strong data are lacking to determine accurate thresholds for alcohol consumption required to cause fatty liver. Historically, daily alcohol intake of 30 g in men and 20 g in women has been used to distinguish NAFLD from alcoholic fatty liver disease, although the validity of these thresholds is unknown[94]. If liver biopsy specimens are obtained, individuals with alcoholic liver disease tend to have more Mallory's hyaline and acidophil bodies and less glycogenated nuclei than those with NAFLD, although these are not reliable findings[74].

Because the radiologic findings of hepatic steatosis are common to its diverse causes, the differential diagnosis is largely discriminated on clinical and laboratory grounds[95]. Special attention needs to be given to the possible imaging overlap between simple steatosis and advanced fibrosis or early cirrhosis; these disparate conditions are often, but not always, easily distinguished clinically. A major challenge in the differential diagnosis of hepatic steatosis occurs when the radiologic findings of focal fat deposition or focal fat sparing simulate hepatic nodular lesions such as abscess, benign neoplasm, or primary or metastatic malignancy. The diagnosis of focal fat deposition or sparing is supported by their occurrence in typical locations, a wedge shape, the lack of mass effect, and the absence of vascular displacement or distortion inside the lesion. When there is still doubt, MR imaging may be performed[74].

Certain drugs may produce *de novo* steatohepatitis (*e.g.*, amiodarone, perhexiline maleate, diethylaminoethoxyhexestrol) and others may exacerbate NASH (tamoxifen, corticosteroids, diethylstilbestrol, estrogens)[96]. Oxaliplatin and irinotecan administered as preoperative chemotherapy before surgical resection of hepatic metastases have been associated with steatohepatitis, with irinotecan-associated steatohepatitis associated with poorer outcomes after hepatic resection[97,98]. Other conditions capable of eliciting fatty liver include intestinal bypass surgery for weight loss (classically seen with jejunoileal bypass surgery), human immunodeficiency virus (HIV) infection with lipodystrophy, and parenteral nutrition[3]. If any of the these secondary causes of fatty liver are excluded (alcohol, viral hepatitis, drug-induced, jejunoileal bypass surgery, HIV infection, and parenteral nutrition support), a diagnosis of NAFLD can be made[74].

GRADING AND STAGING

The standardized schema of NAFLD staging and grading was published by Brunt and associates in 1999, who assigned the overall grade of mild, marked, or severe (grades 1, 2, and 3, respectively), based on the degree of ballooning degeneration, steatosis and lobular and portal inflammation as listed in Table 2[99]. The Pathology Committee of the National Institute of Diabetes and Digestive and Kidney Diseases sponsored NASH Clinical Research Network maintained features of the Brunt schema for NAFLD grading as found in Table 3[9].

TREATMENT

To date, there are no established treatment guidelines

Table 3 Non-alcoholic fatty liver disease activity score[9]

Score	Steatosis
0	< 5%
1	5%-33%
2	> 33%-66%
3	> 66%
Lobular inflammation (counted in 20 × fields)	
1	< 2 foci
2	2-4 foci
3	> 4 foci
Ballooning	
1	Few
2	Many

and no single approved therapy for NAFLD treatment. Historically, the principal treatment for NAFLD consisted of removal of offending drugs and toxins, weight loss, and control of associated metabolic disorders as hyperlipidemia and diabetes. The focus of NAFLD management is to ameliorate the NASH risk factors (*i.e.*, insulin resistance and obesity), with the objective of preventing disease progression or regression of already established fatty liver or NASH. Lifestyle changes and dietary modification are the main methods for weight management.

Weight management

The ultimate weight management goal is to achieve the ideal body weight. However, significant insulin resistance improvement could be attained by modest weight loss[100].

The National Heart, Lung, and Blood Institute guidelines for weight management in obsess subjects are the best evidence-based treatment guidelines, which generally recommended that the diet should be planned to achieve a daily caloric deficit of 500 to 1000 calories along with an increase in everyday activities[101]. Furthermore, for subjects with a BMI higher than 30 kg/m^2 or with a BMI higher than 27 along with other comorbid conditions (*e.g.*, sleep apnea), pharmacologic weight management with orlistat or sibutramine may be considered as these agents could produce a beneficial effect on NAFLD[99]. However, both vertical banded gastroplasty and proximal gastric bypass have been shown to be safe in NASH subjects[102]. Moreover, the severity of hepatic steatosis, fibrosis and cell injury regresses once the weight stabilizes following these operations[103].

Pharmacologic treatment of NASH

Several drug therapies have been tried in both research and clinical settings, yet no agent has been approved by the Food and Drug Administration for the treatment of NAFLD[61].

Vitamin E

Vitamin E, an inexpensive potent antioxidant, has been examined as a treatment agent for NAFLD in many adult and pediatric studies, with varying results. In all studies, vitamin E was well tolerated, and most studies showed modest improvements in ultrasonographic appearance of the liver, serum aminotransferase levels and histologic findings[97,104,105]. In one published series of 11 pediatric patients with NASH who received vitamin E (d-α-tocopherol), 400 to 1200 IU, ALT improved [105-107].

Lipid-lowering agents

Few small trials assessed the usefulness of lipid-lowering and cytoprotective drugs for NAFLD treatment, with varying results. In one controlled trial, gemfibrozil improved liver chemistry in 74% of NAFLD patients in the treatment group, compared with 30% of untreated control subjects with no available histologic data. So, in general, lipid-lowering agents are not used for NASH treatment[108].

Insulin sensitizers

The association between hyperinsulinemic insulin resistance and NAFLD provides a logical target for treatment. Two classes of drugs have been shown to correct insulin resistance: biguanides (*e.g.*, metformin) and thiazolidinediones. Metformin, a biguanide that reduces hyperinsulinemia and improves hepatic insulin sensitivity, reduces hepatomegaly and hepatic steatosis in *ob/ob* mice[109], but results in human studies have been less impressive[110,111] as in human studies, although ALT improved and liver size decreased, metformin was not consistently found to improve liver histology[110,112].

Ursodeoxycholate

Ursodeoxycholate (UDCA) is a hydrophilic bile acid that is associated with hepatoprotective properties. In one study, UDCA produced improvement in liver enzymes and a decrease in hepatic steatosis. The long-term benefits of UDCA and the optimal dose of UDCA remain to be established[113].

Taurine

Taurine is believed to function as a lipotropic factor and to improve the mobilization of hepatic fat. In another single uncontrolled series, 10 children treated with taurine supplements orally had radiologic resolution of their fatty liver[114].

Betaine

Betaine is a hepatoprotective factor, and liver histology and aminotransferase activity were improved in ten NAFLD subjects who received betaine for one year[115,116]. In a recent randomized placebo-control study, 55 NASH patients received betaine (20 g daily). Patients randomized to betaine had a decrease in steatosis grade without a significant change in intragroup or intergroup differences in NAS or fibrosis stage. Moreover, there was no significant change in adiponectin, insulin, glucose, proinflammatory cytokines, or oxidant stress in NASH

patients receiving betaine therapy[116].

Pentoxifylline

Pentoxifylline antagonizes TNF-α and is orally available for long-term use. In two small pilot studies, ALT improved after several months of treatment at a dose of 400 mg three times a day. In addition, although the drug was well tolerated in one study, 9 of 20 subjects in the other study dropped out because of side effects, especially nausea[117,118].

Losartan

Angiotensin Ⅱ has been implicated in hepatic stellate cell activation and matrix production[119]. In a small pilot study of an angiotensin receptor blocker, losartan, an improvement in ALT was noted[120].

Surgical treatment

Bariatric surgery is the primary surgical intervention for NAFLD in patients with an BMI more than 40 kg/m^2 or of 35 kg/m^2 with comorbidities[121]. Current bariatric surgical techniques include vertical banded gastroplasty, adjustable gastric banding, Roux-en-Y gastric bypass, biliopancreatic bypass, and biliopancreatic diversion with duodenal switch. Based on a recent meta-analysis, bariatric surgery is associated with significant histologic improvements in steatosis, steatohepatitis, and fibrosis, with more than 50% of patients experiencing complete resolution of their fatty liver disease after surgery. Although these results are compelling, these observational studies showed no relationship between histologic improvement and the amount of weight loss[122].

As with other causes of cirrhosis, liver transplantation is a viable option for patients with end-stage liver disease due to fatty liver disease[123]. The outcome of liver transplantation in these patients is good, although NAFLD can recur after liver transplantation[124,125].

REFERENCES

1 **Sonsuz A**, Basaranoglu M, Ozbay G. Relationship between aminotransferase levels and histopathological findings in patients with nonalcoholic steatohepatitis. *Am J Gastroenterol* 2000; **95**: 1370-1371 [PMID: 10811364 DOI: 10.1111/j.1572-0241.2000.02046.x]

2 **Ludwig J**, Viggiano TR, McGill DB, Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc* 1980; **55**: 434-438 [PMID: 7382552]

3 **Angulo P**. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; **346**: 1221-1231 [PMID: 11961152 DOI: 10.1056/NEJMra011775]

4 **Charlton MR**, Kondo M, Roberts SK, Steers JL, Krom RA, Wiesner RH. Liver transplantation for cryptogenic cirrhosis. *Liver Transpl Surg* 1997; **3**: 359-364 [PMID: 9346764 DOI: 10.1002/lt.500030402]

5 **McCullough AJ**. Update on nonalcoholic fatty liver disease. *J Clin Gastroenterol* 2002; **34**: 255-262 [PMID: 11873108 DOI: 10.1097/00004836-200203000-00013]

6 **Sass DA**, Chang P, Chopra KB. Nonalcoholic fatty liver disease: a clinical review. *Dig Dis Sci* 2005; **50**: 171-180 [PMID: 15712657 DOI: 10.1007/s10620-005-1267-z]

7 **McCullough AJ**. Pathophysiology of nonalcoholic steatohepatitis. *J Clin Gastroenterol* 2006; **40** Suppl 1: S17-S29 [PMID: 16540762]

8 **Serfaty L**, Lemoine M. Definition and natural history of metabolic steatosis: clinical aspects of NAFLD, NASH and cirrhosis. *Diabetes Metab* 2008; **34**: 634-637 [PMID: 19195623 DOI: 10.1016/S1262-3636(08)74597-X]

9 **Kleiner DE**, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A, Yeh M, McCullough AJ, Sanyal AJ. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005; **41**: 1313-1321 [PMID: 15915461 DOI: 10.1002/hep.20701]

10 **Patton HM**, Sirlin C, Behling C, Middleton M, Schwimmer JB, Lavine JE. Pediatric nonalcoholic fatty liver disease: a critical appraisal of current data and implications for future research. *J Pediatr Gastroenterol Nutr* 2006; **43**: 413-427 [PMID: 17033514 DOI: 10.1097/01.mpg.0000239995.58388.56]

11 **Clark JM**, Brancati FL, Diehl AM. Nonalcoholic fatty liver disease. *Gastroenterology* 2002; **122**: 1649-1657 [PMID: 12016429 DOI: 10.1053/gast.2002.33573]

12 **Clark JM**. The epidemiology of nonalcoholic fatty liver disease in adults. *J Clin Gastroenterol* 2006; **40** Suppl 1: S5-10 [PMID: 16540768]

13 **Browning JD**, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, Grundy SM, Hobbs HH. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* 2004; **40**: 1387-1395 [PMID: 15565570 DOI: 10.1002/hep.20466]

14 **Ryan CK**, Johnson LA, Germin BI, Marcos A. One hundred consecutive hepatic biopsies in the workup of living donors for right lobe liver transplantation. *Liver Transpl* 2002; **8**: 1114-1122 [PMID: 12474149 DOI: 10.1053/jlts.2002.36740]

15 **Soejima Y**, Shimada M, Suehiro T, Kishikawa K, Yoshizumi T, Hashimoto K, Minagawa R, Hiroshige S, Terashi T, Ninomiya M, Shiotani S, Harada N, Sugimachi K. Use of steatotic graft in living-donor liver transplantation. *Transplantation* 2003; **76**: 344-348 [PMID: 12883190 DOI: 10.1097/01.TP.0000071205.52835.A4]

16 **Schwimmer JB**, Deutsch R, Kahen T, Lavine JE, Stanley C, Behling C. Prevalence of fatty liver in children and adolescents. *Pediatrics* 2006; **118**: 1388-1393 [PMID: 17015527 DOI: 10.1542/peds.2006-1212]

17 **Dunn W**, Schwimmer JB. The obesity epidemic and nonalcoholic fatty liver disease in children. *Curr Gastroenterol Rep* 2008; **10**: 67-72 [PMID: 18417045 DOI: 10.1007/s11894-008-0011-1]

18 **Angulo P**, Lindor KD. Non-alcoholic fatty liver disease. *J Gastroenterol Hepatol* 2002; **17** Suppl: S186-S190 [PMID: 12000605]

19 **Crespo J**, Fernández-Gil P, Hernández-Guerra M, Cayón A, Mayorga M, Domínguez-Diez A, Fernández-Escalante JC, Pons-Romero F. Are there predictive factors of severe liver fibrosis in morbidly obese patients with non-alcoholic steatohepatitis? *Obes Surg* 2001; **11**: 254-257 [PMID: 11433896 DOI: 10.1381/096089201321336548]

20 **Dixon JB**, Bhathal PS, O'Brien PE. Nonalcoholic fatty liver disease: predictors of nonalcoholic steatohepatitis and liver fibrosis in the severely obese. *Gastroenterology* 2001; **121**: 91-100 [PMID: 11438497 DOI: 10.1053/gast.2001.25540]

21 **Beymer C**, Kowdley KV, Larson A, Edmonson P, Dellinger EP, Flum DR. Prevalence and predictors of asymptomatic liver disease in patients undergoing gastric bypass surgery. *Arch Surg* 2003; **138**: 1240-1244 [PMID: 14609874 DOI: 10.1001/archsurg.138.11.1240]

22 **Gholam PM**, Kotler DP, Flancbaum LJ. Liver pathology in morbidly obese patients undergoing Roux-en-Y gastric bypass surgery. *Obes Surg* 2002; **12**: 49-51 [PMID: 11868297 DOI: 10.1381/096089202321144577]

23 **Bugianesi E**, Gastaldelli A, Vanni E, Gambino R, Cassader M, Baldi S, Ponti V, Pagano G, Ferrannini E, Rizzetto M. Insulin resistance in non-diabetic patients with non-alcoholic fatty liver disease: sites and mechanisms. *Diabetologia* 2005; **48**: 634-642 [PMID: 15747110 DOI: 10.1007/s00125-005-1682-x]

24 **Angulo P**, Keach JC, Batts KP, Lindor KD. Independent predictors of liver fibrosis in patients with nonalcoholic steatohepatitis. *Hepatology* 1999; **30**: 1356-1362 [PMID: 10573511 DOI: 10.1002/hep.510300604]

25 **Liangpunsakul S**, Chalasani N. Unexplained elevations in alanine aminotransferase in individuals with the metabolic syndrome: results from the third National Health and Nutrition Survey (NHANES III). *Am J Med Sci* 2005; **329**: 111-116 [PMID: 15767815 DOI: 10.1097/00000441-200503000-00001]

26 **Marchesini G**, Bugianesi E, Forlani G, Cerrelli F, Lenzi M, Manini R, Natale S, Vanni E, Villanova N, Melchionda N, Rizzetto M. Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. *Hepatology* 2003; **37**: 917-923 [PMID: 12668987 DOI: 10.1053/jhep.2003.50161]

27 **Matteoni CA**, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology* 1999; **116**: 1413-1419 [PMID: 10348825]

28 **Schwimmer JB**. Definitive diagnosis and assessment of risk for nonalcoholic fatty liver disease in children and adolescents. *Semin Liver Dis* 2007; **27**: 312-318 [PMID: 17682977 DOI: 10.1055/s-2007-985075]

29 **Ruhl CE**, Everhart JE. Relation of elevated serum alanine aminotransferase activity with iron and antioxidant levels in the United States. *Gastroenterology* 2003; **124**: 1821-9 [DOI: 10.1016/S0016-5085(03)00395-0]

30 **Clark JM**, Brancati FL, Diehl AM. The prevalence and etiology of elevated aminotransferase levels in the United States. *Am J Gastroenterol* 2003; **98**: 960-967 [PMID: 12809815 DOI: 10.1111/j.1572-0241.2003.07486.x]

31 **Fan JG**, Zhu J, Li XJ, Chen L, Li L, Dai F, Li F, Chen SY. Prevalence of and risk factors for fatty liver in a general population of Shanghai, China. *J Hepatol* 2005; **43**: 508-514 [PMID: 16006003 DOI: 10.1016/j.jhep.2005.02.042]

32 **Amarapurkar DN**, Hashimoto E, Lesmana LA, Sollano JD, Chen PJ, Goh KL; Asia-Pacific Working Party on NAFLD. How common is non-alcoholic fatty liver disease in the Asia-Pacific region and are there local differences? *J Gastroenterol Hepatol* 2007; **22**: 788-793 [PMID: 17565631 DOI: 10.1111/j.1440-1746.2007.05042.x]

33 **Moretto M**, Kupski C, Mottin CC, Repetto G, Garcia Toneto M, Rizzolli J, Berleze D, de Souza Brito CL, Casagrande D, Colossi F. Hepatic steatosis in patients undergoing bariatric surgery and its relationship to body mass index and co-morbidities. *Obes Surg* 2003; **13**: 622-624 [PMID: 12940291 DOI: 10.1381/096089203322190853]

34 **Hsiao PJ**, Kuo KK, Shin SJ, Yang YH, Lin WY, Yang JF, Chiu CC, Chuang WL, Tsai TR, Yu ML. Significant correlations between severe fatty liver and risk factors for metabolic syndrome. *J Gastroenterol Hepatol* 2007; **22**: 2118-2123 [PMID: 18031368 DOI: 10.1111/j.1440-1746.2006.04698.x]

35 **Stranges S**, Dorn JM, Muti P, Freudenheim JL, Farinaro E, Russell M, Nochajski TH, Trevisan M. Body fat distribution, relative weight, and liver enzyme levels: a population-based study. *Hepatology* 2004; **39**: 754-763 [PMID: 14999694 DOI: 10.1002/hep.20149]

36 **Church TS**, Kuk JL, Ross R, Priest EL, Biltoft E, Blair SN. Association of cardiorespiratory fitness, body mass index, and waist circumference to nonalcoholic fatty liver disease. *Gastroenterology* 2006; **130**: 2023-2030 [PMID: 16762625 DOI: 10.1053/j.gastro.2006.03.019]

37 **Cheung O**, Kapoor A, Puri P, Sistrun S, Luketic VA, Sargeant CC, Contos MJ, Shiffman ML, Stravitz RT, Sterling RK, Sanyal AJ. The impact of fat distribution on the severity of nonalcoholic fatty liver disease and metabolic syndrome. *Hepatology* 2007; **46**: 1091-1100 [PMID: 17610277 DOI: 10.1002/hep.21803]

38 **Targher G**, Bertolini L, Padovani R, Rodella S, Tessari R, Zenari L, Day C, Arcaro G. Prevalence of nonalcoholic fatty liver disease and its association with cardiovascular disease among type 2 diabetic patients. *Diabetes Care* 2007; **30**: 1212-1218 [PMID: 17277038 DOI: 10.2337/dc06-2247]

39 **El-Serag HB**, Tran T, Everhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. *Gastroenterology* 2004; **126**: 460-468 [PMID: 14762783 DOI: 10.1053/j.gastro.2003.10.065]

40 **Mofrad P**, Contos MJ, Haque M, Sargeant C, Fisher RA, Luketic VA, Sterling RK, Shiffman ML, Stravitz RT, Sanyal AJ. Clinical and histologic spectrum of nonalcoholic fatty liver disease associated with normal ALT values. *Hepatology* 2003; **37**: 1286-1292 [PMID: 12774006 DOI: 10.1053/jhep.2003.50229]

41 **Davila JA**, Morgan RO, Shaib Y, McGlynn KA, El-Serag HB. Diabetes increases the risk of hepatocellular carcinoma in the United States: a population based case control study. *Gut* 2005; **54**: 533-539 [PMID: 15753540 DOI: 10.1136/gut.2004.052167]

42 **Day CP**, James OF. Steatohepatitis: a tale of two "hits"? *Gastroenterology* 1998; **114**: 842-845 [PMID: 9547102 DOI: 10.1016/S0016-5085(98)70599-2]

43 **Papandreou D**, Rousso I, Mavromichalis I. Update on non-alcoholic fatty liver disease in children. *Clin Nutr* 2007; **26**: 409-415 [PMID: 17449148 DOI: 10.1016/j.clnu.2007.02.002]

44 **Rolo AP**, Teodoro JS, Palmeira CM. Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis. *Free Radic Biol Med* 2012; **52**: 59-69 [PMID: 22064361 DOI: 10.1016/j.freeradbiomed.2011.10.003]

45 **Chitturi S**, Farrell GC. Etiopathogenesis of nonalcoholic steatohepatitis. *Semin Liver Dis* 2001; **21**: 27-41 [PMID: 11296694 DOI: 10.1055/s-2001-12927]

46 **Pessayre D**, Fromenty B. NASH: a mitochondrial disease. *J Hepatol* 2005; **42**: 928-940 [PMID: 15885365 DOI: 10.1016/j.jhep.2005.03.004]

47 **Le TH**, Caldwell SH, Redick JA, Sheppard BL, Davis CA, Arseneau KO, Iezzoni JC, Hespenheide EE, Al-Osaimi A, Peterson TC. The zonal distribution of megamitochondria with crystalline inclusions in nonalcoholic steatohepatitis. *Hepatology* 2004; **39**: 1423-1429 [PMID: 15122772 DOI: 10.1002/hep.20202]

48 **Albano E**, Mottaran E, Vidali M, Reale E, Saksena S, Occhino G, Burt AD, Day CP. Immune response towards lipid peroxidation products as a predictor of progression of non-alcoholic fatty liver disease to advanced fibrosis. *Gut* 2005; **54**: 987-993 [PMID: 15951547 DOI: 10.1136/gut.2004.057968]

49 **Ganz M**, Szabo G. Immune and inflammatory pathways in NASH. *Hepatol Int* 2013; **7**: 771-781 [PMID: 24587847 DOI: 10.1007/s12072-013-9468-6]

50 **Bilzer M**, Roggel F, Gerbes AL. Role of Kupffer cells in host defense and liver disease. *Liver Int* 2006; **26**: 1175-1186 [PMID: 17105582 DOI: 10.1111/j.1478-3231.2006.01342.x]

51 **Nijhuis J**, Rensen SS, Slaats Y, van Dielen FM, Buurman WA, Greve JW. Neutrophil activation in morbid obesity, chronic activation of acute inflammation. *Obesity (Silver Spring)* 2009; **17**: 2014-2018 [PMID: 19390527 DOI: 10.1038/oby.2009.113]

52 **Rensen SS**, Slaats Y, Nijhuis J, Jans A, Bieghs V, Driessen A, Malle E, Greve JW, Buurman WA. Increased hepatic myeloperoxidase activity in obese subjects with nonalcoholic steatohepatitis. *Am J Pathol* 2009; **175**: 1473-1482 [PMID: 19729473 DOI: 10.2353/ajpath.2009.080999]

53 **Radaeva S**, Sun R, Jaruga B, Nguyen VT, Tian Z, Gao B. Natural killer cells ameliorate liver fibrosis by killing activated stellate cells in NKG2D-dependent and tumor necrosis factor-related apoptosis-inducing ligand-dependent manners. *Gastroenterology* 2006; **130**: 435-452 [PMID: 16472598 DOI: 10.1053/j.gastro.2005.10.055]

54 **Csak T**, Dolganiuc A, Kodys K, Nath B, Petrasek J, Bala S, Lippai D, Szabo G. Mitochondrial antiviral signaling protein defect links impaired antiviral response and liver injury in steatohepatitis in mice. *Hepatology* 2011; **53**: 1917-1931 [PMID: 21425308 DOI: 10.1002/hep.24301]

55 **Farrell GC**. Non-alcoholic steatohepatitis: what is it, and why is it important in the Asia-Pacific region? *J Gastroenterol Hepatol* 2003; **18**: 124-138 [PMID: 12542595 DOI: 10.1046/j.1440-1746.2003.02989.x]

56 **Chitturi S**, Farrell GC, George J. Non-alcoholic steatohepatitis in the Asia-Pacific region: future shock? *J Gastroenterol Hepatol* 2004; **19**:

368-374 [PMID: 15012772 DOI: 10.1111/j.1440-1746.2003.03252.x]
57 **Starley BQ**, Calcagno CJ, Harrison SA. Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection. *Hepatology* 2010; **51**: 1820-1832 [PMID: 20432259 DOI: 10.1002/hep.23594]
58 **Cohen JC**, Horton JD, Hobbs HH. Human fatty liver disease: old questions and new insights. *Science* 2011; **332**: 1519-1523 [PMID: 21700865 DOI: 10.1126/science.1204265]
59 **Hsu CS**, Kao JH. Non-alcoholic fatty liver disease: an emerging liver disease in Taiwan. *J Formos Med Assoc* 2012; **111**: 527-535 [PMID: 23089687 DOI: 10.1016/j.jfma.2012.07.002]
60 **Argo CK**, Caldwell SH. Epidemiology and natural history of non-alcoholic steatohepatitis. *Clin Liver Dis* 2009; **13**: 511-531 [PMID: 19818302 DOI: 10.1016/j.cld.2009.07.005]
61 **Boyer TD**, Manns MP, Sanyal AJ. Zakim& Boyer's Hepatology: A Textbook of Liver Disease. In: Puri P, Sanyal AJ. Nonalcoholic Fatty Liver Disease. Saunders: an imprint of Elsevier Inc, 2012: 941-968
62 **Gariani K**, Philippe J, Jornayvaz FR. Non-alcoholic fatty liver disease and insulin resistance: from bench to bedside. *Diabetes Metab* 2013; **39**: 16-26 [PMID: 23266468 DOI: 10.1016/j.diabet.2012.11.002]
63 **Hanley AJ**, Williams K, Festa A, Wagenknecht LE, D'Agostino RB, Haffner SM. Liver markers and development of the metabolic syndrome: the insulin resistance atherosclerosis study. *Diabetes* 2005; **54**: 3140-3147 [PMID: 16249437 DOI: 10.2337/diabetes.54.11.3140]
64 **Hanley AJ**, Williams K, Festa A, Wagenknecht LE, D'Agostino RB, Kempf J, Zinman B, Haffner SM; insulin resistance atherosclerosis study. Elevations in markers of liver injury and risk of type 2 diabetes: the insulin resistance atherosclerosis study. *Diabetes* 2004; **53**: 2623-2632 [PMID: 15448093 DOI: 10.2337/diabetes.53.10.2623]
65 **Stranges S**, Trevisan M, Dorn JM, Dmochowski J, Donahue RP. Body fat distribution, liver enzymes, and risk of hypertension: evidence from the Western New York Study. *Hypertension* 2005; **46**: 1186-1193 [PMID: 16203871 DOI: 10.1161/01.HYP.0000185688.81320.4d]
66 **Tanné F**, Gagnadoux F, Chazouillères O, Fleury B, Wendum D, Lasnier E, Lebeau B, Poupon R, Serfaty L. Chronic liver injury during obstructive sleep apnea. *Hepatology* 2005; **41**: 1290-1296 [PMID: 15915459 DOI: 10.1002/hep.20725]
67 **Ramesh S**, Sanyal AJ. Evaluation and management of non-alcoholic steatohepatitis. *J Hepatol* 2005; **42** Suppl: S2-12 [PMID: 15777569]
68 **Chitturi S**, Abeygunasekera S, Farrell GC, Holmes-Walker J, Hui JM, Fung C, Karim R, Lin R, Samarasinghe D, Liddle C, Weltman M, George J. NASH and insulin resistance: Insulin hypersecretion and specific association with the insulin resistance syndrome. *Hepatology* 2002; **35**: 373-379 [PMID: 11826411 DOI: 10.1053/jhep.2002.30692]
69 **Feldman M**, Friedman LS, Brandt LJ. Sleisenger and Fordtran's Gastrointestinal and Liver Disease. In: Reid AE. Nonalcoholic Fatty Liver Disease. Saunders: an imprint of Elsevier Inc, 2010: 1401-1411
70 **Adams LA**, Lindor KD, Angulo P. The prevalence of autoantibodies and autoimmune hepatitis in patients with nonalcoholic Fatty liver disease. *Am J Gastroenterol* 2004; **99**: 1316-1320 [PMID: 15233671 DOI: 10.1111/j.1572-0241.2004.30444.x]
71 **Bugianesi E**, Manzini P, D'Antico S, Vanni E, Longo F, Leone N, Massarenti P, Piga A, Marchesini G, Rizzetto M. Relative contribution of iron burden, HFE mutations, and insulin resistance to fibrosis in nonalcoholic fatty liver. *Hepatology* 2004; **39**: 179-187 [PMID: 14752836 DOI: 10.1002/hep.20023]
72 **Karcaaltincaba M**, Akhan O. Imaging of hepatic steatosis and fatty sparing. *Eur J Radiol* 2007; **61**: 33-43 [PMID: 17118603 DOI: 10.1016/j.ejrad.2006.11.005]
73 **Hamer OW**, Aguirre DA, Casola G, Lavine JE, Woenckhaus M, Sirlin CB. Fatty liver: imaging patterns and pitfalls. *Radiographics* 2006; **26**: 1637-1653 [PMID: 17102041 DOI: 10.1148/rg.266065004]
74 **Mazhar SM**, Patton HM, Scuderi RT, Yokoo T, Fari CS, Sirlin CB. Fatty Liver Disease. In: Sahani DV, Samir AE. Abdominal Imaging. Saunders: an imprint of Elsevier Inc, 2011: 595-606
75 **Rofsky NM**, Fleishaker H. CT and MRI of diffuse liver disease. *Semin Ultrasound CT MR* 1995; **16**: 16-33 [PMID: 7718279 DOI: 10.1016/0887-2171(95)90012-8]
76 **Charatcharoenwitthaya P**, Lindor KD. Role of radiologic modalities in the management of non-alcoholic steatohepatitis. *Clin Liver Dis* 2007; **11**: 37-54, viii [PMID: 17544971 DOI: 10.1016/j.cld.2007.02.014]
77 **Thomas EL**, Hamilton G, Patel N, O'Dwyer R, Doré CJ, Goldin RD, Bell JD, Taylor-Robinson SD. Hepatic triglyceride content and its relation to body adiposity: a magnetic resonance imaging and proton magnetic resonance spectroscopy study. *Gut* 2005; **54**: 122-127 [PMID: 15591516 DOI: 10.1136/gut.2003.036566]
78 **Elias J**, Altun E, Zacks S, Armao DM, Woosley JT, Semelka RC. MRI findings in nonalcoholic steatohepatitis: correlation with histopathology and clinical staging. *Magn Reson Imaging* 2009; **27**: 976-987 [PMID: 19356874 DOI: 10.1016/j.mri.2009.02.002]
79 **Yokoo T**, Bydder M, Hamilton G. Hepatic fat quantification by low flip-angle multi-echo gradient-echo MR imaging: a clinical study with validation with MR spectroscopy. Presented before the annual meeting of the International Society for Magnetic Resonance in Medicine. Canada: Toronto, 2008: 706
80 **Wong VW**, Vergniol J, Wong GL, Foucher J, Chan HL, Le Bail B, Choi PC, Kowo M, Chan AW, Merrouche W, Sung JJ, de Lédinghen V. Diagnosis of fibrosis and cirrhosis using liver stiffness measurement in nonalcoholic fatty liver disease. *Hepatology* 2010; **51**: 454-462 [PMID: 20101745 DOI: 10.1002/hep.23312]
81 **Castéra L**, Foucher J, Bernard PH, Carvalho F, Allaix D, Merrouche W, Couzigou P, de Lédinghen V. Pitfalls of liver stiffness measurement: a 5-year prospective study of 13,369 examinations. *Hepatology* 2010; **51**: 828-835 [PMID: 20063276]
82 **Lall CG**, Aisen AM, Bansal N, Sandrasegaran K. Nonalcoholic fatty liver disease. *AJR Am J Roentgenol* 2008; **190**: 993-1002 [PMID: 18356447 DOI: 10.2214/AJR.07.2052]
83 **Cortez-Pinto H**, Baptista A, Camilo ME, De Moura MC. Nonalcoholic steatohepatitis--a long-term follow-up study: comparison with alcoholic hepatitis in ambulatory and hospitalized patients. *Dig Dis Sci* 2003; **48**: 1909-1913 [PMID: 14627331 DOI: 10.1023/A:1026152415917]
84 **Boden G**. Fatty acid-induced inflammation and insulin resistance in skeletal muscle and liver. *Curr Diab Rep* 2006; **6**: 177-181 [PMID: 16898568 DOI: 10.1007/s11892-006-0031-x]
85 **Barshop NJ**, Francis CS, Schwimmer JB, Lavine JE. Nonalcoholic fatty liver disease as a comorbidity of childhood obesity. *Ped Health* 2009; **3**: 271-281 [PMID: 20556232 DOI: 10.2217/phe.09.21]
86 **Roberts EA**. Non-alcoholic steatohepatitis in children. *Clin Liver Dis* 2007; **11**: 155-172, x [PMID: 17544977 DOI: 10.1016/j.cld.2007.02.008]
87 **Carter-Kent C**, Yerian LM, Brunt EM, Angulo P, Kohli R, Ling SC, Xanthakos SA, Whitington PF, Charatcharoenwitthaya P, Yap J, Lopez R, McCullough AJ, Feldstein AE. Nonalcoholic steatohepatitis in children: a multicenter clinicopathological study. *Hepatology* 2009; **50**: 1113-1120 [PMID: 19637190 DOI: 10.1002/hep.23133]
88 **Ozturk Y**, Soylu OB. Fatty liver in childhood. *World J Hepatol* 2014; **6**: 33-40 [PMID: 24653792]
89 **Schwenger KJ**, Allard JP. Clinical approaches to non-alcoholic fatty liver disease. *World J Gastroenterol* 2014; **20**: 1712-1723 [PMID: 24587650 DOI: 10.3748/wjg.v20.i7.1712]
90 **Cotler SJ**, Kanji K, Keshavarzian A, Jensen DM, Jakate S. Prevalence and significance of autoantibodies in patients with non-alcoholic steatohepatitis. *J Clin Gastroenterol* 2004; **38**: 801-804 [PMID: 15365409 DOI: 10.1097/01.mcg.0000139072.38580.a0]
91 **Itai Y**, Saida Y. Pitfalls in liver imaging. *Eur Radiol* 2002; **12**: 1162-1174 [PMID: 11976863 DOI: 10.1007/s00330-001-1178-0]
92 **Rinella ME**, McCarthy R, Thakrar K, Finn JP, Rao SM, Koffron AJ, Abecassis M, Blei AT. Dual-echo, chemical shift gradient-echo magnetic resonance imaging to quantify hepatic steatosis: Implications for living liver donation. *Liver Transpl* 2003; **9**: 851-856 [PMID: 12884199 DOI: 10.1053/jlts.2003.50153]

93 **Pilleul F**, Chave G, Dumortier J, Scoazec JY, Valette PJ. Fatty infiltration of the liver. Detection and grading using dual T1 gradient echo sequences on clinical MR system. *Gastroenterol Clin Biol* 2005; **29**: 1143-1147 [PMID: 16505760 DOI: 10.1016/S0399-8320(05)82179-7]

94 **Lelbach WK**. Epidemiology of alcoholic liver disease. *Prog Liver Dis* 1976; **5**: 494-515 [PMID: 775553]

95 **Kawamori Y**, Matsui O, Takahashi S, Kadoya M, Takashima T, Miyayama S. Focal hepatic fatty infiltration in the posterior edge of the medial segment associated with aberrant gastric venous drainage: CT, US, and MR findings. *J Comput Assist Tomogr* 1996; **20**: 356-359 [PMID: 8626889 DOI: 10.1097/00004728-199605000-00004]

96 **Patton HM**, Patel K, Behling C, Bylund D, Blatt LM, Vallée M, Heaton S, Conrad A, Pockros PJ, McHutchison JG. The impact of steatosis on disease progression and early and sustained treatment response in chronic hepatitis C patients. *J Hepatol* 2004; **40**: 484-490 [PMID: 15123364 DOI: 10.1016/j.jhep.2003.11.004]

97 **Hasegawa T**, Yoneda M, Nakamura K, Makino I, Terano A. Plasma transforming growth factor-beta1 level and efficacy of alpha-tocopherol in patients with non-alcoholic steatohepatitis: a pilot study. *Aliment Pharmacol Ther* 2001; **15**: 1667-1672 [PMID: 11564008 DOI: 10.1046/j.1365-2036.2001.01083.x]

98 **Fernandez FG**, Ritter J, Goodwin JW, Linehan DC, Hawkins WG, Strasberg SM. Effect of steatohepatitis associated with irinotecan or oxaliplatin pretreatment on resectability of hepatic colorectal metastases. *J Am Coll Surg* 2005; **200**: 845-853 [PMID: 15922194 DOI: 10.1016/j.jamcollsurg.2005.01.024]

99 **Brunt EM**, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol* 1999; **94**: 2467-2474 [PMID: 10484010 DOI: 10.1111/j.1572-0241.1999.01377.x]

100 **Niskanen L**, Uusitupa M, Sarlund H, Siitonen O, Paljärvi L, Laakso M. The effects of weight loss on insulin sensitivity, skeletal muscle composition and capillary density in obese non-diabetic subjects. *Int J Obes Relat Metab Disord* 1996; **20**: 154-160 [PMID: 8646252]

101 Executive summary of the clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults. *Arch Intern Med* 1998; **158**: 1855-1867 [PMID: 9759681]

102 **Luyckx FH**, Desaive C, Thiry A, Dewé W, Scheen AJ, Gielen JE, Lefèbvre PJ. Liver abnormalities in severely obese subjects: effect of drastic weight loss after gastroplasty. *Int J Obes Relat Metab Disord* 1998; **22**: 222-226 [PMID: 9539189 DOI: 10.1038/sj.ijo.0800571]

103 **Luyckx FH**, Scheen AJ, Desaive C, Thiry A, Lefébvre PJ. Parallel reversibility of biological markers of the metabolic syndrome and liver steatosis after gastroplasty-induced weight loss in severe obesity. *J Clin Endocrinol Metab* 1999; **84**: 4293 [PMID: 10566691 DOI: 10.1210/jcem.84.11.6171-4]

104 **Kugelmas M**, Hill DB, Vivian B, Marsano L, McClain CJ. Cytokines and NASH: a pilot study of the effects of lifestyle modification and vitamin E. *Hepatology* 2003; **38**: 413-419 [PMID: 12883485 DOI: 10.1053/jhep.2003.50316]

105 **Harrison SA**, Torgerson S, Hayashi P, Ward J, Schenker S. Vitamin E and vitamin C treatment improves fibrosis in patients with nonalcoholic steatohepatitis. *Am J Gastroenterol* 2003; **98**: 2485-2490 [PMID: 14638353 DOI: 10.1111/j.1572-0241.2003.08699.x]

106 **Sanyal AJ**, Mofrad PS, Contos MJ, Sargeant C, Luketic VA, Sterling RK, Stravitz RT, Shiffman ML, Clore J, Mills AS. A pilot study of vitamin E versus vitamin E and pioglitazone for the treatment of nonalcoholic steatohepatitis. *Clin Gastroenterol Hepatol* 2004; **2**: 1107-1115 [PMID: 15625656 DOI: 10.1016/S1542-3565(04)00457-4]

107 **Lavine JE**. Vitamin E treatment of nonalcoholic steatohepatitis in children: a pilot study. *J Pediatr* 2000; **136**: 734-738 [PMID: 10839868 DOI: 10.1016/S0022-3476(00)05040-X]

108 **Basaranoglu M**, Acbay O, Sonsuz A. A controlled trial of gemfibrozil in the treatment of patients with nonalcoholic steatohepatitis. *J Hepatol* 1999; **31**: 384 [PMID: 10453959 DOI: 10.1016/S0168-8278(99)80243-8]

109 **Lin HZ**, Yang SQ, Chuckaree C, Kuhajda F, Ronnet G, Diehl AM. Metformin reverses fatty liver disease in obese, leptin-deficient mice. *Nat Med* 2000; **6**: 998-1003 [PMID: 10973319 DOI: 10.1038/79697]

110 **Marchesini G**, Brizi M, Bianchi G, Tomassetti S, Zoli M, Melchionda N. Metformin in non-alcoholic steatohepatitis. *Lancet* 2001; **358**: 893-894 [PMID: 11567710 DOI: 10.1016/S0140-6736(01)06042-1]

111 **Nair S**, Diehl AM, Wiseman M, Farr GH, Perrillo RP. Metformin in the treatment of non-alcoholic steatohepatitis: a pilot open label trial. *Aliment Pharmacol Ther* 2004; **20**: 23-28 [PMID: 15225167 DOI: 10.1111/j.1365-2036.2004.02025.x]

112 **Ozturk ZA**, Kadayifci A. Insulin sensitizers for the treatment of non-alcoholic fatty liver disease. *World J Hepatol* 2014; **6**: 199-206 [PMID: 24799988 DOI: 10.4254/wjh.v6.i4.199]

113 **Angulo P**. Use of ursodeoxycholic acid in patients with liver disease. *Curr Gastroenterol Rep* 2002; **4**: 37-44 [PMID: 11825540 DOI: 10.1007/s11894-002-0036-9]

114 **Obinata K**, Maruyama T, Hayashi M, Watanabe T, Nittono H. Effect of taurine on the fatty liver of children with simple obesity. *Adv Exp Med Biol* 1996; **403**: 607-613 [PMID: 8915401 DOI: 10.1007/978-1-4899-0182-8_67]

115 **Abdelmalek MF**, Angulo P, Jorgensen RA, Sylvestre PB, Lindor KD. Betaine, a promising new agent for patients with nonalcoholic steatohepatitis: results of a pilot study. *Am J Gastroenterol* 2001; **96**: 2711-2717 [PMID: 11569700 DOI: 10.1111/j.1572-0241.2001.04129.x]

116 **Abdelmalek MF**, Sanderson SO, Angulo P, Soldevila-Pico C, Liu C, Peter J, Keach J, Cave M, Chen T, McClain CJ, Lindor KD. Betaine for nonalcoholic fatty liver disease: results of a randomized placebo-controlled trial. *Hepatology* 2009; **50**: 1818-1826 [PMID: 19824078 DOI: 10.1002/hep.23239]

117 **Adams LA**, Zein CO, Angulo P, Lindor KD. A pilot trial of pentoxifylline in nonalcoholic steatohepatitis. *Am J Gastroenterol* 2004; **99**: 2365-2368 [PMID: 15571584 DOI: 10.1111/j.1572-0241.2004.40064.x]

118 **Satapathy SK**. Beneficial effects of tumor necrosis factor-alpha inhibition by pentoxifylline on clinical, biochemical, and metabolic parameters of patients with nonalcoholic steatohepatitis. *Am J Gastroenterol* 2004; **99**: 1946-1952

119 **Bataller R**, Sancho-Bru P, Ginès P, Lora JM, Al-Garawi A, Solé M, Colmenero J, Nicolás JM, Jiménez W, Weich N, Gutiérrez-Ramos JC, Arroyo V, Rodés J. Activated human hepatic stellate cells express the renin-angiotensin system and synthesize angiotensin II. *Gastroenterology* 2003; **125**: 117-125 [PMID: 12851877 DOI: 10.1016/S0016-5085(03)00695-4]

120 **Yokohama S**, Yoneda M, Haneda M, Okamoto S, Okada M, Aso K, Hasegawa T, Tokusashi Y, Miyokawa N, Nakamura K. Therapeutic efficacy of an angiotensin II receptor antagonist in patients with nonalcoholic steatohepatitis. *Hepatology* 2004; **40**: 1222-1225 [PMID: 15382153 DOI: 10.1002/hep.20420]

121 **Belfort R**, Harrison SA, Brown K, Darland C, Finch J, Hardies J, Balas B, Gastaldelli A, Tio F, Pulcini J, Berria R, Ma JZ, Dwivedi S, Havranek R, Fincke C, DeFronzo R, Bannayan GA, Schenker S, Cusi K. A placebo-controlled trial of pioglitazone in subjects with nonalcoholic steatohepatitis. *N Engl J Med* 2006; **355**: 2297-2307 [PMID: 17135584 DOI: 10.1056/NEJMoa060326]

122 NIH conference. Gastrointestinal surgery for severe obesity. Consensus Development Conference Panel. *Ann Intern Med* 1991; **115**: 956-961 [PMID: 1952493]

123 **Mummadi RR**, Kasturi KS, Chennareddygari S, Sood GK. Effect of bariatric surgery on nonalcoholic fatty liver disease: systematic review and meta-analysis. *Clin Gastroenterol Hepatol* 2008; **6**: 1396-1402 [PMID: 18986848 DOI: 10.1016/j.cgh.2008.08.012]

124 **Contos MJ**, Cales W, Sterling RK, Luketic VA, Shiffman ML, Mills AS, Fisher RA, Ham J, Sanyal AJ. Development of nonalcoholic fatty liver disease after orthotopic liver transplantation

for cryptogenic cirrhosis. *Liver Transpl* 2001; **7**: 363-373 [PMID: 11303298 DOI: 10.1053/jlts.2001.23011]

125 **Burke A**, Lucey MR. Non-alcoholic fatty liver disease, non-alcoholic steatohepatitis and orthotopic liver transplantation. *Am J Transplant* 2004; **4**: 686-693 [PMID: 15084161 DOI: 10.1111/j.1600-6143.2004.00432.x]

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REVIEW

Progress and prospects of engineered sequence-specific DNA modulating technologies for the management of liver diseases

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Abstract

Liver diseases are one of the leading causes of mortality in the world. The hepatic illnesses, which include inherited metabolic disorders, hemophilias and viral hepatitides, are complex and currently difficult to treat. The maturation of gene therapy has heralded new avenues for developing effective intervention for these diseases. DNA modification using gene therapy is now possible and available technology may be exploited to achieve long term therapeutic benefit. The ability to edit DNA sequences specifically is of paramount importance to advance gene therapy for application to liver diseases. Recent development of technologies that allow for this has resulted in rapid advancement of gene therapy to treat several chronic illnesses. Improvements in application of derivatives of zinc finger proteins (ZFPs), transcription activator-like effectors (TALEs), homing endonucleases (HEs) and clustered regularly interspaced palindromic repeats (CRISPR) and CRISPR associated (Cas) systems have been particularly important. These sequence-specific technologies may be used to modify genes permanently and also to alter gene transcription for therapeutic purposes. This review describes progress in development of ZFPs, TALEs, HEs and CRISPR/Cas for application to treating liver diseases.

Key words: Gene therapy; Zinc fingers; Transcription activator-like effectors; Clustered regularly interspaced short palindromic repeats; Homing endonucleases; Liver diseases

Core tip: The treatment of liver diseases is varied and often complicated. Gene editing is being developed to treat a variety of chronic disorders and has exciting potential for curing hepatic diseases. Engineering of derivatives of zinc finger proteins, transcription activator-like effectors, homing endonucleases and clustered regularly interspaced palindromic repeats potentially enables sequence-specific gene editing. These DNA binding proteins may be used to alter genes permanently or to influence the epigenetic status of liver cells for

therapeutic benefit.

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LIVER DISEASES AND THE APPLICATION OF GENE THERAPY

The liver plays an important role in metabolism and the etiology of numerous inherited and acquired disorders and can be traced to this organ. Liver diseases are varied and suitable options for therapy are often limited[1]. Treatment outcomes are dependent not only on patient history and disease type, but also disease progression and prior treatment[2]. Gene therapy, which offers the potential for improving currently available therapy for liver diseases, is defined as the introduction of nucleic acids into cells to treat a disease. Gene therapy may cause: (1) substitution or correction of genetic material; (2) changes in gene expression through augmentation; (3) post-transcriptional regulation; (4) cell death as a result of expression of toxic genes; and (5) transcriptional repression or activation (Figure 1)[3]. Applications of the approach may be expanded through use in concert with cell-based therapies. Collectively these versatile technologies have the potential to improve efficacy and expand the repertoire of strategies for management of liver diseases. Gene therapy is a rapidly developing field of research that has application to treatment and prophylaxis of many liver diseases, such as is caused by persistent hepatitis B virus (HBV) infection. Selected examples of gene therapy for various liver diseases are shown in Tables 1 and 2.

Although the application of gene therapy is far reaching, not all liver diseases are currently amenable to this type of treatment. Reversal of certain hepatic disorders, such as alcohol induced cirrhosis, is not feasible at present. With other diseases, such as tuberculosis of the liver, suitable technology is not yet available to effect treatment. For these reasons gene therapy is focused on developing treatment for hereditary monogenic diseases, certain viral infections, some acquired and complex genetic cellular gene defects. Complex genetic diseases are caused by multiple gene mutations which accumulate over time. An example of such a disease is hepatocellular carcinoma (HCC)[4]. Hereditary monogenic diseases are the result of a single inherited gene defect[5,6] and include disorders such as Crigler-Najjar syndrome type 1[5] and tyrosinemia type 1[6]. These diseases are caused by mutations in the genes encoding uridine 5'-diphospho-glucouronosyl transferase[7] and fumarylacetoacetate hydrolase (Fah) respectively[6], and result in hepatic accumulation of toxic metabolites. Other hereditary monogenic diseases such as hemophilia A and B, caused by gene mutations in coagulation factors Ⅷ[8] and Ⅸ[9] respectively, result in excessive bleeding and associated extra hepatic manifestations[10]. Viral infections of the liver include hepatitis B[11] and hepatitis C[12]. These viral hepatitides are major risk factors for chronic liver diseases, with carriers exhibiting increased susceptibility to cirrhosis and hepatocellular carcinoma[11,12]. Gene therapy can be applied to treating these diseases by altering gene expression through gene editing, augmentation of gene expression, transcriptional repressive or activational mechanisms (Figure 1)[3].

Availability of DNA editing tools will expand the application of gene therapy to the treatment of liver diseases. Currently available technologies have limitations. For example, application of RNA interference-based silencing to treatment of HBV infection may not be sufficiently durable and specific to achieve a safe curative effect. Genome modification technologies provide the means for overcoming some of these shortcomings. Currently gene editing tools are based on engineered derivatives of zinc finger proteins (ZFPs)[13], transcription activator-like effectors (TALEs)[14], homing endonucleases (HEs)[15] and clustered regularly interspaced palindromic repeat (CRISPR) and CRISPR associated (Cas) protein arrays[16,17]. This review will explore the application of these sequence-specific DNA modification technologies to treatment of liver diseases.

SEQUENCE-SPECIFIC DNA EDITING TECHNOLOGIES: A TOOL FOR GENE THERAPY

The ability to sequence entire genomes has facilitated identification of genetic defects that underlie diseases, and this has assisted with prevention and cure of illness. Sequence-specific DNA editing technologies thus present a novel, versatile tool for modifying the genome and treating disease. Early applications of this approach were hampered by several factors. These included low efficiency, the need for complicated screening to identify suitable gene editors and the potential for adverse effects caused by lack of specificity[16,26]. During the past decade, characterization of derivatives of sequence-specific proteins and RNA-guided nucleases[27], which include ZFPs[28], TALEs[29], HEs[30,31] CRISPR/Cas[17] has allowed investigators to develop tools to edit almost any gene.

ZFPs

ZFP arrays rely on the sequence-specific interaction of the His_2-Cys_2 protein motif present in individual fingers with specific sequences in the target DNA[32]. Zinc finger (ZF) domains constitute one of the most common DNA-binding motifs in eukaryotes and prokaryotes. Moreover they are the second most commonly encoded family of proteins in the human genome. Each finger consists

Table 1 Selected examples of liver-specific gene therapies for complex genetic and hereditary monogenic disorders

Disease	Therapy	Rationale	Stage of development	Ref.
Gene therapy for complex genetic diseases				
Hepatocellular carcinoma	Recombinant human adenovirus type 5 administration followed by TACE	Adenovirus is highly infectious and when it is used in conjunction with TACE it improves tissue penetration and thus tumor shrinkage	Clinical (phase Ⅰ and Ⅱ)	[18]
Gene therapy for hereditary monogenic diseases				
Crigler-Najjar syndrome type Ⅰ	AAV neonatal mouse *hUGT1A1* gene transfer	AAV has low immunogenicity and is highly infectious in hepatocytes. Thus, in this study expression of bilirubin UDP glucuronosyl-transferase was augmented a large number of hepatocytes following transduction with *hUGT1A1*	Preclinical	[19]
Familial hyper-cholesterolemia	Hepatocytes corrected with retroviruses expressing the low-density lipoprotein receptor	The transplantation of hepatocytes allows the slow repopulation of the liver with a desirable phenotype. In this study this method was used to introduce hepatocytes expressing the low-density lipoprotein receptor	Clinical (phase Ⅰ)	[20]
Hemophilia A	Recombinant factor Ⅷ fused to Fc domain of IgG1 (rFⅧFc)	Coagulation factor replacement therapy requires the regular replacement of factor Ⅷ (FⅧ) with recombinant FⅧ products or plasma-derived concentrates. The use of a long-lasting recombinant FⅧ protein would reduce the need for frequent injections. The fusion of the human Fc domain of IgG1 to F Ⅷ extends the half-life of FⅧ and may reduce the injection frequency by 50% when compared with current treatments	Clinical (phase Ⅲ)	[21]

TACE: Trans-catheter arterial chemoembolization; AAV: Adeno-associated virus; UDP: Uridine 5'-diphospho.

Table 2 Selected examples of liver-specific gene therapies for viral hepatitides

Disease	Therapy	Rationale	Disadvantages	Stage of development	Ref.
HBV	Expression of anti-HBV primary microRNA (pri-miR)-122- and pri-miR-31-based mimics	Using artificial HBV-targeting pri-miRNAs it was possible to silence viral genes selectively, thus reducing their expression and causing inhibition of viral replication	Expression of pri-miRs must be maintained over prolonged periods	Preclinical	[22]
HBV	Administration of increasing doses of HB-110 DNA vaccine with fixed doses of adefovir dipivoxil	HB-110 is a DNA vaccine used in chronic hepatitis B infections. This vaccine induces antigen production and stimulates the immune response; helping to clear infected cells from the liver. Adefovir dipivoxil is a chemical therapeutic which blocks reverse transcriptase preventing viral replication. When used in conjunction, adefovir dipivoxil prevents viral replication and thus infection of healthy hepatocytes while the stimulated immune system clears infected hepatocytes, thus reducing the symptoms of chronic HBV	Possibility of escape mutants and viral rebound after therapeutic withdrawal	Clinical (phase Ⅰ)	[23]
HBV	Administration of a single dose ARC-520 with entecavir	ARC-520 is an RNAi therapeutic that will be used in combination with the inhibitor of HBV DNA polymerase, entecavir, in patients with chronic HBV infection. The decline of HBsAg along with other measures will be evaluated to determine the efficacy of treatment in response to a single dose of ARC-520	The efficacy of RNAi-therapeutics must be maintained over prolonged periods	Clinical (phase Ⅱa)	[24]
HCV	Administration of Miravirsen to target miR-122 with LNA–modified oligonucleotide	miR-122 expression is specific to the liver and plays an important role in the regulation of HCV replication. Disrupting miRNAs can be difficult but one of the tools used to silence their activity are LNA-modified oligonucleotides. Their bridge modification significantly increases their hybridization properties, making disassociation more difficult. In this study they produced a LNA-oligonucleotide which is complementary to miR-122, inhibiting its activity; this decreases HCV replication and reduces infection	LNA-oligonucleotide expression needs to be maintained for long periods	Clinical (phase Ⅱ)	[25]

HBV: Hepatitis B virus; HCV: Hepatitis C virus; RNAi: RNA interference; HBsAg: Hepatitis B surface antigen; LNA: Locked nucleic acid.

of 30 amino acids with 3 amino acids from an α-helical region interacting with 3 consecutive base pairs (bp) in the major groove of the target DNA[13]. Naturally DNA-binding domains comprise tandem repeats of up to three fingers, and thus allow recognition of up to 9 bp of the target sequence. The ability to link more than 3 adjacent fingers together has been useful for application of this technology and was made possible through the

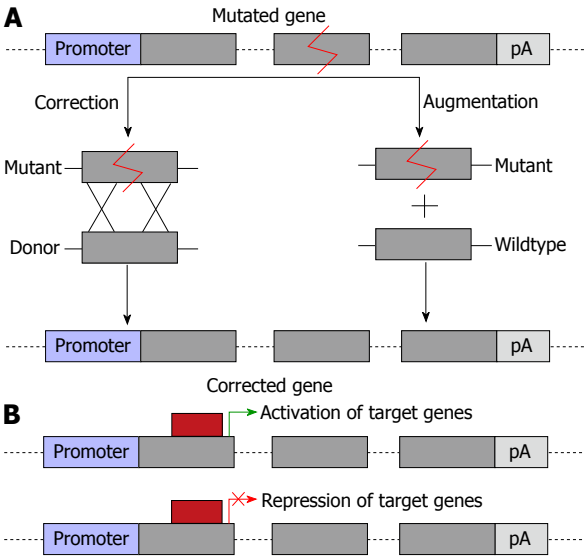

Figure 1 Potential outcomes of gene therapy. A: Correction of mutations or augmentation of gene function by introduction of a second copy of the gene of interest into a safe harbor locus; B: Changes to transcriptional regulation of a gene of interest, allowing for correction of gene function either by transcriptional activation or suppression. The grey boxes represent exons and linking lines represent introns.

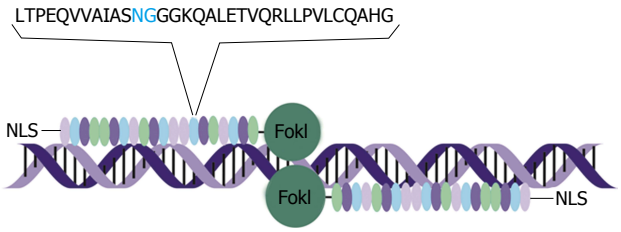

Figure 2 Schematic representation of transcription activator-like effector nuclease interaction with DNA. A pair of transcription activator-like effector (TALE) nucleases interacts with its target through a tandem arrangement of DNA-binding modules, represented by oval structures in the schematic. Each DNA-binding module comprises 34 amino acids, with a repeat variable diresidue (RVD), in blue, at amino acids 12 and 13. The RVD specifies the DNA base to be targeted. There is a nuclear localisation signal on the N-terminal and a FokI effector bound to the C-terminal. As well as FokI nuclease domains, TALEs may also be bound to transcriptional regulators such as Krüppel-associated box or VP16/64. NLS: Nuclear localisation signal.

discovery of a structurally conserved linker region of sequence amino acid TGEKP[33]. This conserved linker region provides additional stability to the protein-DNA complexes[33]. It is possible to build modular arrays that recognize 9-18 bp, which would provide sufficient specificity for targeting within the human genome[34,35]. Through use of libraries consisting of combinations of ZFs that recognize almost all 64 possible nucleotide triplets, it was possible to select ZFPs with the intended target binding specificity[13,36]. A drawback of the approach was that combinations of the fingers did not always bind to targets with intended affinity[13,36]. This phenomenon is the result of context dependency of the individual fingers[13,36]. That is, binding affinity of individual fingers is influenced by neighboring modules, and strength of interaction of the fingers with their intended cognates depends on properties of surrounding sequences of the ZFP[13,36]. The development of the Oligomerized Pool ENgineering protocols, which provides a publically available tool for the development of ZFPs, improved the ability of researchers to select proteins with good target-binding properties[37]. Subsequently, an approach was developed that takes into account the effect of neighboring individual ZFs when generating an array. This method, context dependent assembly (CoDA), selects for active arrays by using combinations derived from known existing functional ZFPs[38]. In CoDA the N and C-terminal fingers from different arrays, which have a common middle finger, can be joined together to make new active ZFPs. The method is rapid and does not require a selection process, thus reducing the screening required for identification of functional ZFPs[35,38]. As a result of the influence of context dependency, and the experimentally observed affinity of ZFPs for G-rich sequences, not all DNA sequences are suitable targets for binding by ZFPs. It has been estimated that one pair of efficient ZFPs may be generated for approximately every 100 bp of the human genome[16]. The context dependency of the ZFs also has implications for off-target activity. Tools that aid in target selection and prediction of off-target activity have been developed to assist with generating functional ZFPs[28,39]. Despite the difficulties associated with use of ZFPs they are currently the most widely applied of the genome editing technologies. This is illustrated by their recent application in preclinical and clinical settings in which ZF nucleases (ZFNs) were used to disrupt the human immunodeficiency virus (HIV) co-receptor CC-chemokine receptor 5 gene in $CD4^+$ T cells to produce cells that are resistant to HIV infection[40-42].

TALEs

TALEs are a unique family of DNA-binding proteins that have been isolated from the phytopathogenic bacteria of Xanthomonas species and Ralstonia solanacearum[43]. The characterization of native TALEs led to discovery of the DNA-binding region, which is a highly repetitive protein sequence[43-45]. Each repeat comprises 33-35 amino acids[43] that collectively constitute a protein of approximately 122 kDa[46]. The repeats of the TALE each recognize and bind to a single nucleotide. The nucleotide specificity is conferred by two consecutive variable residues at positions 12 and 13 of the repeated unit, and are termed the repeat variable diresidues (RVDs) (Figure 2). Each RVD interacts specifically with either A, T, G or C nucleotides[47], and a simple code determines nucleotide binding. There are 20 known RVDs and certain RVDs are found more commonly than others in native TALEs[47]. Individual RVD recognition of its cognate nucleotide is independent of both the preceding and succeeding RVDs[43,47]. This lack of context dependence is a distinct advantage over ZFPs, and allows for convenient construction of TALE subunits that recognize specific DNA sequences. A requirement of efficient TALE binding is the presence of a T nucleotide

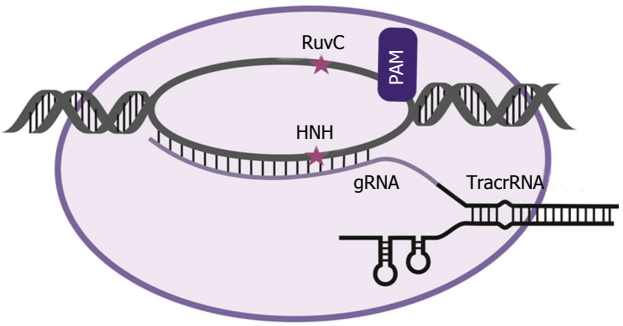

Figure 3 Schematic representation of the clustered regularly interspaced palindromic repeats/clustered regularly interspaced palindromic repeats associated 9 complex and its interaction with DNA. With the CRISPR/Cas system, a 20 nucleotide guide RNA is processed and bound to the Cas9 protein. When adjacent to a proto-spacer–associated motif element, the guide recognises and binds a complementary sequence in the DNA to direct target cleavage by the Cas9 protein. A catalytically inactive Cas9 protein may also be coupled to other effectors such as Krüppel-associated box or VP16/64 domains. CRISPR/Cas: Clustered regularly interspaced palindromic repeats/CRISPR associated. CRISPR: Clustered regularly interspaced palindromic repeats; Cas: CRISPR associated; tracrRNA: Trans-activating CRISPR RNA; gRNA: RNA guide.

at the base 0 position of the cognate (T^0)[48]. Substitution of T^0 with another nucleotide reduces the binding of the TALE[49] and places a minor restriction on the number of target sites within the genome that can be recognized by custom TALEs. TALE recognition sequences are up to 18 nucleotides in length and alterations at a single nucleotide can have a significant impact on binding efficiency. However, the average binding contribution of individual RVDs is minimal. Nevertheless, choice of RVD may have an impact on the electrostatic interactions between the TALE and DNA, which in turn may influence the stability of the DNA-TALE complex. The length of the target sequence is approximately equal to the number of RVD repeats and single repeats each bind to 1 nucleotide of DNA[48]. Mismatches at the 5′ end of the recognized sequence are more detrimental to TALE binding than they are at the 3′ end of a target[47]. This suggests that the generation of synthetic designer TALEs should be biased to favor selection of unique targets within 5′ regions[47]. Apart from RVD choice and target sequence selection, other factors influencing TALE binding efficacy include modifications to the DNA target and chromatin structure variations[47]. Valton *et al*[50] showed that TALE arrays cannot bind 5-methylcytosine (5mC) residues, which may pose barrier to application in mammalian systems where DNA methylation commonly occurs as a transcriptional regulatory mechanism[51]. The RVDs NG and N* both have some affinity for 5mC, and these RVDs may thus be incorporated into engineered TALEs to target DNA that is methylated[50]. Development of web-based tools, which take all of these parameters into account, has simplified TALE array design and facilitated their more wide spread application[52]. TALEs are large proteins, and with addition of the nuclease or transcriptional regulation domains, DNA encoding the engineered DNA binding proteins may be as long as 4 kb[43,53,54]. This makes them significantly larger than ZFPs and considerably larger than HEs. Although the TALE binding arrays have been shown to be robust and easily programmable, the size of the TALEs makes their delivery challenging for therapeutic application.

CRISPR/Cas

CRISPR/Cas systems are naturally RNA-mediated adaptive defense mechanisms that are found in some prokaryotic organisms. Using short CRISPR RNAs (crRNAs) and Cas proteins, the prokaryote is able to identify and eliminate invading DNA elements[55]. CRISPR/Cas complexes identify targeted genomic loci using a 20 nucleotide RNA guide (gRNA), which is complementary to its target DNA sequence. CRISPR/Cas systems are classified into three types (Ⅰ-Ⅲ) on the basis of the structure and sequence of their Cas proteins[56]. All three have three essential components: the CRISPR array, the upstream leader sequence and the cas genes[57]. The most commonly used CRISPR/Cas system for gene editing applications is type Ⅱ CRISPR/Cas[58]. The CRISPR array is composed of identical repeats that are 23-47 bp in length[59]. The CRISPR/Cas leader region acts as a promoter for the transcription of the CRISPR array[60]. The cas genes encode the Cas proteins. This protein contains RuvC-like and HNH-like catalytic domains, which cleave the targeted DNA. The HNH-like domain cleaves the strand that is complementary to the gRNA, while the RuvC region cuts the other non-complementary DNA strand of the target[61]. Recognition of the target sequence by the Cas proteins is facilitated by presence of the proto-spacer-associated motif (PAM). This sequence is usually 2-4 nucleotides long and flanks the target site. It is absent from the endogenous loci and thus prevents CRISPR/Cas auto cleavage and adds specificity to targeting[60].

The type Ⅱ CRISPR/Cas systems use the Cas9 protein, which recognizes a PAM sequence of 5′NGG3′. In type Ⅱ systems a small non-coding RNA, known as the trans-activating crRNA (tracrRNA), which is partially complementary to the CRISPR repeats, forms an RNA duplex with crRNA. This RNA hybrid is recognized and processed to form mature gRNA and, subsequently, associates with Cas9. The complex, including the tracrRNA, recognizes invading DNA and inactivates it by cleavage[60,62] (Figure 3). When using an engineered CRISPR/Cas system cognates for new gRNAs are identified within the targeted gene. Binding sites for these artificial gRNAs are preceded by the PAM, which is required by the associated Cas protein. Optimization of the CRISPR/Cas guide architecture, to enhance specificity and broaden their application, is a highly active field of research[63]. As with other gene editing technologies, the CRISPR/Cas system faces several developmental challenges, including potential off-target activity which may result in adverse mutation.

HEs

HEs are named as a result of their native endonuclease and homing activities. Homing is the transfer of introns

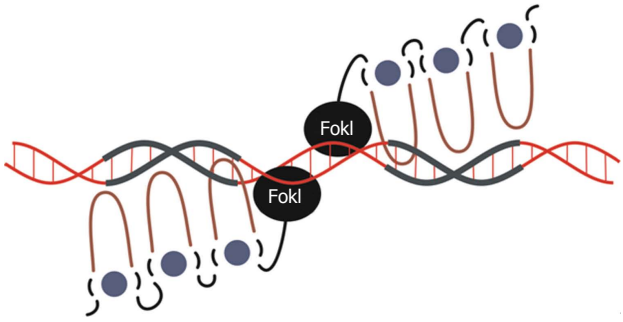

Figure 4 Schematic representation of zinc finger nuclease interactions with DNA. Each zinc finger interacts with 3-4 bp in the major groove of the DNA double helix. The Hys2Cys2 side chains (depicted as the loop) bind to the DNA, which is facilitated by the interaction between the zinc finger protein (ZFP) and a Zinc ion (depicted as green circle) which stabilizes the protein structure. As well as FokI nuclease domains, ZFPs may also be bound to other effectors such as Krüppel-associated box or VP16/64.

or inteins into alleles that themselves do not have introns or inteins[64]. These endonucleases are encoded by open reading frames (ORFs) within the mobile sequence. They avoid disrupting the genetic function of the host by exclusively moving the mobile sequences into inteins and introns. HEs have an ability to recognize DNA target sites of 14-44 bp, which correspond to the intron/intein insertion site. They create a double-strand break (DSB) or single-stranded nick to promote insertion of sequences encoding the HEs within introns or inteins into the target allele[15]. Group I introns, large self-splicing ribozymes, encode the most well characterized HEs. In the case of group I introns, the HEs are translated prior to transposition of the mobile element[15]. The resulting endonuclease is specific for the "intronless" target site. Host-mediated homology-directed repair (HDR) results in the unidirectional transfer of the HE-encoding intron into the cleaved allele with disruption of the homing site. Although less well studied, the process for HEs comprising inteins is likely to be similar to that of HEs encoded within group I introns[65]. Six families of group I HEs have been identified, namely: LAGLIDADG; GIY-YIG; HNH; His-Cys box; EDxHD and PD-(D/E) xK[15]. These families are classified according to the presence of conserved amino acid sequence motifs within their active sites. The largest family of the HEs is the LAGLIDADG HEs (LHEases). LHEases generate staggered DSBs in the DNA at the target site to produce four nucleotide 3′ overhangs[15] which induce DNA repair.

HEs are attractive gene editing tools because of their high sequence specificity and were applied as gene modifiers in murine cells 20 years ago[66]. Since then, studies have been performed targeting human genes[67] and certain monogenic diseases[30]. Theoretically, their high sequence specificity should reduce off-target effects, which is supported by their limited toxicity when expressed in cells over prolonged periods[31]. A further advantage to HEs is that they are significantly smaller than ZFPs and TALEs, which simplifies their delivery. However, engineering HEs is significantly more difficult than it is for ZFPs, or TALEs[16]. This is because enzymatic and target recognition sequences are located within the same domains of the HEs, and altering target specificity without compromising enzymatic function is complicated to achieve[15]. Despite these limitations, studies continue to advance the development of HEs[68,69]. It is possible that improvements in use of HEs may lead to their use for gene editing purposes and for liver-specific applications. However, with advances in other gene editing technologies use of HEs are currently not in favor.

USE OF SEQUENCE-SPECIFIC GENE EDITING TECHNOLOGIES FOR TREATMENT OF LIVER DISEASES

Although gene editing as a mode of treating liver diseases is still limited, application of the technologies to management of other diseases has useful implications for hepatology. Gene editing initially focused on creation of DNA-modifying nucleases derived from ZFPs[28,70], TALEs[29,71], CRISPR/Cas[17,58] and HEs[15]. The native endonuclease activity of CRISPR/Cas and HEs means that no additional effectors are required for their application as nucleases. However TALEs and ZFPs require that nuclease domains be added to the DNA-binding regions, and FokI is most commonly used. FokI is a type Ⅱ S restriction enzyme that recognizes its target site as a monomer and nicks a single-strand of DNA[72], but requires dimerization for double-stranded DNA-cleaving activity[73]. The implication of this is that a ZFN and TALE nuclease (TALEN) pairs are required to effect complete cleavage of the duplex. To engineer a site-specific ZFN or TALEN, independent subunits need to be designed to nick each strand of the DNA target. To achieve intended cleavage of the DNA duplex, the subunits need to be delivered to a target cell simultaneously[16] (Figures 2 and 4). This is not necessarily a limitation as the addition of a second nuclease improves sequence-specificity, which is especially desirable in a clinical setting.

DNA-modifying nucleases enable genome modification by introducing DSBs in the DNA and stimulation of the mutagenic non-homologous end joining (NHEJ) and high fidelity HDR pathways[26] (Figure 5). Repeated activation of NHEJ typically results in generation of small insertions or deletions (indels) at a targeted site that may be used to disable specific genes (Figure 5)[26]. Introduction of a gene modifying nuclease along with donor DNA (plasmid, single-stranded or double-stranded oligonucleotides), which has homology to sites flanking the target, induces HDR[74,75]. By activating HDR it is possible to make precise alterations to the genome: correction of single point mutations, facilitation of integration of single and multiple transgenes into specific loci can be achieved[76,77] (Figure 5). As a result permanent correction, silencing or augmentation of cellular genes may be achieved. When two duplex DNA-cleaving nucleases are simultaneously introduced

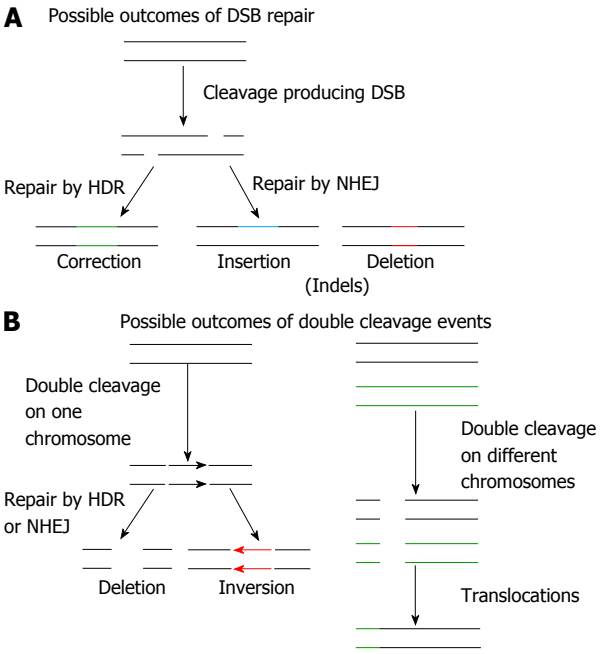

Figure 5 Potential effects of using genome editing endonucleases. A: Nuclease induced double strand breaks (DSB) may be repaired by homology directed repair (HDR), resulting in corrections that may be to a single base pair or thousands of base pairs. Repair by non-homologous end joining (NHEJ) may result in non-specific insertions and deletions (indels); B: The introduction of two nucleases targeting the same chromosome may result in large deletions or inversions of the DNA. Chromosomal translocations may occur when two DSBs are introduced on different chromosomes.

into a cell, inversions, translocations, large insertions and deletions may be induced within the intervening sequences (Figure 5).

Early investigations focused on the nuclease activity of gene modifiers. However, their potential for off-target mutagenic effects, accumulation of cytotoxic DSBs and reliance on host repair machinery, led investigators to explore therapeutic utility of transcriptional activators and repressors[16] to modulate genes selectively without changing the sequence of the DNA[71]. A commonly used transcriptional repressor is the Krüppel-associated box (KRAB) domain, which is a 75 amino acid motif originally found on the N-terminus of ZFPs[78,79]. The Herpes simplex virus type I-derived 65 kDa VP16[80] domain, or its synthetic tetrameric derivative VP64[81], are commonly used to effect transcriptional activation.

TALEs, ZFPs and CRISPR/Cas constructs have all been used to create artificial transcription factors[82,83]. ZFPs or TALEs may be fused to an activator or repressor domain to create ZF-transcription factors or TALE-transcription factors. These transcription factors have the advantage of functioning as monomeric proteins, although multiple transcription factors might improve their efficacy. Modification of CRISPR/Cas arrays to act as artificial transcription factors is achieved by modifying the Cas9 endonuclease to render it catalytically inactive, and form the so-called dead Cas9 (dCas9). dCas9 may still be recruited by gRNAs to target specific DNA sites[84], but when modified with an effector domain such as KRAB or VP64, it is able to alter the epigenetic state of its target[85].

In addition to deciding on which type of gene modifier to apply for therapy, there are disease-specific considerations that must be evaluated before a new treatment may be developed. These include an assessment of the proportion of hepatocytes that needs to be treated, duration of expression of a gene modifier, the potential for off-target activity and the type of gene alteration that is required to attain a therapeutic effect. Thus, treatment of a viral hepatitis would be vastly different from that required for a hereditary or complex genetic disorder.

APPLICATION OF GENE EDITING TECHNOLOGIES TO THE TREATMENT OF VIRAL HEPATITIS

Zimmerman *et al*[86] investigated the ability of ZFPs to target the episomal covalently closed circular DNA (cccDNA) reservoir of duck HBV (DHBV). This replication intermediate is a template for the transcription of viral genes and the production of pre-genomic RNA[86]. The persistence of cccDNA is responsible for chronic HBV infection and currently there are no available therapies that directly target this replication intermediate. The anti-DHBV ZFPs were designed to target the enhancer 1 region of the cccDNA, as this element controls transcription from both the core and surface promoters. After introduction of the ZFPs into cells in culture, a decrease in total viral RNA and pre-genomic RNA with ZFPa (61.2% and 57.2% respectively) and ZFPb (45.3% and 73.5% respectively) was observed. Furthermore, a reduction in DHBV core and surface protein expression and production of virus progeny was also reported. Importantly, the introduction of the ZFPs did not result in any significant toxicity[86]. In 2010 Cradick *et al*[87] evaluated the ability of ZFNs to target and disrupt HBV DNA. The ZFNs used were designed by searching the HBV genome for recognition sites for pairs of three-finger ZFNs separated by six nucleotides. One pair of ZFNs was selected from 18. This ZFN pair cleaved within the HBV core ORF and reduced the pre-genomic RNA by 29%. About 26% of targeted DNA was linearized in the presence of the ZFNs and approximately 10% showed aberrant re-ligation. The re-ligated products all showed indels and subsequent disruption of viral replication markers[87]. Although this study demonstrated the ability of ZFNs to cleave HBV DNA targets, there was no evidence to suggest that the ZFN pair was able to disable the HBV cccDNA.

Zhao *et al*[88] evaluated the use of a ZF-artificial repressor (ATF) to down-regulate an integrated hepatitis B X gene (HBx) in the Hep3B hepatocellular carcinoma cell line. During normal HBV infection, HBx gene integration is associated with the progression of chronic HBV to HCC and is responsible for the dysregulation

of expression of a number of cellular genes. The ZFP-based artificial repressor recognized 18 bp in the enhancer 1 region of the HBx gene and used the KRAB domain to effect knockdown. Investigators saw a marked decrease in luciferase production from an HBx reporter construct. When the ATF was stably expressed in the Hep3B cell line there was significant cell cycle arrest. In a cell lines without the integrated HBx gene no cell cycle arrest was observed[88]. This study presents a novel approach to inhibiting HCC progression caused by HBV and illustrates the potential of applying gene editing technologies to the functional repression of disease associated genes.

In 2013, Bloom *et al*[89] were the first group to demonstrate the potential of anti-HBV TALENs for treatment of chronic HBV infection. The investigators successfully designed four pairs of TALENs. Two of these pairs target the surface (S) and core (C) ORFs of HBV. The S and C targeting TALENs efficiently caused indels in these ORFs including those found within the cccDNA of HepG2.2.15 cells which stably replicates HBV[90]. In this assay, the cccDNA was isolated using a hirt extraction coupled to use of an ATPase dependent nuclease, which selectively degrades nicked or linear DNA. Following this isolation, deep sequencing was used to evaluate targeted disruption of the TALEN cleavage sites. The S TALEN disrupted 31% of the cccDNA, while the C TALEN disrupted 12% of the HBV cccDNA. Hydrodynamic injection (HDI) was used to introduce HBV DNA into mice to simulate HBV replication *in vivo*. A significant reduction in markers of viral replication following the HDI-mediated introduction of the TALENs was observed in this model. The S TALEN effected knockdown of HBV surface antigen (HBsAg) by more than 90% on both days 3 and 5 after HDI. Circulating viral particle equivalents decreased by approximately 70% with the introduction of either the S or C TALEN. Immunohistochemical detection of the HBV core antigen in the liver of treated mice was only decreased with the addition of the C TALEN. These results not only showed that the TALENs had an effect in a murine model of HBV infection but that the effect of each TALEN was limited to its specific target. Furthermore, intrahepatic mRNA concentrations remained constant after TALEN treatment, demonstrating that inhibition of viral replication markers was the result of inactivating the targeted genes rather than transcriptional repression. It was noted that the other two pairs of TALENs, targeting the polymerase ORF (P1 and P2) did not result in target cleavage despite reducing HBsAg expression. These observations suggested that the P1 and P2 TALENs may function through a different mechanism of action, such as by targeted transcriptional repression. Measurements of aspartate aminotransferase and alanine amino-transferase activity in the murine liver indicated that there were minimal toxic effects associated with TALEN treatment[89]. However, as cccDNA is not produced by mice, the evaluation of TALEN mediated cccDNA disruption *in vivo* could not be carried out in this study.

A subsequent study by Chen *et al*[91] supported the findings made by Bloom *et al*[89]. Chen *et al*[91] designed a unique set of TALENs against conserved regions within HBV genomic DNA. Application of their TALEN pairs resulted in the reduction of HBV core, surface and e antigen expression; viral knockdown and a reduction in the cccDNA. cccDNA was produced at low levels in Huh7 cells using a transfection method. cccDNA levels were evaluated by quantitative polymerase chain reaction using HBV cccDNA specific primers, which were designed to amplify DNA across the gap that is present in relaxed circular DNA (rcDNA). This allowed for discrimination between the two HBV replication intermediates. cccDNA was reduced by approximately 10%-20% with TALENs-L1/R1 and twofold in TALENs-L2/R2. Furthermore, a synergistic effect on the inhibition of HBV transcription was observed when TALENs and interferon-á were co-administered[91]. The studies by Bloom *et al*[89] and Chen *et al*[91] provide evidence for TALEN applications to treating chronic diseases that have a DNA reservoir within the human liver. While the findings of these studies are promising the need to test TALEN efficacy in a model that simulates the human condition more closely remains important. The prolonged nuclease activity required, which would ensure eradication of all of the cccDNA, and thus successful elimination of HBV infection may also result in unwanted side effects. Therefore, the development complementary technologies to combat HBV such as TALE transcriptional regulators which may be used in their place or in conjunction with their TALEN counterparts should also be considered.

Most recently, Lin *et al*[92] evaluated the potential of a CRISPR/Cas approach to eradicating HBV replication intermediates (rcDNA and cccDNA) both *in vitro* and *in vivo*. The investigators designed gRNAs to the HBV A genotype and of the eight gRNAs, four had good anti-HBV activity. Improved efficacy was observed when the gRNAs and Cas9 were co-expressed from the same plasmid, with the most active gRNA reducing intracellular HBsAg levels by 96%. This gRNA targeted the surface region and was designated as the S1 guide. When Lin *et al*[92] evaluated effects of gRNAs in combination, anti-HBV efficacy was augmented and large deletions in the viral DNA could be introduced between the two gRNA recognition sites. The ability to delete larger sections of DNA has implications for the eradication of integrated HBV genomes, and has been successful when using a set of CRISPR/Cas gRNAs to eliminate HIV-1 provirus infection[92]. To evaluate the ability of the CRISPR/Cas gRNAs to eliminate HBV-cccDNA, the investigators used a DHBV replication model with new DHBV-specific gRNAs. With this avian model of HBV replication, more cccDNA is formed in transfected cells. Results indicated that cccDNA and rcDNA production was significantly diminished. This study is the first to demonstrate the application of CRISPR/Cas constructs against HBV. This early evidence suggests that as with TALENs and ZFNs, CRISPR/Cas

may be a useful tool against viral hepatitis[93].

APPLICATION OF GENE EDITING TECHNOLOGIES *IN VIVO* AND *EX VIVO*

In 2011, Li *et al*[94] investigated the potential of ZFNs for gene editing of liver progenitor cells and the correction of gene mutations responsible for hemophilia B in the murine model. In this study ZFNs targeting intron 1 of the human *F9* gene were shown to induce DSB and HDR at the intended sites in both the human erythroleukemia K-562 cells and the Hep3B human hepatocyte line[94]. An *in vivo* study was performed in which the ZFNs and a gene targeting vector with a donor sequence were delivered to the liver using an adeno-associated virus (AAV), serotype 8 vector. The ZFNs and gene targeting vector mediated correction and resultant prolonged clotting times of the mice. When liver regeneration was induced in these animals, the effects of the treatment were maintained[94].

The ability to repair genetic defects has been furthered by improvements in technologies related to use of induced pluripotent stem cells (iPSCs). There is potential for the application of patient-derived iPSCs for the correction of underlying genetic errors while autologous transplant reduces problems of immune-mediated graft rejection[95,96]. Coupling this methodology to gene editing may be used to correct gene defects and induce disease-resistant phenotypes. After modification *ex vivo*, differentiation into hepatocytes and autologous transplant, it may be feasible to generate livers that no longer manifest a disease phenotype.

Rio *et al*[97] were the first to demonstrate the potential of DNA modification technologies to correct DNA repair deficiency syndromes. In this study, a pair of ZFNs targeting the AAV 1 locus were introduced into iPSCs using recombinant AAVs. These ZFNs, introduced by an integrase defective lentiviral vector, induced cleavage of the target and improved integration of a new Fanconi anemia, complementation group A cassette by HDR. The ZFNs were central to the correction of fanconi anaemia (FA) phenotype. Corrected human iPSCs were re-programmed, re-differentiated and introduced into the bone marrow of FA sufferers. These re-differentiated cells were able to produce disease-free liver cells[97].

In an earlier study, Yusa *et al*[98], used the piggyBac transposon and ZFN-based genome modification in human iPSCs to achieve the biallelic correction of the Z mutation (342Glu to Lys) associated with á1-antitrypsin deficiency. ZFN pairs were designed to target the site of a single point mutation in the á1-antitrypsin gene. Following expression of the ZFN pair and introduction of donor constructs, 11% of the screened iPSC colonies exhibited biallelic excision and repair. Subsequent sequencing analyses showed that the Z mutation had been corrected on both alleles in these colonies. When the human iPSCs were transplanted and differentiated they produced normal liver cells, with no á1-antitrypsin deficiency[98]. In 2013, Choi *et al*[99] compared the efficacy of the ZFN pair used in the study by Yusa *et al*[98] to the efficacy of TALEN pairs in patient-derived iPSCs. The TALEN pair was designed to target regions adjacent to the Z mutation of the á1-antitrypsin gene. Following introduction of the TALEN pair and donor sequences, all 66 iPSC clones showed the desired corrective integration. Twenty five to thirty three percent of these clones lacked the endogenous allele, suggesting the simultaneous targeting of both alleles, which was confirmed by subsequent sequence analyses. When comparing the efficacy of the TALENs to the ZFNs, Choi *et al*[99] demonstrated that their TALEN pair achieved comparable or higher gene targeting efficiencies (100% single allele cleavage efficiency with 25%-33% biallelic targeting) than were observed with ZFNs pair (54% single allele cleavage efficiency with 4% biallelic targeting). These studies highlight the potential for gene editing technologies in the correction of inborn genetic disorders while illustrating the point that gene editor choice has an impact on therapeutic outcome.

In a study by Yin *et al*[100] the CRISPR/Cas system was used to correct a Fah U1 mutation in the hepatocytes of a murine model for the hereditary monogenic disease, tyrosinemia. The correction of the G to A splicing mutation, which restores correct processing of mRNA transcripts, in the endogenous Fah locus was achieved by HDR. A single guide RNA targeting the Fah gene, was introduced in conjunction with the Cas9 protein and a 199 nucleotide single-stranded DNA donor. The DNA donor contained the correcting wild-type sequence which was flanked on both sides by sequences that were homologous to the DNA of the mutant gene. The correction of the Fah gene resulted in expression of the wild-type Fah protein in murine hepatocytes. Subsequent proliferation of the Fah-positive hepatocytes and replacement of diseased cells in mice improved their survival[100].

CURRENT LIMITATIONS AND FUTURE PROSPECTS OF SEQUENCE-SPECIFIC DNA MODIFYING TECHNOLOGIES FOR THE TREATMENT OF LIVER DISEASES

The treatment of liver diseases is often complex, which has been a motivating factor for new and better technologies. While gene editing does not provide a cure-all therapeutic for liver diseases, the approach has potential for application to monogenic disorders and some viral hepatic infections. However, three significant challenges need to be addressed before clinical application of gene editing for hepatic diseases is realized: ensuring (1) specific targeting; (2) safe and efficient delivery to target cells; and (3) limited immunogenicity of the gene modifier and its delivery agent are all important.

Off-target activity by gene modifiers may result in cytotoxicity and the repair of DSBs generated from this

activity may result in undesired deletions, inversions, and translocations[28]. Furthermore, the off-target activity from gene modifiers is potentially carcinogenic. When fused to activator, repressor and/or nuclease domains, gene editors may cause inactivation of tumor suppressor genes or activation of oncogenes. To alleviate these concerns, a number of studies evaluating the off-target effects of gene editing technologies have been carried out[101,102]. These investigations have sought to improve the design of the DNA recognition motifs. Pattanayak *et al*[103] developed new design rules to alleviate off-target activity of ZFNs. Other groups have investigated improvement of the architecture, length and composition of the CRISPR/Cas chimeric gRNAs[63,102,104]. New tools which evaluate potential off-target activity in silico have also been described[105], and more sensitive methods of identifying and characterizing off-target activity *in vivo* have been reported[102]. When using exome and deep sequencing, TALENs have been shown to have significantly fewer off-target effects than ZFNs[29,106]. This was corroborated in 2014 when a study by Suzuki *et al*[107] found that TALENs did not increase the overall mutation load of a targeted genome. In 2014 Smith *et al*[108] demonstrated that both CRISPR/Cas and TALENs had almost no off-target mutagenic effects in iPSCs. These studies suggest that off-target activity of TALENs and CRISPR/Cas may be more limited than originally anticipated. However, guidelines for acceptable levels of off-target activity need to be developed. Currently, limited off-target activity in introns and non-coding sections of DNA are considered acceptable[29,107,108], but with advancing understanding of the functional roles of non-coding DNA this may not always be true. Development of tools of bioinformatics which aid target choice has simplified the design of recognition sequences with low homology to exons and important regulatory elements[52,105]. It should also be noted that these studies focus on developing technologies with off-target activity below the detection of deep sequencing technologies. Deep sequencing technologies are currently the most sensitive tool available for evaluating off target effects[29,102]. Current limitations in technologies for DNA sequencing mean that this requirement is presently sufficient but will need to be re-examined as more sensitive technologies are developed. Potential off-target activity of therapeutic artificial transcription factors still needs to be evaluated. The growing body of evidence and tools for design and analysis suggests that while off-target effects will need to be evaluated for any clinical application, this need not be a universally limiting factor.

The second obstacle to efficient application of gene editing technologies is their specific delivery to intended target cells. This specific delivery may be further complicated by the need for multiple administrations of a targeted therapy due to their lower efficacy rates (around 50%) in a clinical setting. Viral vectors, which have been widely used in other gene therapy applications, may be employed as an efficient delivery mechanism for gene modifiers. Studies have shown potential for the use of lentivirus-, adenovirus- and baculovirus-based delivery methods, and AAV vectors have also been used to deliver gene editing tools successfully[109,110]. These viral vectors have some limitations with respect to the type of gene editing technology that may be delivered, the specific circumstance under which the vectors may be used and whether delivery may be carried out *in vivo* or *ex vivo*[109,110]. Challenges, such as immunogenicity and potential mutation or integration that may be associated with the use of viral vectors, have led to the exploration of other avenues for delivery. Liu *et al*[111] explored the possibility of conjugating TALENs to cell penetrating peptides such as the Tat protein to facilitate the systemic delivery of TALENs *in vitro*. Gaj *et al*[112] have assessed the applicability of a Salmonella-derived delivery system. Both of these studies were moderately successful. mRNA delivery offers another potential solution, which has the advantage of improving the regulation of the duration of transgene expression. mRNA needs only to be delivered to the cytoplasm, does not need to be transcribed, and therefore enables realization of the therapeutic effect more quickly. Moreover, since mRNA is unable to integrate into the host genome concerns about exogenous DNA integration are alleviated[113]. mRNA may be delivered using non-viral vectors which are less immunogenic than viral vectors and recombinant proteins[114]. They are also amenable to large scale preparation that is required for clinical use. There are some TALEN and ZFN studies which have applied this approach, although with moderate success to date[115,116]. The vectors used for delivery of sequences encoding gene editors may be engineered to target specific tissues. Using different serotypes, receptors or lipids it is possible to ensure specific delivery of the transgene payload. This flexibility also allows for modification of the delivery vehicle to avoid immune detection, should repeat administrations be necessary in a clinical setting. The choice of delivery system will eventually depend on a combination of the type of gene editing technology being used, its specific application, requirements for dose regulation, duration of expression and minimizing of off target effects.

The potential of an immune reaction to gene editors may be a concern for application of the technology. This immunostimulation may result from either the gene modifier itself[117] or from the mechanism of delivery[118]. In either case it is important to assess immune activation as it may diminish efficacy following repeat administrations and cause toxicity. Immune stimulation has significantly hampered gene therapies in the past[119] and will be important for clinical application of gene editing.

CONCLUSION

This review has discussed ZFPs, TALEs, CRISPR/Cas and HEs highlighting their advantages and disadvantages for applications to the treatment of liver diseases.

Gene editing has a promising future as a clinical therapeutic and as these techniques continue to be refined, off-target activity will become more limited and delivery more feasible. Several studies have already demonstrated that these technologies may be used in a tissue- and patient-specific manner to disable, augment and correct gene function. Other studies have established their potential to expand the use of iPSCs in the treatment of disease. Ultimately gene modifiers and cell-based therapies will increase the number of disorders that may be permanently corrected and alter the way in which inborn liver diseases and viral hepatic infections are treated. These technologies present a novel, versatile tool for the treatment and cure of many hepatic illnesses.

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REFERENCES

1 **Williams R**. Global challenges in liver disease. *Hepatology* 2006; **44**: 521-526 [PMID: 16941687 DOI: 10.1002/hep.21347]

2 **Jacobs F**, Gordts SC, Muthuramu I, De Geest B. The liver as a target organ for gene therapy: state of the art, challenges, and future perspectives. *Pharmaceuticals* (Basel) 2012; **5**: 1372-1392 [PMID: 24281341 DOI: 10.3390/ph5121372]

3 **Blum HE**. Molecular therapy and prevention of liver diseases. *Adv Med Sci* 2007; **52**: 29-36 [PMID: 18217387 DOI: 10.1007/s12250-008-2938-7]

4 **Kan Z**, Zheng H, Liu X, Li S, Barber TD, Gong Z, Gao H, Hao K, Willard MD, Xu J, Hauptschein R, Rejto PA, Fernandez J, Wang G, Zhang Q, Wang B, Chen R, Wang J, Lee NP, Zhou W, Lin Z, Peng Z, Yi K, Chen S, Li L, Fan X, Yang J, Ye R, Ju J, Wang K, Estrella H, Deng S, Wei P, Qiu M, Wulur IH, Liu J, Ehsani ME, Zhang C, Loboda A, Sung WK, Aggarwal A, Poon RT, Fan ST, Wang J, Hardwick J, Reinhard C, Dai H, Li Y, Luk JM, Mao M. Whole-genome sequencing identifies recurrent mutations in hepatocellular carcinoma. *Genome Res* 2013;**23**: 1422-1433 [PMID: 23788652 DOI: 10.1101/gr.154492.113]

5 **Crigler JF**, Najjar VA. Congenital familial nonhemolytic jaundice with kernicterus. *Pediatrics* 1952; **10**: 169-180 [PMID: 12983120]

6 **Lindblad B**, Lindstedt S, Steen G. On the enzymic defects in hereditary tyrosinemia. *Proc Natl Acad Sci USA* 1977; **74**: 4641-4645 [PMID: 270706]

7 **Tukey RH**, Strassburg CP. Human UDP-glucuronosyltransferases: metabolism, expression, and disease. *Annu Rev Pharmacol Toxicol* 2000; **40**: 581-616 [PMID: 10836148 DOI: 10.1146/annurev.pharmtox.40.1.581]

8 **Graham JB**, Miller CH, Reisner HM, Elston RC, Olive JA. The phenotypic range of hemophilia A carriers. *Am J Hum Genet* 1976; **28**: 482-488 [PMID: 984044]

9 **Yang MY**, Ragni MV. Clinical manifestations and management of labor and delivery in women with factor IX deficiency. *Haemophilia* 2004; **10**: 483-490 [PMID: 15357775 DOI: 10.1111/j.1365-2516.2004.00946.x]

10 **Wahlberg T**. Carriers and noncarriers of haemophilia A. Evaluation of bleeding symptoms registered by a self-administered questionnaire with binary (no/yes) questions. *Thromb Res* 1982; **25**: 415-422 [PMID: 7071812 DOI: 10.1016/0049-3848(82)90131-1]

11 **McMahon BJ**. Chronic hepatitis B virus infection. *Med Clin North Am* 2014; **98**: 39-54 [PMID: 24266913 DOI: 10.1016/j.mcna.2013.08.004]

12 **Rosen HR**. Clinical practice. Chronic hepatitis C infection. *N Engl J Med* 2011; **364**: 2429-2438 [PMID: 21696309 DOI: 10.1056/NEJMcp1006613]

13 **Urnov FD**, Rebar EJ, Holmes MC, Zhang HS, Gregory PD. Genome editing with engineered zinc finger nucleases. *Nat Rev Genet* 2010; **11**: 636-646 [PMID: 20717154 DOI: 10.1038/nrg2842]

14 **Maeder ML**, Linder SJ, Reyon D, Angstman JF, Fu Y, Sander JD, Joung JK. Robust, synergistic regulation of human gene expression using TALE activators. *Nat Methods* 2013; **10**: 243-245 [PMID: 23396285 DOI: 10.1038/nmeth.2366]

15 **Stoddard BL**. Homing endonucleases from mobile group I introns: discovery to genome engineering. *Mob DNA* 2014; **5**: 7 [PMID: 24589358 DOI: 10.1186/1759-8753-5-7]

16 **Gaj T**, Gersbach CA, Barbas CF. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol* 2013; **31**: 397-405 [PMID: 23664777 DOI: 10.1016/j.tibtech.2013.04.004]

17 **Bikard D**, Marraffini LA. Control of gene expression by CRISPR-Cas systems. *F1000Prime Rep* 2013; **5**: 47 [PMID: 24273648 DOI: 10.12703/P5-47]

18 **Dong J**, Li W, Dong A, Mao S, Shen L, Li S, Gong X, Wu P. Gene therapy for unresectable hepatocellular carcinoma using recombinant human adenovirus type 5. *Med Oncol* 2014; **31**: 95 [PMID: 24990099 DOI: 10.1007/s12032-014-0095-4]

19 **Bortolussi G**, Zentillin L, Vaníkova J, Bockor L, Bellarosa C, Mancarella A, Vianello E, Tiribelli C, Giacca M, Vitek L, Muro AF. Life-long correction of hyperbilirubinemia with a neonatal liver-specific AAV-mediated gene transfer in a lethal mouse model of Crigler-Najjar Syndrome. *Hum Gene Ther* 2014; **25**: 844-855 [PMID: 25072305 DOI: 10.1089/hum.2013.233]

20 **Grossman M**, Raper SE, Kozarsky K, Stein EA, Engelhardt JF, Muller D, Lupien PJ, Wilson JM. Successful ex vivo gene therapy directed to liver in a patient with familial hypercholesterolaemia. *Nat Genet* 1994; **6**: 335-341 [PMID: 8054972 DOI: 10.1038/ng0494-335]

21 **Mahlangu J**, Powell JS, Ragni MV, Chowdary P, Josephson NC, Pabinger I, Hanabusa H, Gupta N, Kulkarni R, Fogarty P, Perry D, Shapiro A, Pasi KJ, Apte S, Nestorov I, Jiang H, Li S, Neelakantan S, Cristiano LM, Goyal J, Sommer JM, Dumont JA, Dodd N, Nugent K, Vigliani G, Luk A, Brennan A, Pierce GF. Phase 3 study of recombinant factor VIII Fc fusion protein in severe hemophilia A. *Blood* 2014; **123**: 317-325 [PMID: 24227821 DOI: 10.1182/blood-2013-10-529974]

22 **Ely A**, Naidoo T, Mufamadi S, Crowther C, Arbuthnot P. Expressed anti-HBV primary microRNA shuttles inhibit viral replication efficiently in vitro and in vivo. *Mol Ther* 2008; **16**: 1105-1112 [PMID: 18431360 DOI: 10.1038/mt.2008.82]

23 **Yoon SK**, Seo YB, Im SJ, Bae SH, Song MJ, You CR, Jang JW, Yang SH, Suh YS, Song JS, Kim BM, Kim CY, Jeong SH, Sung YC. Safety and immunogenicity of therapeutic DNA vaccine with antiviral drug in chronic HBV patients and its immunogenicity in mice. *Liver Int* 2015; **35**: 805-815 [PMID: 24620920 DOI: 10.1111/liv.12530]

24 **Arrowhead Research**. Arrowhead begins dosing in phase 2a trial of rnai therapeutic arc-520 in chronic hepatitis b patients. [Updated 2014 March 24]. Available from: URL: http: //www.arrowheadresearch.com/press-releases/arrowhead-begins-dosing-phase-2a-trial-rnai-therapeutic-arc-520-chronic-hepatitis-b

25 **Janssen HL**, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, Patel K, van der Meer AJ, Patick AK, Chen A, Zhou Y, Persson R, King BD, Kauppinen S, Levin AA, Hodges MR. Treatment of HCV infection by targeting microRNA. *N Engl J Med* 2013; **368**: 1685-1694 [PMID: 23534542 DOI: 10.1056/NEJMoa1209026]

26 **Johnson RD**, Jasin M. Double-strand-break-induced homologous recombination in mammalian cells. *Biochem Soc Trans* 2001; **29**: 196-201 [PMID: 11356153 DOI: 10.1042/bst0290196]

27 **Segal DJ**, Meckler JF. Genome engineering at the dawn of the golden age. *Annu Rev Genomics Hum Genet* 2013; **14**: 135-158 [PMID: 23701416 DOI: 10.1146/annurev-genom-091212-153435]

28 **Lee HJ**, Kweon J, Kim E, Kim S, Kim JS. Targeted chromosomal duplications and inversions in the human genome using zinc finger nucleases. *Genome Res* 2012; **22**: 539-548 [PMID: 22183967 DOI: 10.1101/gr.129635.111]

29 **Kim Y**, Kweon J, Kim A, Chon JK, Yoo JY, Kim HJ, Kim S, Lee C, Jeong E, Chung E, Kim D, Lee MS, Go EM, Song HJ, Kim H, Cho N, Bang D, Kim S, Kim JS. A library of TAL effector nucleases spanning the human genome. *Nat Biotechnol* 2013; **31**: 251-258 [PMID: 23417094 DOI: 10.1038/nbt.2517]

30 **Arnould S**, Chames P, Perez C, Lacroix E, Duclert A, Epinat JC, Stricher F, Petit AS, Patin A, Guillier S, Rolland S, Prieto J, Blanco FJ, Bravo J, Montoya G, Serrano L, Duchateau P, Pâques F. Engineering of large numbers of highly specific homing endonucleases that induce recombination on novel DNA targets. *J Mol Biol* 2006; **355**: 443-458 [PMID: 16310802 DOI: 10.1016/j.jmb.2005.10.065]

31 **Aubert M**, Boyle NM, Stone D, Stensland L, Huang ML, Magaret AS, Galetto R, Rawlings DJ, Scharenberg AM, Jerome KR. In vitro Inactivation of Latent HSV by Targeted Mutagenesis Using an HSV-specific Homing Endonuclease. *Mol Ther Nucleic Acids* 2014; **3**: e146 [PMID: 24496438 DOI: 10.1038/mtna.2013.75]

32 **Urnov FD**, Miller JC, Lee YL, Beausejour CM, Rock JM, Augustus S, Jamieson AC, Porteus MH, Gregory PD, Holmes MC. Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* 2005; **435**: 646-651 [PMID: 15806097 DOI: 10.1038/nature03556]

33 **Laity JH**, Dyson HJ, Wright PE. DNA-induced alpha-helix capping in conserved linker sequences is a determinant of binding affinity in Cys(2)-His(2) zinc fingers. *J Mol Biol* 2000; **295**: 719-727 [PMID: 10656784 DOI: 10.1006/jmbi.1999.3406]

34 **Alwin S**, Gere MB, Guhl E, Effertz K, Barbas CF, Segal DJ, Weitzman MD, Cathomen T. Custom zinc-finger nucleases for use in human cells. *Mol Ther* 2005; **12**: 610-617 [PMID: 16039907 DOI: 10.1016/j.ymthe.2005.06.094]

35 **Bhakta MS**, Henry IM, Ousterout DG, Das KT, Lockwood SH, Meckler JF, Wallen MC, Zykovich A, Yu Y, Leo H, Xu L, Gersbach CA, Segal DJ. Highly active zinc-finger nucleases by extended modular assembly. *Genome Res* 2013; **23**: 530-538 [PMID: 23222846 DOI: 10.1101/gr.143693.112]

36 **Ramirez CL**, Foley JE, Wright DA, Müller-Lerch F, Rahman SH, Cornu TI, Winfrey RJ, Sander JD, Fu F, Townsend JA, Cathomen T, Voytas DF, Joung JK. Unexpected failure rates for modular assembly of engineered zinc fingers. *Nat Methods* 2008; **5**: 374-375 [PMID: 18446154 DOI: 10.1038/nmeth0508-374]

37 **Maeder ML**, Thibodeau-Beganny S, Sander JD, Voytas DF, Joung JK. Oligomerized pool engineering (OPEN): an 'open-source' protocol for making customized zinc-finger arrays. *Nat Protoc* 2009; **4**: 1471-1501 [PMID: 19798082 DOI: 10.1038/nprot.2009.98]

38 **Maeder ML**, Thibodeau-Beganny S, Osiak A, Wright DA, Anthony RM, Eichtinger M, Jiang T, Foley JE, Winfrey RJ, Townsend JA, Unger-Wallace E, Sander JD, Müller-Lerch F, Fu F, Pearlberg J, Göbel C, Dassie JP, Pruett-Miller SM, Porteus MH, Sgroi DC, Iafrate AJ, Dobbs D, McCray PB, Cathomen T, Voytas DF, Joung JK. Rapid "open-source" engineering of customized zinc-finger nucleases for highly efficient gene modification. *Mol Cell* 2008; **31**: 294-301 [PMID: 18657511 DOI: 10.1016/j.molcel.2008.06.016]

39 **Lee HJ**, Kim E, Kim JS. Targeted chromosomal deletions in human cells using zinc finger nucleases. *Genome Res* 2010; **20**: 81-89 [PMID: 19952142 DOI: 10.1101/gr.099747.109]

40 **Tebas P**, Stein D, Tang WW, Frank I, Wang SQ, Lee G, Spratt SK, Surosky RT, Giedlin MA, Nichol G, Holmes MC, Gregory PD, Ando DG, Kalos M, Collman RG, Binder-Scholl G, Plesa G, Hwang WT, Levine BL, June CH. Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. *N Engl J Med* 2014; **370**: 901-910 [PMID: 24597865 DOI: 10.1056/NEJMoa1300662]

41 **Maier DA**, Brennan AL, Jiang S, Binder-Scholl GK, Lee G, Plesa G, Zheng Z, Cotte J, Carpenito C, Wood T, Spratt SK, Ando D, Gregory P, Holmes MC, Perez EE, Riley JL, Carroll RG, June CH, Levine BL. Efficient clinical scale gene modification via zinc finger nuclease-targeted disruption of the HIV co-receptor CCR5. *Hum Gene Ther* 2013; **24**: 245-258 [PMID: 23360514 DOI: 10.1089/hum.2012.172]

42 **Perez EE**, Wang J, Miller JC, Jouvenot Y, Kim KA, Liu O, Wang N, Lee G, Bartsevich VV, Lee YL, Guschin DY, Rupniewski I, Waite AJ, Carpenito C, Carroll RG, Orange JS, Urnov FD, Rebar EJ, Ando D, Gregory PD, Riley JL, Holmes MC, June CH. Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. *Nat Biotechnol* 2008; **26**: 808-816 [PMID: 18587387 DOI: 10.1038/nbt1410]

43 **Boch J**, Bonas U. Xanthomonas AvrBs3 family-type III effectors: discovery and function. *Annu Rev Phytopathol* 2010; **48**: 419-436 [PMID: 19400638 DOI: 10.1146/annurev-phyto-080508-081936]

44 **White FF**, Yang B. Host and pathogen factors controlling the rice-Xanthomonas oryzae interaction. *Plant Physiol* 2009; **150**: 1677-1686 [PMID: 19458115 DOI: 10.1104/pp.109.139360]

45 **Bai J**, Choi SH, Ponciano G, Leung H, Leach JE. Xanthomonas oryzae pv. oryzae avirulence genes contribute differently and specifically to pathogen aggressiveness. *Mol Plant Microbe Interact* 2000; **13**: 1322-1329 [PMID: 11106024 DOI: 10.1094/MPMI.2000.13.12.1322]

46 **Scholze H**, Boch J. TAL effectors are remote controls for gene activation. *Curr Opin Microbiol* 2011; **14**: 47-53 [PMID: 21215685 DOI: 10.1016/j.mib.2010.12.001]

47 **Meckler JF**, Bhakta MS, Kim MS, Ovadia R, Habrian CH, Zykovich A, Yu A, Lockwood SH, Morbitzer R, Elsäesser J, Lahaye T, Segal DJ, Baldwin EP. Quantitative analysis of TALE-DNA interactions suggests polarity effects. *Nucleic Acids Res* 2013; **41**: 4118-4128 [PMID: 23408851 DOI: 10.1093/nar/gkt085]

48 **Boch J**, Scholze H, Schornack S, Landgraf A, Hahn S, Kay S, Lahaye T, Nickstadt A, Bonas U. Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* 2009; **326**: 1509-1512 [PMID: 19933107 DOI: 10.1126/science.1178811]

49 **Römer P**, Recht S, Lahaye T. A single plant resistance gene promoter engineered to recognize multiple TAL effectors from disparate pathogens. *Proc Natl Acad Sci USA* 2009; **106**: 20526-20531 [PMID: 19910532 DOI: 10.1073/pnas.0908812106]

50 **Valton J**, Dupuy A, Daboussi F, Thomas S, Maréchal A, Macmaster R, Melliand K, Juillerat A, Duchateau P. Overcoming transcription activator-like effector (TALE) DNA binding domain sensitivity to cytosine methylation. *J Biol Chem* 2012; **287**: 38427-38432 [PMID: 23019344 DOI: 10.1074/jbc.C112.408864]

51 **Sun N**, Liang J, Abil Z, Zhao H. Optimized TAL effector nucleases (TALENs) for use in treatment of sickle cell disease. *Mol Biosyst* 2012; **8**: 1255-1263 [PMID: 22301904 DOI: 10.1039/c2mb05461b]

52 **Heigwer F**, Kerr G, Walther N, Glaeser K, Pelz O, Breinig M, Boutros M. E-TALEN: a web tool to design TALENs for genome engineering. *Nucleic Acids Res* 2013; **41**: e190 [PMID: 24003033 DOI: 10.1093/nar/gkt789]

53 **Morbitzer R**, Elsaesser J, Hausner J, Lahaye T. Assembly of custom TALE-type DNA binding domains by modular cloning. *Nucleic Acids Res* 2011; **39**: 5790-5799 [PMID: 21421566 DOI: 10.1093/nar/gkr151]

54 **Mussolino C**, Morbitzer R, Lütge F, Dannemann N, Lahaye T, Cathomen T. A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity. *Nucleic Acids Res* 2011; **39**: 9283-9293 [PMID: 21813459 DOI: 10.1093/nar/gkr597]

55 **Deveau H**, Barrangou R, Garneau JE, Labonté J, Fremaux C, Boyaval P, Romero DA, Horvath P, Moineau S. Phage response to CRISPR-encoded resistance in Streptococcus thermophilus. *J Bacteriol* 2008; **190**: 1390-1400 [PMID: 18065545 DOI: 10.1128/JB.01412-07]

56 **Makarova KS**, Haft DH, Barrangou R, Brouns SJ, Charpentier E, Horvath P, Moineau S, Mojica FJ, Wolf YI, Yakunin AF, van der Oost J, Koonin EV. Evolution and classification of the CRISPR-Cas systems. *Nat Rev Microbiol* 2011; **9**: 467-477 [PMID: 21552286 DOI: 10.1038/nrmicro2577]

57 **Westra ER**, Nilges B, van Erp PB, van der Oost J, Dame RT, Brouns SJ. Cascade-mediated binding and bending of negatively

supercoiled DNA. *RNA Biol* 2012; **9**: 1134-1138 [PMID: 22954644 DOI: 10.4161/rna.21410]
58 **Cong L**, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F. Multiplex genome engineering using CRISPR/Cas systems. *Science* 2013; **339**: 819-823 [PMID: 23287718 DOI: 10.1126/science.1231143]
59 **Mulepati S**, Bailey S. Structural and biochemical analysis of nuclease domain of clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein 3 (Cas3). *J Biol Chem* 2011; **286**: 31896-31903 [PMID: 21775431 DOI: 10.1074/jbc.M111.270017]
60 **Deltcheva E**, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J, Charpentier E. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* 2011; **471**: 602-607 [PMID: 21455174 DOI: 10.1038/nature09886]
61 **Sapranauskas R**, Gasiunas G, Fremaux C, Barrangou R, Horvath P, Siksnys V. The Streptococcus thermophilus CRISPR/Cas system provides immunity in Escherichia coli. *Nucleic Acids Res* 2011; **39**: 9275-9282 [PMID: 21813460 DOI: 10.1093/nar/gkr606]
62 **Richter H**, Zoephel J, Schermuly J, Maticzka D, Backofen R, Randau L. Characterization of CRISPR RNA processing in Clostridium thermocellum and Methanococcus maripaludis. *Nucleic Acids Res* 2012; **40**: 9887-9896 [PMID: 22879377 DOI: 10.1093/nar/gks737]
63 **Sternberg SH**, Redding S, Jinek M, Greene EC, Doudna JA. DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature* 2014; **507**: 62-67 [PMID: 24476820 DOI: 10.1038/nature13011]
64 **Dujon B**. Sequence of the intron and flanking exons of the mitochondrial 21S rRNA gene of yeast strains having different alleles at the omega and rib-1 loci. *Cell* 1980; **20**: 185-197 [PMID: 6156002 DOI: 10.1016/0092-8674(80)90246-9]
65 **Chevalier BS**, Stoddard BL. Homing endonucleases: structural and functional insight into the catalysts of intron/intein mobility. *Nucleic Acids Res* 2001; **29**: 3757-3774 [PMID: 11557808 DOI: 10.1093/nar/29.18.3757]
66 **Rouet P**, Smih F, Jasin M. Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. *Proc Natl Acad Sci USA* 1994; **91**: 6064-6068 [PMID: 8016116 DOI: 10.1073/pnas.91.13.6064]
67 **Silva GH**, Belfort M, Wende W, Pingoud A. From monomeric to homodimeric endonucleases and back: engineering novel specificity of LAGLIDADG enzymes. *J Mol Biol* 2006; **361**: 744-754 [PMID: 16872628 DOI: 10.1016/j.jmb.2006.06.063]
68 **Grosse S**, Huot N, Mahiet C, Arnould S, Barradeau S, Clerre DL, Chion-Sotinel I, Jacqmarcq C, Chapellier B, Ergani A, Desseaux C, Cédrone F, Conseiller E, Pâques F, Labetoulle M, Smith J. Meganuclease-mediated Inhibition of HSV1 Infection in Cultured Cells. *Mol Ther* 2011; **19**: 694-702 [PMID: 21224832 DOI: 10.1038/mt.2010.302]
69 **Ulge UY**, Baker DA, Monnat RJ. Comprehensive computational design of mCreI homing endonuclease cleavage specificity for genome engineering. *Nucleic Acids Res* 2011; **39**: 4330-4339 [PMID: 21288879 DOI: 10.1093/nar/gkr022]
70 **Segal DJ**, Beerli RR, Blancafort P, Dreier B, Effertz K, Huber A, Koksch B, Lund CV, Magnenat L, Valente D, Barbas CF. Evaluation of a modular strategy for the construction of novel polydactyl zinc finger DNA-binding proteins. *Biochemistry* 2003; **42**: 2137-2148 [PMID: 12590603 DOI: 10.1021/bi026806o]
71 **Zhang F**, Cong L, Lodato S, Kosuri S, Church GM, Arlotta P. Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription. *Nat Biotechnol* 2011; **29**: 149-153 [PMID: 21248753 DOI: 10.1038/nbt.1775]
72 **Skowron P**, Kaczorowski T, Tucholski J, Podhajska AJ. Atypical DNA-binding properties of class-IIS restriction endonucleases: evidence for recognition of the cognate sequence by a FokI monomer. *Gene* 1993; **125**: 1-10 [PMID: 8166773 DOI: 10.1016/0378-1119(93)90738-O]
73 **Bitinaite J**, Wah DA, Aggarwal AK, Schildkraut I. FokI dimerization is required for DNA cleavage. *Proc Natl Acad Sci USA* 1998; **95**: 10570-10575 [PMID: 9724744 DOI: 10.1073/pnas.95.18.10570]
74 **Zu Y**, Tong X, Wang Z, Liu D, Pan R, Li Z, Hu Y, Luo Z, Huang P, Wu Q, Zhu Z, Zhang B, Lin S. TALEN-mediated precise genome modification by homologous recombination in zebrafish. *Nat Methods* 2013; **10**: 329-331 [PMID: 23435258 DOI: 10.1038/nmeth.2374]
75 **Certo MT**, Gwiazda KS, Kuhar R, Sather B, Curinga G, Mandt T, Brault M, Lambert AR, Baxter SK, Jacoby K, Ryu BY, Kiem HP, Gouble A, Paques F, Rawlings DJ, Scharenberg AM. Coupling endonucleases with DNA end-processing enzymes to drive gene disruption. *Nat Methods* 2012; **9**: 973-975 [PMID: 22941364 DOI: 10.1038/nmeth.2177]
76 **Orlando SJ**, Santiago Y, DeKelver RC, Freyvert Y, Boydston EA, Moehle EA, Choi VM, Gopalan SM, Lou JF, Li J, Miller JC, Holmes MC, Gregory PD, Urnov FD, Cost GJ. Zinc-finger nuclease-driven targeted integration into mammalian genomes using donors with limited chromosomal homology. *Nucleic Acids Res* 2010; **38**: e152 [PMID: 20530528 DOI: 10.1093/nar/gkq512]
77 **Chen F**, Pruett-Miller SM, Huang Y, Gjoka M, Duda K, Taunton J, Collingwood TN, Frodin M, Davis GD. High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases. *Nat Methods* 2011; **8**: 753-755 [PMID: 21765410 DOI: 10.1038/nmeth.1653]
78 **Bellefroid EJ**, Marine JC, Matera AG, Bourguignon C, Desai T, Healy KC, Bray-Ward P, Martial JA, Ihle JN, Ward DC. Emergence of the ZNF91 Krüppel-associated box-containing zinc finger gene family in the last common ancestor of anthropoidea. *Proc Natl Acad Sci USA* 1995; **92**: 10757-10761 [PMID: 7479878 DOI: 10.1073/pnas.92.23.10757]
79 **Margolin JF**, Friedman JR, Meyer WK, Vissing H, Thiesen HJ, Rauscher FJ. Krüppel-associated boxes are potent transcriptional repression domains. *Proc Natl Acad Sci USA* 1994; **91**: 4509-4513 [PMID: 8183939 DOI: 10.1073/pnas.91.10.4509]
80 **Sadowski I**, Ma J, Triezenberg S, Ptashne M. GAL4-VP16 is an unusually potent transcriptional activator. *Nature* 1988; **335**: 563-564 [PMID: 3047590 DOI: 10.1038/335563a0]
81 **Beerli RR**, Segal DJ, Dreier B, Barbas CF. Toward controlling gene expression at will: specific regulation of the erbB-2/HER-2 promoter by using polydactyl zinc finger proteins constructed from modular building blocks. *Proc Natl Acad Sci USA* 1998; **95**: 14628-14633 [PMID: 9843940 DOI: 10.1073/pnas.95.25.14628]
82 **Beerli RR**, Dreier B, Barbas CF. Positive and negative regulation of endogenous genes by designed transcription factors. *Proc Natl Acad Sci USA* 2000; **97**: 1495-1500 [PMID: 10660690 DOI: 10.1073/pnas.040552697]
83 **Beerli RR**, Barbas CF. Engineering polydactyl zinc-finger transcription factors. *Nat Biotechnol* 2002; **20**: 135-141 [PMID: 11821858 DOI: 10.1038/nbt0202-135]
84 **Gasiunas G**, Barrangou R, Horvath P, Siksnys V. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc Natl Acad Sci USA* 2012; **109**: E2579-E2586 [PMID: 22949671 DOI: 10.1073/pnas.1208507109]
85 **Gilbert LA**, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, Stern-Ginossar N, Brandman O, Whitehead EH, Doudna JA, Lim WA, Weissman JS, Qi LS. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* 2013; **154**: 442-451 [PMID: 23849981 DOI: 10.1016/j.cell.2013.06.044]
86 **Zimmerman KA**, Fischer KP, Joyce MA, Tyrrell DL. Zinc finger proteins designed to specifically target duck hepatitis B virus covalently closed circular DNA inhibit viral transcription in tissue culture. *J Virol* 2008; **82**: 8013-8021 [PMID: 18524822 DOI: 10.1128/JVI.00366-08]
87 **Cradick TJ**, Keck K, Bradshaw S, Jamieson AC, McCaffrey AP. Zinc-finger nucleases as a novel therapeutic strategy for targeting hepatitis B virus DNAs. *Mol Ther* 2010; **18**: 947-954 [PMID: 20160705 DOI: 10.1038/mt.2010.20]
88 **Zhao X**, Zhao Z, Guo J, Huang P, Zhu X, Zhou X, Yang Z, Zhao L, Xu L, Xu J, Fu L, Zhang J, Zhang X, Dong Y, Huang G, Wang Q, Li B, Song X, Yang X, Liu S, Yi S, Yu T, Yu C, Hou L, Li J, Chen W. Creation of a six-fingered artificial transcription factor that

represses the hepatitis B virus HBx gene integrated into a human hepatocellular carcinoma cell line. *J Biomol Screen* 2013; **18**: 378-387 [PMID: 23042077 DOI: 10.1177/1087057112463066]

89 **Bloom K**, Ely A, Mussolino C, Cathomen T, Arbuthnot P. Inactivation of hepatitis B virus replication in cultured cells and in vivo with engineered transcription activator-like effector nucleases. *Mol Ther* 2013; **21**: 1889-1897 [PMID: 23883864 DOI: 10.1038/mt.2013.170]

90 **Sells MA**, Zelent AZ, Shvartsman M, Acs G. Replicative intermediates of hepatitis B virus in HepG2 cells that produce infectious virions. *J Virol* 1988; **62**: 2836-2844 [PMID: 2839705]

91 **Chen J**, Zhang W, Lin J, Wang F, Wu M, Chen C, Zheng Y, Peng X, Li J, Yuan Z. An efficient antiviral strategy for targeting hepatitis B virus genome using transcription activator-like effector nucleases. *Mol Ther* 2014; **22**: 303-311 [PMID: 24025750 DOI: 10.1038/mt.2013.212]

92 **Lin SR**, Yang HC, Kuo YT, Liu CJ, Yang TY, Sung KC, Lin YY, Wang HY, Wang CC, Shen YC, Wu FY, Kao JH, Chen DS, Chen PJ. The CRISPR/Cas9 System Facilitates Clearance of the Intrahepatic HBV Templates In Vivo. *Mol Ther Nucleic Acids* 2014; **3**: e186 [PMID: 25137139 DOI: 10.1038/mtna.2014.38]

93 **Ebina H**, Misawa N, Kanemura Y, Koyanagi Y. Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus. *Sci Rep* 2013; **3**: 2510 [PMID: 23974631 DOI: 10.1038/srep02510]

94 **Li H**, Haurigot V, Doyon Y, Li T, Wong SY, Bhagwat AS, Malani N, Anguela XM, Sharma R, Ivanciu L, Murphy SL, Finn JD, Khazi FR, Zhou S, Paschon DE, Rebar EJ, Bushman FD, Gregory PD, Holmes MC, High KA. In vivo genome editing restores haemostasis in a mouse model of haemophilia. *Nature* 2011; **475**: 217-221 [PMID: 21706032 DOI: 10.1038/nature10177]

95 **Tolar J**, McGrath JA, Xia L, Riddle MJ, Lees CJ, Eide C, Keene DR, Liu L, Osborn MJ, Lund TC, Blazar BR, Wagner JE. Patient-specific naturally gene-reverted induced pluripotent stem cells in recessive dystrophic epidermolysis bullosa. *J Invest Dermatol* 2014; **134**: 1246-1254 [PMID: 24317394 DOI: 10.1038/jid.2013.523]

96 **Armijo E**, Soto C, Davis BR. HIV/AIDS: modified stem cells in the spotlight. *Cell Mol Life Sci* 2014; **71**: 2641-2649 [PMID: 24509823 DOI: 10.1007/s00018-014-1572-9]

97 **Rio P**, Baños R, Lombardo A, Quintana-Bustamante O, Alvarez L, Garate Z, Genovese P, Almarza E, Valeri A, Díez B, Navarro S, Torres Y, Trujillo JP, Murillas R, Segovia JC, Samper E, Surralles J, Gregory PD, Holmes MC, Naldini L, Bueren JA. Targeted gene therapy and cell reprogramming in Fanconi anemia. *EMBO Mol Med* 2014; **6**: 835-848 [PMID: 24859981 DOI: 10.15252/emmm.201303374]

98 **Yusa K**, Rashid ST, Strick-Marchand H, Varela I, Liu PQ, Paschon DE, Miranda E, Ordóñez A, Hannan NR, Rouhani FJ, Darche S, Alexander G, Marciniak SJ, Fusaki N, Hasegawa M, Holmes MC, Di Santo JP, Lomas DA, Bradley A, Vallier L. Targeted gene correction of α1-antitrypsin deficiency in induced pluripotent stem cells. *Nature* 2011; **478**: 391-394 [PMID: 21993621 DOI: 10.1038/nature10424]

99 **Choi SM**, Kim Y, Shim JS, Park JT, Wang RH, Leach SD, Liu JO, Deng C, Ye Z, Jang YY. Efficient drug screening and gene correction for treating liver disease using patient-specific stem cells. *Hepatology* 2013; **57**: 2458-2468 [PMID: 23325555 DOI: 10.1002/hep.26237]

100 **Yin H**, Xue W, Chen S, Bogorad RL, Benedetti E, Grompe M, Koteliansky V, Sharp PA, Jacks T, Anderson DG. Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. *Nat Biotechnol* 2014; **32**: 551-553 [PMID: 24681508 DOI: 10.1038/nbt.2884]

101 **Fu Y**, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK, Sander JD. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat Biotechnol* 2013; **31**: 822-826 [PMID: 23792628 DOI: 10.1038/nbt.2623]

102 **Cho SW**, Kim S, Kim Y, Kweon J, Kim HS, Bae S, Kim JS. Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Res* 2014; **24**: 132-141 [PMID: 24253446 DOI: 10.1101/gr.162339.113]

103 **Pattanayak V**, Ramirez CL, Joung JK, Liu DR. Revealing off-target cleavage specificities of zinc-finger nucleases by in vitro selection. *Nat Methods* 2011; **8**: 765-770 [PMID: 21822273 DOI: 10.1038/nmeth.1670]

104 **Chen B**, Gilbert LA, Cimini BA, Schnitzbauer J, Zhang W, Li GW, Park J, Blackburn EH, Weissman JS, Qi LS, Huang B. Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell* 2013; **155**: 1479-1491 [PMID: 24360272 DOI: 10.1016/j.cell.2013.12.001]

105 **Sander JD**, Ramirez CL, Linder SJ, Pattanayak V, Shoresh N, Ku M, Foden JA, Reyon D, Bernstein BE, Liu DR, Joung JK. In silico abstraction of zinc finger nuclease cleavage profiles reveals an expanded landscape of off-target sites. *Nucleic Acids Res* 2013; **41**: e181 [PMID: 23945932 DOI: 10.1093/nar/gkt716]

106 **Ding Q**, Lee YK, Schaefer EA, Peters DT, Veres A, Kim K, Kuperwasser N, Motola DL, Meissner TB, Hendriks WT, Trevisan M, Gupta RM, Moisan A, Banks E, Friesen M, Schinzel RT, Xia F, Tang A, Xia Y, Figueroa E, Wann A, Ahfeldt T, Daheron L, Zhang F, Rubin LL, Peng LF, Chung RT, Musunuru K, Cowan CA. A TALEN genome-editing system for generating human stem cell-based disease models. *Cell Stem Cell* 2013; **12**: 238-251 [PMID: 23246482 DOI: 10.1016/j.stem.2012.11.011]

107 **Suzuki K**, Yu C, Qu J, Li M, Yao X, Yuan T, Goebl A, Tang S, Ren R, Aizawa E, Zhang F, Xu X, Soligalla RD, Chen F, Kim J, Kim NY, Liao HK, Benner C, Esteban CR, Jin Y, Liu GH, Li Y, Izpisua Belmonte JC. Targeted gene correction minimally impacts whole-genome mutational load in human-disease-specific induced pluripotent stem cell clones. *Cell Stem Cell* 2014; **15**: 31-36 [PMID: 24996168 DOI: 10.1016/j.stem.2014.06.016]

108 **Smith C**, Gore A, Yan W, Abalde-Atristain L, Li Z, He C, Wang Y, Brodsky RA, Zhang K, Cheng L, Ye Z. Whole-genome sequencing analysis reveals high specificity of CRISPR/Cas9 and TALEN-based genome editing in human iPSCs. *Cell Stem Cell* 2014; **15**: 12-13 [PMID: 24996165 DOI: 10.1016/j.stem.2014.06.011]

109 **Holkers M**, Maggio I, Liu J, Janssen JM, Miselli F, Mussolino C, Recchia A, Cathomen T, Gonçalves MA. Differential integrity of TALE nuclease genes following adenoviral and lentiviral vector gene transfer into human cells. *Nucleic Acids Res* 2013; **41**: e63 [PMID: 23275534 DOI: 10.1093/nar/gks1446]

110 **Zhu H**, Lau CH, Goh SL, Liang Q, Chen C, Du S, Phang RZ, Tay FC, Tan WK, Li Z, Tay JC, Fan W, Wang S. Baculoviral transduction facilitates TALEN-mediated targeted transgene integration and Cre/LoxP cassette exchange in human-induced pluripotent stem cells. *Nucleic Acids Res* 2013; **41**: e180 [PMID: 23945944 DOI: 10.1093/nar/gkt721]

111 **Liu J**, Gaj T, Patterson JT, Sirk SJ, Barbas CF. Cell-penetrating peptide-mediated delivery of TALEN proteins via bioconjugation for genome engineering. *PLoS One* 2014; **9**: e85755 [PMID: 24465685 DOI: 10.1371/journal.pone.0085755]

112 **Gaj T**, Guo J, Kato Y, Sirk SJ, Barbas CF. Targeted gene knockout by direct delivery of zinc-finger nuclease proteins. *Nat Methods* 2012; **9**: 805-807 [PMID: 22751204 DOI: 10.1038/nmeth.2030]

113 **Van Tendeloo VF**, Ponsaerts P, Berneman ZN. mRNA-based gene transfer as a tool for gene and cell therapy. *Curr Opin Mol Ther* 2007; **9**: 423-431 [PMID: 17932806]

114 **Bettinger T**, Carlisle RC, Read ML, Ogris M, Seymour LW. Peptide-mediated RNA delivery: a novel approach for enhanced transfection of primary and post-mitotic cells. *Nucleic Acids Res* 2001; **29**: 3882-3891 [PMID: 11557821 DOI: 10.1093/nar/29.18.3882]

115 **Sung YH**, Baek IJ, Kim DH, Jeon J, Lee J, Lee K, Jeong D, Kim JS, Lee HW. Knockout mice created by TALEN-mediated gene targeting. *Nat Biotechnol* 2013; **31**: 23-24 [PMID: 23302927 DOI: 10.1038/nbt.2477]

116 **Doyon Y**, McCammon JM, Miller JC, Faraji F, Ngo C, Katibah GE, Amora R, Hocking TD, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Amacher SL. Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat Biotechnol* 2008; **26**: 702-708 [PMID: 18500334 DOI: 10.1038/nbt1409]

117 **Zhao Q**, Temsamani J, Iadarola PL, Jiang Z, Agrawal S. Effect of

different chemically modified oligodeoxynucleotides on immune stimulation. *Biochem Pharmacol* 1996; **51**: 173-182 [PMID: 8615886 DOI: 10.1016/0006-2952(95)02177-9]

118 **Bessis N**, GarciaCozar FJ, Boissier MC. Immune responses to gene therapy vectors: influence on vector function and effector mechanisms. *Gene Ther* 2004; **11** Suppl 1: S10-S17 [PMID: 15454952 DOI: 10.1038/sj.gt.3302364]

119 **Marshall E**. Gene therapy death prompts review of adenovirus vector. *Science* 1999; **286**: 2244-2245 [PMID: 10636774 DOI: 10.1126/science.286.5448.2244]

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REVIEW

Liver cirrhosis in hepatic vena cava syndrome (or membranous obstruction of inferior vena cava)

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Abstract

Hepatic vena cava syndrome (HVCS) also known as membranous obstruction of inferior vena cava reported mainly from Asia and Africa is an important cause of hepatic venous outflow obstruction (HVOO) that is complicated by high incidence of liver cirrhosis (LC) and moderate to high incidence of hepatocellular carcinoma (HCC). In the past the disease was considered congenital and was included under Budd-Chiari syndrome (BCS). HVCS is a chronic disease common in developing countries, the onset of which is related to poor hygienic living condition. The initial lesion in the disease is a bacterial infection induced localized thrombophlebitis in hepatic portion of inferior vena cava at the site where hepatic veins open which on resolution transforms into stenosis, membrane or thick obstruction, and is followed by development of cavo-caval collateral anastomosis. The disease is characterized by long asymptomatic period and recurrent acute exacerbations (AE) precipitated by clinical or subclinical bacterial infection. AE is managed with prolonged oral antibiotic. Development of LC and HCC in HVCS is related to the severity and frequency of AEs and not to the duration of the disease or the type or severity of the caval obstruction. HVOO that develops during severe acute stage or AE is a pre-cirrhotic condition. Primary BCS on the other hand is a rare disease related to prothrombotic disorders reported mainly among Caucasians that clinically manifest as acute, subacute disease or as fulminant hepatic failure; and is managed with life-long anticoagulation, porto-systemic shunt/endovascular angioplasty and stent or liver transplantation. As epidemiology, etiology and natural history of HVCS are different from classical BCS, it is here, recognized as a separate disease entity, a third primary cause of HVOO after sinusoidal obstruction syndrome and BCS. Understanding of the natural history has made early diagnosis of HVCS possible. This paper describes epidemiology, natural history and diagnosis of HVCS and discusses the pathogenesis of LC in the disease and mentions distinctive clinical features of HVCS related LC.

Key words: Hepatic venous outflow obstruction; Budd-Chiari syndrome; Hepatic inferior vena cava disease; Bacterial infection; Hepatocellular carcinoma

Core tip: Previously considered congenital and diagnosed late hepatic vena cava syndrome (HVCS) is a dynamic life-long disease related to bacterial infection that begins insidiously often in childhood and leads to development of cirrhosis and hepatocellular carcinoma. Localized stenosis, the sequel of the initial lesion persists life-long makes it vulnerable to subsequent bacterial infection which is followed by thrombosis in inferior vena cava

(IVC) and intra-hepatic veins resulting in recurrent ischemic liver damage. As it frequently occurs as a co-morbid condition in patients with chronic hepatitis B or C infection or alcohol use, patients with cirrhosis in developing countries should be assessed for presence of HVCS by ultrasonography of IVC.

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INTRODUCTION

Cirrhosis develops in liver diseases where recurrent loss of hepatocytes is followed by fibrosis and formation of regenerative nodules. The process distorts the architecture of the liver, and results in porto-systemic shunting of blood and impairment of hepatic function. Alcoholic liver disease and chronic hepatitis B and hepatitis C viral infections are common causes of cirrhosis in the world. These diseases cause veno-portal type of cirrhosis where bridging fibrosis develop between hepatic veins (HV) and portal tract (PT). Hepatic venous outflow obstruction (HVOO) is an interesting condition associated with a distinctive type of liver cirrhosis (LC) where fibrous bridges develop between terminal hepatic veins, with minimal fibrosis between HV and PT called reversed lobulation or non-portal or veno-centric cirrhosis[1,2]. Both veno-centric and veno-portal cirrhosis occur in HVOO but veno-centric cirrhosis is not seen in other conditions[2]. Three primary diseases of HVOO are sinusoidal obstruction syndrome (SOS), caused by toxic damage of sinusoids by pyrrolizidine alkaloid or myeloablative therapy[3]; Budd-Chiari syndrome (BCS) caused by thrombosis of HV related to prothrombotic disorders[4]; and bacterial infection initiated primary disease of the hepatic portion of the inferior vena cava (IVC) the hepatic vena cava syndrome (HVCS)[5].

SOS was previously called veno-occlusive disease[1]. Till 1950s before the advent of chemotherapy the only cause of SOS was pyrrolizidine alkaloid ingestion as herbal tea or food contaminated with seeds of plants like Senecio, Crotalaria and Heliotropium. The disease then occurred in developing countries as sporadic cases and as outbreaks[6,7]. The disease is now rare and is seen in persons with myeloablative therapy[3]. BCS is also a rare disease, often occurs as acute or fulminant type[8] reported mainly from the West with a prevalence of 1:10000[4] that occurs predominantly in young Caucasian female. HVCS is an important cause of HVOO in Asia and Africa[9-25] and is associated with high incidence of LC and moderate to high incidence of hepatocellular carcinoma (HCC).

HVCS at present is diagnosed late after development of complete obstruction of the IVC or after development of LC or HCC[12-14]. Understanding of the natural history of the disease and use of ultrasonography and color Doppler (USG) examination of IVC and liver in people with bacterial infection has helped to recognize the disease at early stage[26]. The disease is endemic in Nepal[22], and may also be common in other developing countries. The aim of this article is to draw attention to this under-diagnosed bacterial infection induced disease that is complicated by LC and HCC. Pathogenesis of LC and its distinctive features in HVCS are described, and difference between classical BCS and HVCS are mentioned.

HVCS AND BUDD-CHIARI SYNDROME ARE TWO DIFFERENT DISEASES

HVCS is often described under BCS or under various names as membranous obstruction of inferior vena cava (MOVC), hepatic vena cava disease, or coarctation of inferior vena cava or hepatocavopathy. The popularly used term MOVC is a misnomer as the lesion in the chronic disease reported from Asia and Africa had either thick localized stenosis or thick obstruction[12,24,27].

BCS reported from the West is caused by prothrombotic disorders[28] where thrombosis occurs predominantly in hepatic veins. Some prothrombotic conditions like factor V Leiden cause thrombosis at the supra-hepatic portion of the IVC that transformed into a thin membrane on resolution[29]. The natural history of BCS is different from HVCS. Classical BCS manifest clinically as acute and subacute disease or as fulminant hepatic failure[4,8] and is managed with lifelong anticoagulation, porto-systemic shunt/endovascular angioplasty and stent or liver transplantation[30,31]. Its inclusion under BCS[32,33] has caused much confusion and led to adoption of treatment of BCS in HVCS[15].

HVCS is not related to prothrombotic disorders[15,34]. The disease occurs in people living in poor hygienic condition[17,22,26] and affects both sexes of all age groups including children[23,35-37]. It is a chronic disease characterized by insidious onset, long asymptomatic period, recurrent acute exacerbations (AE) and development of cavo-caval collaterals. Thrombolytic or anticoagulant therapy is not effective in HVCS[38,39]. Acute disease and AEs are managed with prolonged high dose oral antibiotic with diuretics where necessary. Developments of extensive collaterals in chronic patients results in establishment of circulatory equilibrium making surgery or endovascular procedure to correct obstructive lesion in the IVC superfluous. The etiology, natural history and management of HVCS are thus different from BCS. Okuda *et al*[40] in 1998 proposed to separate it from BCS. It is here recognized as a third primary disease causing HVOO, after SOS and BCS under the name "HVCS".

EPIDEMIOLOGY OF HVCS

The incidence of congestive cirrhosis due to HVCS in autopsied cases of HCC in Japan in 1921 was 8.1% (9 out of 110 cases)[41], which in 1986-1987 dropped to 0.1% among 2982 cases[42]. An epidemiological survey conducted in Japan in 1989 by a national study group detected 300 cases in the whole country, with 21 new cases occurring annually[42]. These studies indicated to occurrence of the disease in Japan, whose prevalence had declined recently. An epidemiological survey of the disease carried out in Dongping county of Shandong province in China in 1980s showed the prevalence of the disease to be 6.5 per 100000[43]. And large series of surgically operated cases in China[16,17] till recently showed that IVC disease (94.4%) predominated in that country compared to hepatic vein obstruction (5.5%). In South Africa the frequency of HVCS diagnosed indirectly from liver biopsy was 7.1% among black patients with liver disease[24]. HVCS is endemic in Nepal, where it is a common cause of ascites[44] and LC[37,45]. Liver biopsies performed in 430 patients in 1990 to 1997 showed evidence of HVCS in 158 (36.7%), 126 had congestive changes and 32 had congestive cirrhosis[34]. Okuda[46] observed that the prevalence of HVCS in different countries was inversely related to the standard of hygiene and suggested that the causative factor of the disease perhaps lay in opportunity for frequent bacterial infection[27]. In India besides HVCS, recently BCS related to prothrombotic disorders had been described[47,48].

PATHOGENESIS OF HVCS

HVCS was previously considered a congenital disease[12,15,19,25,49]. Much discussion ensued on the subject and consensus had developed on the acquired nature of the disease[5,34,40,50,51]. Observation of transformation of thrombosis in IVC into a membrane led Okuda *et al*[40] to propose thrombosis theory. Thrombosis was considered idiopathic, as it was not related to prothrombotic disorders[40,52]. Later recognition of acute stage of the disease associated with bacteremia or bacterial infection[34] and transformation of acute localized thrombosis formed during bacterial infection into stenosis and complete obstruction[5], and high prevalence of the disease among people living in poor hygienic conditions in China and Nepal[17,22] led to suggestion that the initial lesion probably was bacterial infection induced thrombophlebitis[5]. Occurrence of fever chills and bacteremia within a few hours after cavogram, absence of thrombo-embolic phenomenon[53]; and past autopsy studies that detected bacteria in the thrombus in IVC[35,54] and the histology of the lesion in IVC that showed features of thrombophlebitis[55], all supported the hypothesis.

The initial localized thrombophlebitis in IVC occurred typically at the site where hepatic veins open[5,34]. Subsequent resolution transformed the lesion into stenosis or complete obstruction (Figure 1). An important feature of the disease is the occurrence of recurrent AE[10,22,54-56] precipitated by clinical or subclinical bacterial infection[53,54]. During AE fresh thrombus is deposited at the site of the lesion in IVC (Figure 2)[10,45]. AE may be subclinical or when thrombus so formed obstructs the hepatic orifices it manifests as HVOO with hepatomegaly and ascites (Figure 2D). Resolutions of thrombosis formed during recurrent AEs eventually convert the segment of the IVC into a thick obliteration of various types[12,51,57]. During AE thrombus is also deposited in intra-hepatic veins, resolution of which leads to development of intimal thickening, segmental stenosis or membrane within the vein and at orifices of big veins. These changes that had been described in autopsy studies[51,52] are also observed in USG examination of the patients (Figure 3). The obliterative lesion in the IVC and HV is followed by development of collaterals anastomosis. Deep cavo-caval collaterals like dilated ascending lumbar, azygos and hemiazygos veins are most constant and are better outlined by cavogram[57]. Superficial cava-caval collaterals are seen as dilated superficial veins in the body trunk with upward flow. It is observed only in about 25% of the patients. Collaterals also develop between obstructed and patent intra-hepatic veins and veins around the liver (Figure 4).

NATURAL HISTORY OF HVCS

The acute stage of the disease is frequently unrecognized or misdiagnosed. The disease becomes chronic with long asymptomatic period and recurrent AE. Intermittent upper abdomen discomfort usually after food or exertion or intermittent mild ankle edema is common. The disease is usually diagnosed following fortuitous detection of hepatomegaly or splenomegaly or dilated superficial veins in the body trunk, or during AE[58,59]. Features of AE include fever, mild jaundice or mild alanine transaminase (ALT) elevation. Severe AE is characterized by prolonged fever or jaundice followed by ascites (due to HVOO), edema legs, or pleural effusion[60] or puffy face or variceal bleeding (due to transient portal hypertension). Ascites in HVCS is associated with bacterial peritonitis[44]. In between AEs patients remain well with normal or minimal elevation of bilirubin or ALT.

Patients with long standing disease may develop varicose veins or signs of poor circulation in legs like increase pigmentation or poorly healing ulcer in sheen. Other features of the chronic disease include proteinurea, sterility or failure to sustain pregnancy to full term[10,17]. Clinical manifestation of the HVCS is thus protean and its diagnosis depends on the detection of the localized lesion in hepatic portion of the IVC.

The disease is compatible with long survival[9,37]. Death in early stage is from AE related septicemia, renal failure or bleeding from esophageal varices and in later stage is from HCC or natural cause[9,11,26]. HVCS is complicated by high incidence (70%) of liver cirrhosis (Table 1) and moderate (about 10%) to high (> 20%)

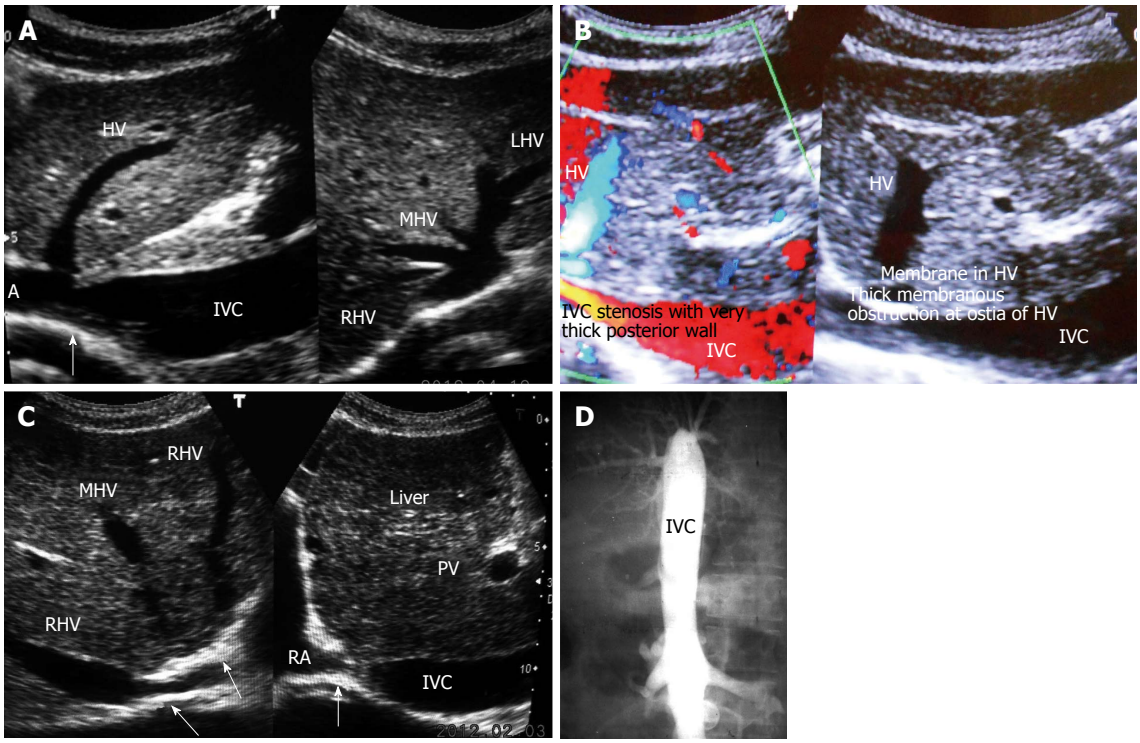

Figure 1 Inferior vena cava obstruction. A: Ultrasonography showing stenosis of inferior vena cava (IVC) at cavo-atrial junction. Note patent orifices hepatic vein (HV)-right HV (RHV), middle HV (MHV) and left HV (LHV); B: Color Doppler ultrasonography of a patient with liver cirrhosis showing IVC stenosis, membranes in HV; C: Ultrasonography of a patient with liver cirrhosis showing complete obstruction of IVC at cavo-atrial junction and obstruction at orifices of MHV and LHV. Note dilated hepatic veins; D: Cavogram showing complete obstruction of the IVC. PV: Portal vein; RA: Right atrium.

Table 1 Incidence of liver cirrhosis in hepatic vena cava syndrome

Ref.	Country	No. of patients	LC (%)
Nakamura *et al*[9]	Japan	7	7 (100)
Nakamura *et al*[9]	From Japanese literature	64	64 (100)
Takeuchi *et al*[10]	Japan	7	5 (71)
Nakamura *et al*[11]	Japan	13[1]	13 (100)
Ono *et al*[12]	Japan	18	17 (94.0)[2]
Gentil-Kocher *et al*[36]	France	22	3 (13.6%)
Kage *et al*[51]	Japan	17	6 (35.2)[3]
Shrestha[45]	Nepal	56[1]	44 (78.5)
Shrestha *et al*[37]	Nepal[4]	178	49 (27.5)

[1]Long term follow-up cases; [2]Of 18 biopsied cases 17 showed LC and 1 congestion; [3]Autopsied cases, reminder 4 had congestion and 7 congestive fibrosis; [4]Children. LC: Liver cirrhosis.

incidence of HCC[9,24,45,46]. HVCS thus is a lifelong disease with a potential to develop recurrent ascites, portal hypertension and LC or HCC. In endemic areas HVCS occurs as co-morbid conditions with other acute and chronic liver diseases as chronic hepatitis B, chronic hepatitis C and alcoholic liver disease[14,61].

DIAGNOSIS OF HVCS

Diagnosis is made by identification of the obliterative lesion in the hepatic portion of the IVC-thrombosis, stenosis or complete obstruction by imaging procedure. Early diagnosis before development of complete IVC obstruction is possible. Ultrasonography and color Doppler is specific and sensitive in the diagnosis of the lesion and is the investigation of first choice. The procedure is not only non-invasive and cost-effective but is easily available in developing countries where the disease is common. It yields better result when used by the clinician himself as a part of initial and follow-up clinical examination. Detection of intra-hepatic veins thrombosis and its sequel intimal thickening, stenosis or membrane (Figure 3) and collaterals in and around the liver (Figure 4), and detection of cava-caval collaterals supports the diagnosis.

Inferior vena cavogram (cavogram) and other imaging procedures as MRI; or liver biopsy are used to confirm the diagnosis. Cavogram may miss the diagnosis in patients with minimal stenosis or in advanced stage when contrast medium rapidly runs off into large collaterals and fails to outline the caval obstruction[24]. Liver biopsy during AE may show acute centrilobular congestion. Biopsy findings vary greatly depending on the phase of the disease. It varies from normal or minimal changes like sinusoidal dilatation, central vein dilatation or fibrosis or obstruction; to thrombosis or endophlebitis of sublobular vein; or congestive fibrosis or cirrhosis[36,51,52,58].

Recognition of AE is important. USG is sensitive in the diagnosis of AE. Detection of "recent" thrombus in the IVC at the site of old lesion indicates AE (Figure

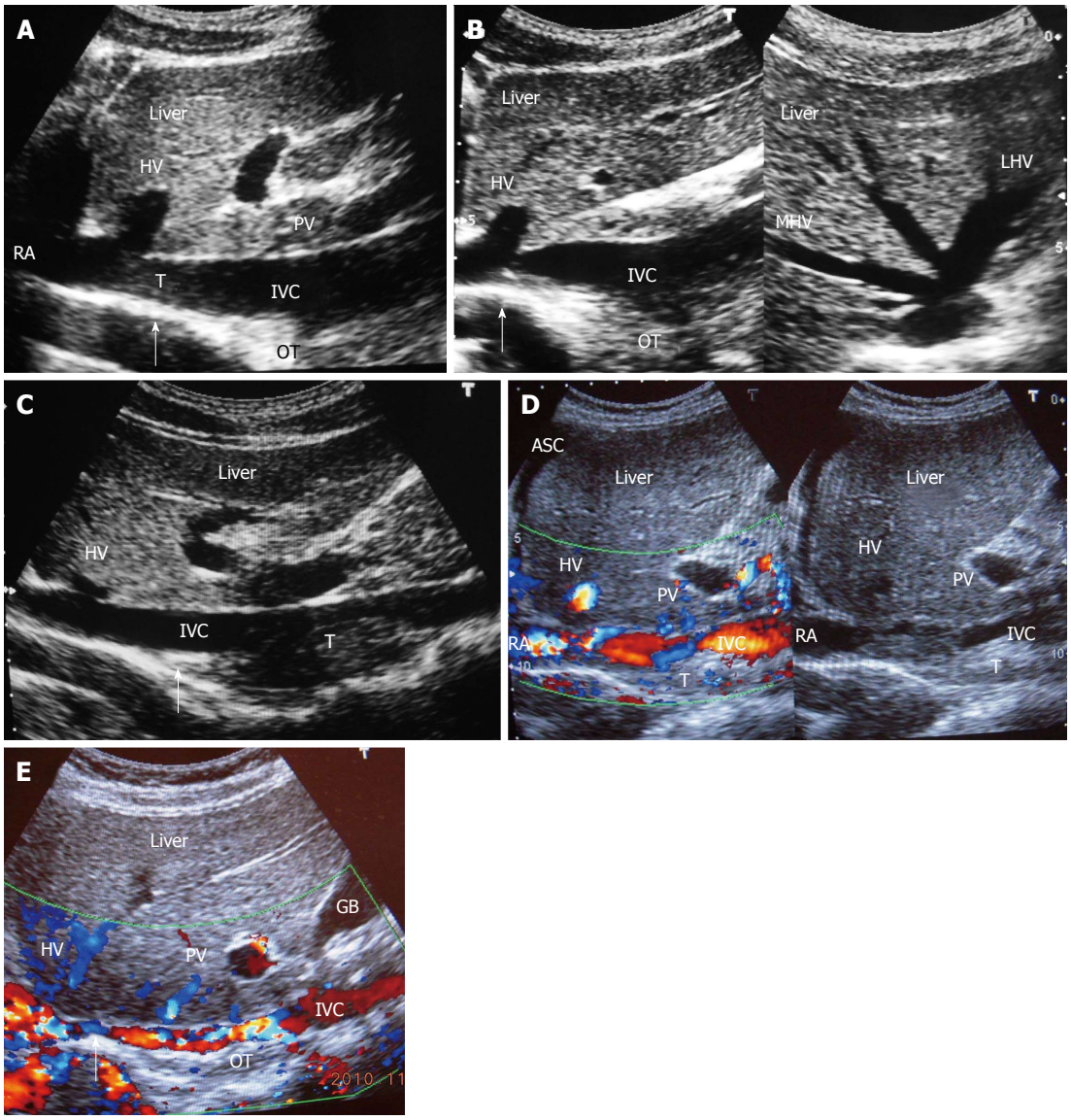

Figure 2 Ultrasonography showing thrombi of different ages in inferior vena cava due to recurrent acute exacerbations. A: Ultrasonography showing mild stenosis of inferior vena cava (IVC) with thick echoic posterior wall at cavo-atrial junction. It shows recent thrombus (T) and old organized thrombus (OT) deposited during recurrent acute exacerbation (AE); B: Ultrasonography showing stenosis of IVC at cavo-atrial junction, with OT along posterior wall just distal to it. Middle hepatic vein (MHV) and left hepatic vein (LHV) hepatic veins are patent; C: Ultrasonography showing mild stenosis of IVC at cavo-atrial junction and thrombus of different ages along the posterior wall of the IVC; D: Ultrasonography showing features of HV outflow obstruction-hepatomegaly and ascites (ASC) in a patient with IVC stenosis near cavo-atrial junction and IVC filled with recent and old organized T; E: Ultrasonographic evidence of recurrent AE; Color Doppler ultrasonography showing layers of linear old OT along posterior wall of the hepatic portion of the IVC narrowing its lumen. Arrow indicates to the site of initial lesion in IVC. USG also shows segmental stenosis of HV. PV: Portal vein; HV: Hepatic vein; RA: Right atrium; GB: Gall bladder.

2A). Presence of old organized thrombi of different ages along posterior wall of the IVC just distal to the initial lesion indicates to occurrence of recurrent AE (Figure 2). Severe AE is recognized in USG by presence of ascites, hepatomegaly and recent thrombus in IVC obstructing hepatic vein orifices (Figure 2D). Ascitic fluid has high protein content, high serum ascitic albumin gradient and evidence of bacterial peritonitis[44]. Neutrophil leukocytosis, increased level of C-reactive protein and bacteremia occur during severe AE. Severe AE is a pre-cirrhotic condition. Severe AE is followed by development of LC within 6 mo[45]. Recognition and early treatment of severe AE is thus important.

CIRRHOSIS IN HVCS

Clinical features of cirrhosis are determined by three vectors: (1) portal hypertension (PH); (2) extent of parenchymal failure; and (3) features of the original disease that had caused cirrhosis. Relative importance of these three vectors differs in different diseases causing cirrhosis and in the stage of the cirrhosis. In alcoholic cirrhosis, symptoms of parenchymal failure or PH dominate the clinical picture. Vascular spiders, gynaecomastia and coagulopathy are common. In LC due to chronic viral infection ascites, jaundice and bacterial infection develop late and their presence often

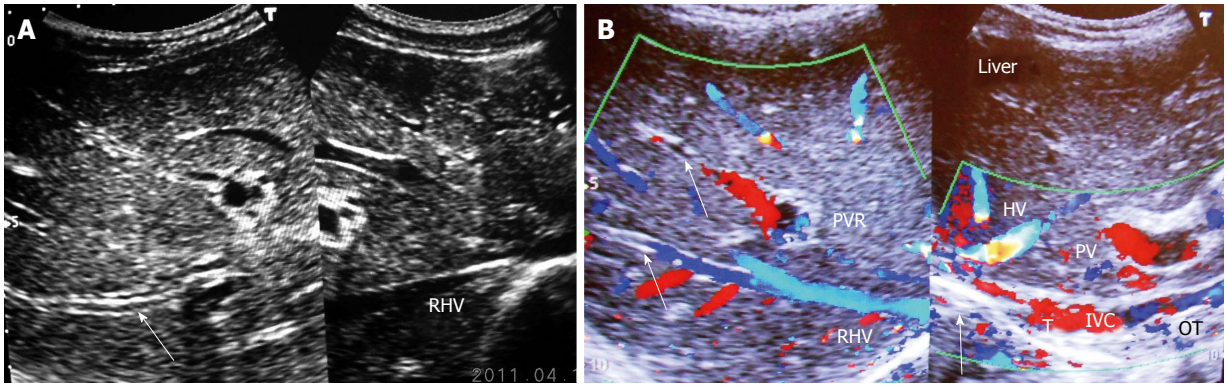

Figure 3 Ultrasonography showing thrombosed intra-hepatic veins. A: Ultrasonography showing diffuse thrombosed and echoic walls of large and medium-sized intra-hepatic veins (one of which is indicated by an arrow) that occurred during acute exacerbation. Right hepatic vein (RHV) orifice is narrowed; B: Color Doppler Ultrasonography of patient with cirrhosis. It shows long segment stenosis of inferior vena cava (IVC) with recent thrombus (T) and old organized thrombi (OT) on thick posterior wall. Arrow shows thrombosed large and medium-sized intra-hepatic veins. HV: Hepatic vein; PV: Portal vein; PVR: Portal vein radical.

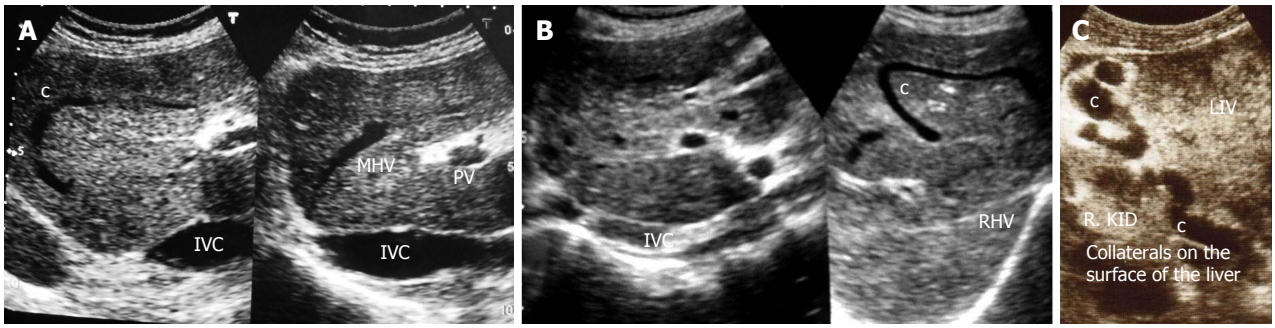

Figure 4 Ultrasonography showing collaterals. A: Ultrasonography of a patient with liver cirrhosis due to complete inferior vena cava (IVC) obstruction at cavo-atrial junction showing obstruction of middle hepatic vein (MHV) and a intra-hepatic collateral (c); B: Ultrasonography of a young girl with liver cirrhosis: IVC is filled with old organized thrombi of different ages, right hepatic vein (RHV) is thrombosed and a large intra-hepatic collateral is seen; C: Ultrasonography showing dilated collaterals on the surface of liver (LIV) close to right kidney (R. KID) in a patient with liver cirrhosis. PV: Portal vein.

indicates presence of severe hepatocellular damage and an indication for liver transplantation[62,63]. In HVCS related LC vascular spiders, palmer erythema, coagulopathy are uncommon. Hepatomegaly is seen in 75% and splenomegaly in 25% of the cases. Symptoms of AE dominate the clinical picture. Recurrent jaundice, ascites with bacterial peritonitis and pleural effusion is common and occur early. These are related to AE and not to severity of hepatocellular damage, and responds to medical treatment. Even patient with advanced cirrhosis develop ascites due to bacterial infection induced AE (Figure 5A) that responds to medical treatment. LC due to HVCS in general has better prognosis with long survival[45] if infection is prevented or AE treated with prolonged high dose oral antibiotic.

HVCS related LC is characterized by a few distinctive USG features that help in its diagnosis. Hepatic veins are frequently dilated (Figures 1C and 5B), whereas in LC due to other causes these appear attenuated. Other distinctive features include presence of echoic intra-hepatic vein wall (Figure 4A and B), intra-hepatic and extra-hepatic collaterals (Figure 4), membrane in HVs (Figure 1B), obstruction at the ostia of hepatic veins (Figure 1B and C), calcified foci in liver (Figure 5C) and presence of thick or thick edematous gall bladder wall and thick visceral and parietal peritoneum (Figure 5D). These signs are related to infection and infection induced vascular obstruction. Thick or thick edematous gall bladder wall had been reported earlier and was labeled as acalculous cholecystitis[8]. Color Doppler study showed that calcified focus occurred at the wall of the hepatic vein.

CIRRHOSIS IN CHILDREN DUE TO HVCS

Cirrhosis in children is interesting, as alcohol, chronic hepatitis B and C the common causes of the disease in adult are often not the important issue in children. There is a distinct geographical pattern in the etiology of cirrhosis in children. Common cause of cirrhosis in children in 1950s in West Indies was SOS caused by pyrrolizidine alkaloid[64], and in India in 1950-1990s it was Indian childhood cirrhosis[65,66]. Chronic hepatitis and metabolic disorders such as Wilsons's disease are rare causes of cirrhosis in children the world over[67]. HVCS occurs in children and LC (Figure 6) had been reported among them[23,36]. In Nepal it was a common cause of cirrhosis in children and affected predominantly children

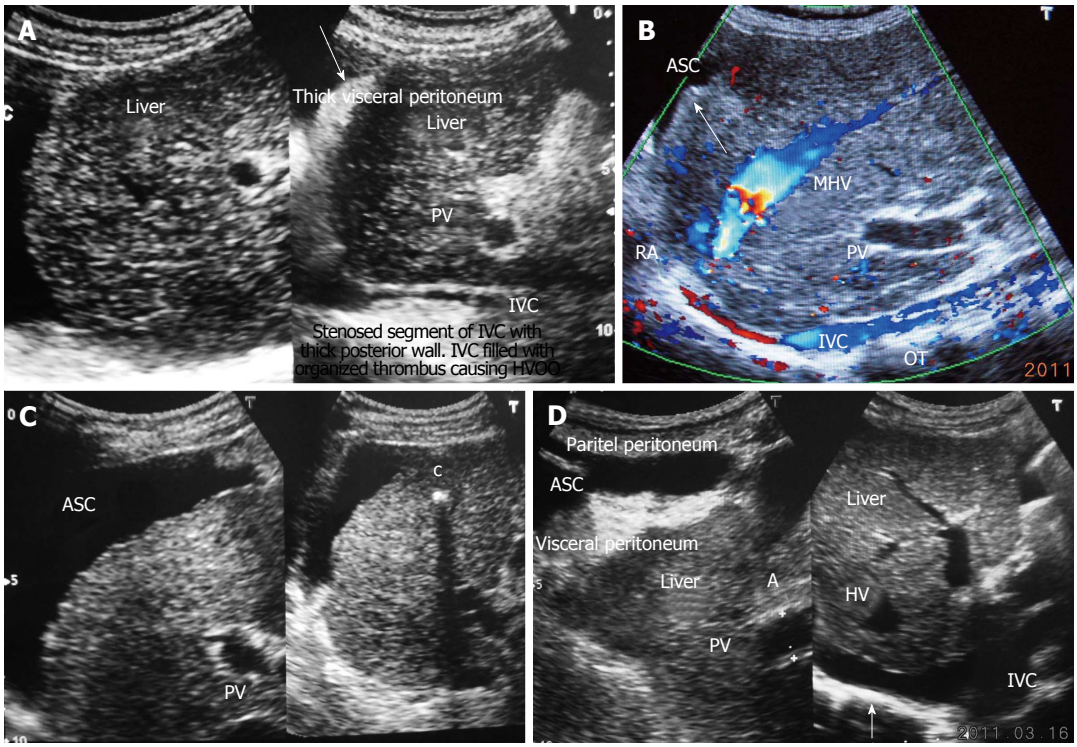

Figure 5 Ultrasonography showing ascites and evidence of chronic peritonitis in a patient with cirrhosis. A: Acute exacerbation in a patient with liver cirrhosis showing hepatic portion of the inferior vena cava (IVC) filled with organizing thrombus and ascites with thick visceral peritoneum-indicating presence of bacterial peritonitis; B: Ultrasonography of a patient with liver cirrhosis due to HVCS: showing long segment stenosis of IVC with thick old organized thrombus (OT) along the posterior wall of the hepatic portion of the IVC. Note the presence of ascites (ASC) and irregular margin of the liver indicated by an arrow. Middle hepatic vein (MHV) is obstructed at its orifice and shows distal segmental stenosis; C: Ultrasonography of a patient with liver cirrhosis due to HVCS showing inferior vena cava stenosis, a calcified focus (c) in the liver and ascites; D: Ultrasonography of patient with liver cirrhosis due to HVCS showing IVC stenosis with organized thrombus on posterior wall and ASC and thick visceral peritoneum, suggestive of chronic bacterial peritonitis. PV: Portal vein; HVCS: Hepatic vena cava syndrome; RA: Right atrium; HV: Hepatic vein.

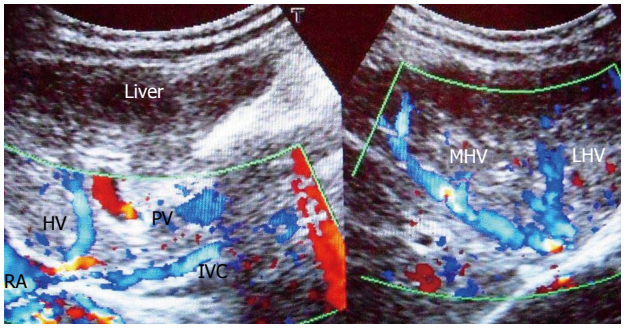

Figure 6 Liver cirrhosis in a young girl due to hepatic vena cava syndrome. Note long segment obstruction of the inferior vena cava (IVC) at cavo-atrial junction and irregular narrowing of its hepatic portion. Middle hepatic vena (MHV) and left hepatic vena (LHV) are dilated with irregular caliber. PV: Portal vein; HV: Hepatic vein; RA: Right atrium.

Table 2 Comparison of frequency of acute exacerbations in hepatic vena cava syndrome: Among patients who did and did not develop liver cirrhosis and hepatocellular carcinoma[1]

	Incidence of AE	*P* value
Who did not develop LC/HCC	3.2 ± 3.2	
Patient who developed LC	6.5 ± 4.5	0.017
Patients who developed HCC	11.5 + 3.0[1]	< 0.001
56 patients of HVCS seen in the period 1990-1997		
Followed up for 14.8 ± 9 yr		
LC developed in 44 (78.5%). HCC developed in 6 (10.7%)		

[1]Quarter of the AE in HCC group was severe. Severe AE: Development of ascites due to HVOO. Modified from Shrestha *et al*[45]. HVCS: Hepatic vena cava syndrome; HCC: Hepatocellular carcinoma; AE: Acute exacerbation; LC: Liver cirrhosis; HVOO: Hepatic venous outflow obstruction.

of poor socio-economic background or those with history of chronic diarrhea or prolonged intermittent fever[37].

PATHOGENESIS OF LIVER CIRRHOSIS IN HVCS

Occurrence of LC in HVCS was recognized since 1878[57]. But its pathogenesis was not clearly understood. Okuda assumed that cirrhosis in HVCS is a late event in the course of the disease, and it resulted from prolonged congestion with loss of hepatocyte followed by failure to regeneration because of continued high intra-hepatic venous pressure[27]. Liver damage in HVCS occurs periodically during AE and there is no prolonged continued damage because of development efficient collateral circulation. Development of cirrhosis is not related to the duration of the disease or the type of caval lesion but to the severity and frequency of the AEs (Table 2)[45]. Further, surgical or endovascular procedures to treat caval obstruction did not prevent development of LC or HCC[15,36].

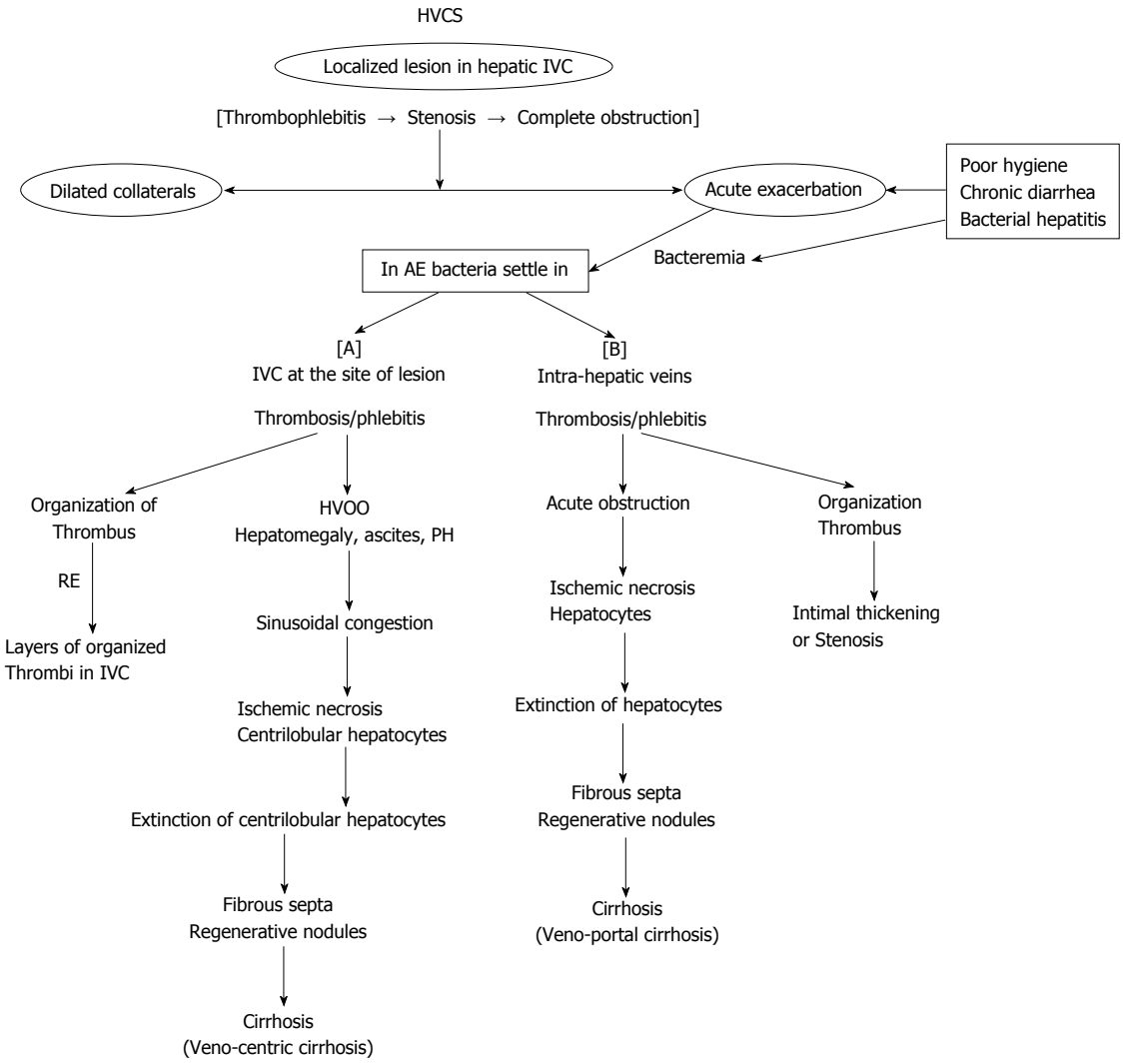

Figure 7 Mechanism of development of cirrhosis in hepatic vena cava syndrome. IVC: Inferior vena cava; HVOO: Hepatic venous outflow obstruction; PH: Portal hypertension; AE: Acute exacerbation; RE: Recurrent AE; HVCS: Hepatic vena cava syndrome.

Severe AE is a pre-cirrhotic condition. Severe AE associated with HVOO and/or thrombosis or endophlebitis of medium-sized intra-hepatic veins are followed by development of cirrhosis within a few months. Two mechanisms (Figure 7) of development cirrhosis in HVCS are described[45]: (1) HVOO: Large thrombosis formed in IVC close to hepatic veins orifices during acute stage or AE results in HVOO (Figure 2D). Severe HVOO causes sudden increases in the sinusoidal pressure that result in sinusoidal congestion and hemorrhage in the space of Disse (Figure 8A). Increase in sinusoidal pressure is followed by reflex reduction of hepatic arterial blood flow. The combined effect of sinusoidal congestion and decrease arterial flow results in ischemic necrosis of hepatocytes around central vein. Apoptosis of the hepatocytes in the congested region is followed by fibrosis (Figure 8B) and regenerative activity in periportal areas which eventually leads to development of venocentric or reversed lobulation cirrhosis within a few months[2,45]; (2) Thrombosis or thrombophlebitis of sublobular or medium-sized hepatic vein (Figure 8C) that occurs in AE is associated ischemic necrosis of hepatocytes drained by the vein[45,58]. Extinction of large areas of hepatocytes is followed by development of fibrous septa within a few weeks[2,68]. Obstruction of hepatic vein branch is more injurious. Combined portal vein and HV obstruction leads to veno-portal cirrhosis. Obstruction of portal vein radicals alone lead to atrophy and secondary nodular hyperplasia but no extinction of hepatocytes as ischemic insult is compensated by arterial flow. This however may lead to development of large regenerative nodules[2]. Development of LC in steatohepatitis and chronic viral infection was also considered to be due to obstruction of small hepatic veins adjacent to hepatic necroinflammation[69]. Mechanism of development of cirrhosis in HVCS thus is more explicit-ischemic necrosis and extinction of hepatic parenchyma secondary to obstruction of hepatic vein and sinusoids by thrombosis or phlebitis during AE.

CONCLUSION

Geo-cultural factors determine the etiology of cirrhosis in a community. In Japan and recently in the West

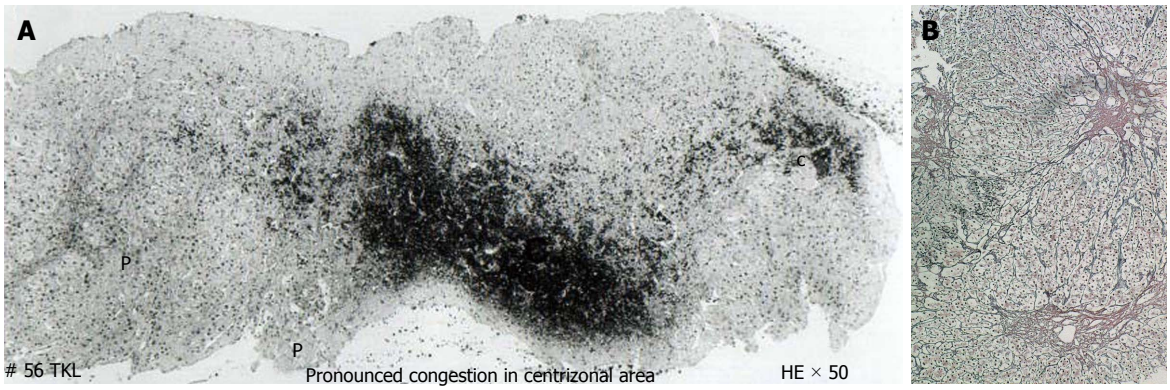

Figure 8 Histology showing fibrosis in centri-lobular areas. A: Histology of liver of a patient with hepatic vena cava syndrome during acute exacerbation showing acute congestive changes around central vein (c) and sparing of liver around portal tract (P) due to hepatic venous outflow obstruction; B: Histology of liver of patient with hepatic vena cava syndrome a few months after development of hepatic venous outflow obstruction during acute exacerbation showing fibrosis around central vein. Histology of liver of a patient with hepatic vena cava syndrome showing the wall of a thrombosed medium sized intra-hepatic vein that occurred during acute exacerbation. HE: Hematoxylin eosin stain.

beside alcohol, chronic hepatitis C related to drug abuse that followed social upheaval following 2nd World War or Vietnam War is an important cause of cirrhosis and HCC[70]. In Asia and Africa besides chronic hepatitis B infection, bacterial infection initiated HVCS is an important cause of cirrhosis both in children and adults. Patients with LC and HCC in developing countries may have two or more co-morbid condition like alcohol, hepatitis B or C and HVCS co-existing together[14,61]. Therefore careful assessment of the cause of cirrhosis is mandatory before planning treatment. This is done based on the understanding of the natural history of the etiologic factors. History of recurrent AE with jaundice and ascites and presence of USG features of IVC lesion and the distinctive features of HVCS related cirrhosis mentioned above helps in the diagnosis of LC due to HVCS. Ascites due to HVOO that occur during AE is a pre-cirrhotic condition. Prognosis of LC due to HVCS is improved by prevention or adequate treatment of AE. AE is precipitated by clinical or subclinical bacterial infection and is treated with high dose prolonged antibiotic.

HVCS still remains an underdiagnosed entity in developing countries[71] or is often diagnosed late after development of cirrhosis or HCC[12-14]. Routine examination of IVC and liver by color Doppler ultrasonography in patients with bacterial infection and liver disease in developing countries is expected to provide a better assessment of its prevalence in the community. Recognition of the early stage of the disease provides opportunity for prevention of cirrhosis and HCC in this disease.

REFERENCES

1 **Bras G**, Jelliffe DB, Stuart KL. Veno-occlusive disease of liver with nonportal type of cirrhosis, occurring in Jamaica. *AMA Arch Pathol* 1954; **57**: 285-300 [PMID: 13147641]

2 **Tanaka M**, Wanless IR. Pathology of the liver in Budd-Chiari syndrome: portal vein thrombosis and the histogenesis of veno-centric cirrhosis, veno-portal cirrhosis, and large regenerative nodules. *Hepatology* 1998; **27**: 488-496 [PMID: 9462648 DOI: 10.1002/hep.510270224]

3 **DeLeve LD**, Shulman HM, McDonald GB. Toxic injury to hepatic sinusoids: sinusoidal obstruction syndrome (veno-occlusive disease). *Semin Liver Dis* 2002; **22**: 27-42 [PMID: 11928077 DOI: 10.1055/s-2002-23204]

4 **Plessier A**, Valla DC. Budd-Chiari syndrome. *Semin Liver Dis* 2008; **28**: 259-269 [PMID: 18814079 DOI: 10.1055/s-0028-1085094]

5 **Shrestha SM**, Shrestha S. Hepatic vena cava disease: Etiologic relation to bacterial infection. *Hepatol Res* 2007; **37**: 196-204 [PMID: 17362302 DOI: 10.1111/j.1872-034X.2007.00012.x]

6 **Mohabbat O**, Younos MS, Merzad AA, Srivastava RN, Sediq GG, Aram GN. An outbreak of hepatic veno-occlusive disease in north-western Afghanistan. *Lancet* 1976; **2**: 269-271 [PMID: 59848]

7 **Tandon HD**, Tandon BN, Tandon R, Nayak NC. A pathological study of the liver in an epidemic outbreak of veno-occlusive disease. *Indian J Med Res* 1977; **65**: 679-684 [PMID: 924565]

8 **Powell-Jackson PR**, Ede RJ, Williams R. Budd-Chiari syndrome presenting as fulminant hepatic failure. *Gut* 1986; **27**: 1101-1105 [PMID: 3758822 DOI: 10.1136/gut.27.9.1101]

9 **Nakamura T**, Nakamura S, Aikawa T, Suzuki O, Onodera A, Karoji N. Obstruction of the inferior vena cava in the hepatic portion and the hepatic veins. Report of eight cases and review of the Japanese literature. *Angiology* 1968; **19**: 479-498 [PMID: 5677579 DOI: 10.1177/000331976801900805]

10 **Takeuchi J**, Takada A, Hasumura Y, Matsuda Y, Ikegami P. Budd-Chiari syndrome associated with obstruction of the inferior vena cava. A report of seven cases. *Am J Med* 1971; **51**: 11-20 [PMID: 5570315 DOI: 10.1016/0002-9343(71)90319-6]

11 **Nakamura S**, Takezawa Y. Obstruction of the inferior vena cava in the hepatic portion and hepatocellular carcinoma. *Tohoku J Exp Med* 1982; **138**: 119-120 [PMID: 6293115 DOI: 10.1620/tjem.138.119]

12 **Ono J**, Sakoda K, Kawada T. Membranous obstruction of the inferior vena cava. *Ann Surg* 1983; **197**: 454-458 [PMID: 6830351]

13 **Shin SH**, Chung YH, Suh DD, Shin JW, Jang MK, Ryu SH, Park NH, Lee HC, Lee YS, Suh DJ. Characteristic clinical features of hepatocellular carcinoma associated with Budd-Chiari syndrome: evidence of different carcinogenic process from hepatitis B virus-associated hepatocellular carcinoma. *Eur J Gastroenterol Hepatol* 2004; **16**: 319-324 [PMID: 15195897 DOI: 10.1097/00042737-200403000-00012]

14 **Gwon D**, Ko GY, Yoon HK, Sung KB, Kim JH, Lee SS, Lee JM, Ohm JY, Shin JH, Song HY. Hepatocellular carcinoma associated with membranous obstruction of the inferior vena cava: incidence, characteristics, and risk factors and clinical efficacy of TACE. *Radiology* 2010; **254**: 617-626 [PMID: 20093533 DOI: 10.1148/radiol.09090738]

15 **Lee BB**, Villavicencio L, Kim YW, Do YS, Koh KC, Lim HK, Lim JH, Ahn KW. Primary Budd-Chiari syndrome: outcome of endovascular management for suprahepatic venous obstruction. *J Vasc Surg* 2006; **43**: 101-108 [PMID: 16414396 DOI: 10.1016/j.jvs.2005.09.003]

16 **Wang ZG**. Management of Budd-Chiari syndrome: experience from 430 cases. *Asian J Surg* 1996; **19**: 23-30

17 **Wang ZG**, Zhang FJ, Li XQ, Meng QY. Management of Budd-Chiari syndrome: what is the best approach? *J Gastroenterol Hepatol* 2004; **19**: S212-S218 [DOI: 10.1111/j.14001746.2004.03677.x]

18 **Datta DV**, Saha S, Singh SA, Gupta BB, Aikat BK, Chhuttani PN. Clinical spectrum of Budd-Chiari syndrome in Chandigrah with particular reference to obstruction of intrahepatic portion of inferior vena cava. *Indian J Med Res* 1972; **60**: 385-402 [PMID: 4659918]

19 **Victor S**, Jayanthi V, Madanagopalan N. Coarctation of the inferior vena cava. *Trop Gastroenterol* 1987; **8**: 127-142 [PMID: 3321651]

20 **Dilawari JB**, Bambery P, Chawla Y, Kaur U, Bhusnurmath SR, Malhotra HS, Sood GK, Mitra SK, Khanna SK, Walia BS. Hepatic outflow obstruction (Budd-Chiari syndrome). Experience with 177 patients and a review of the literature. *Medicine* (Baltimore) 1994; **73**: 21-36 [PMID: 8309360 DOI: 10.1097/00005792-199401000-00003]

21 **Singh V**, Sinha SK, Nain CK, Bambery P, Kaur U, Verma S, Chawla YK, Singh K. Budd-Chiari syndrome: our experience of 71 patients. *J Gastroenterol Hepatol* 2000; **15**: 550-554 [PMID: 10847443 DOI: 10.1046/j.1440-1746.2000.02157.x]

22 **Shrestha SM**, Okuda K, Uchida T, Maharjan KG, Shrestha S, Joshi BL, Larsson S, Vaidya Y. Endemicity and clinical picture of liver disease due to obstruction of the hepatic portion of the inferior vena cava in Nepal. *J Gastroenterol Hepatol* 1996; **11**: 170-179 [PMID: 8672764 DOI: 10.1111/j.1440-1746.tb00056.x]

23 **Awwad S**. The Budd-Chiari syndrome. *J Egypt Med Assoc* 1952; **35**: 650-669 [PMID: 13035825]

24 **Simson IW**. Membranous obstruction of the inferior vena cava and hepatocellular carcinoma in South Africa. *Gastroenterology* 1982; **82**: 171-178 [PMID: 6274728]

25 **Kew MC**, McKnight A, Hodkinson J, Bukofzer S, Esser JD. The role of membranous obstruction of the inferior vena cava in the etiology of hepatocellular carcinoma in Southern African blacks. *Hepatology* 1989; **9**: 121-125 [PMID: 2461892 DOI: 10.1002/hep.1840090121]

26 **Shrestha SM**. Natural history of hepatic vena cava disease: the liver disease due to obstruction of the hepatic portion of inferior vena cava. *Hepatol Int* 2009; **3**: 392-402 [DOI 10.1007/s12072-009-9133-2]

27 **Okuda K**. Inferior vena cava thrombosis at its hepatic portion (obliterative hepatocavopathy). *Semin Liver Dis* 2002; **22**: 15-26 [PMID: 11928076]

28 **Moucari R**, Rautou PE, Cazals-Hatem D, Geara A, Bureau C, Consigny Y, Francoz C, Denninger MH, Vilgrain V, Belghiti J, Durand F, Valla D, Plessier A. Hepatocellular carcinoma in Budd-Chiari syndrome: characteristics and risk factors. *Gut* 2008; **57**: 828-835 [PMID: 18218675 DOI: 10.1136/gut.2007.139477]

29 **Blanshard C**, Dodge G, Pasi J, Ormiston M, Dick R, Burroughs AK. Membranous obstruction of the inferior vena cava in a patient with factor V Leiden: evidence for a post-thrombotic aetiology. *J Hepatol* 1997; **26**: 731-735 [PMID: 9075684]

30 **Ouwendijk RJ**, Koster JC, Wilson JH, Stibbe J, Lameris JS, Visser W, Benhamou JP. Budd-Chiari syndrome in a young patient with anticardiolipin antibodies: need for prolonged anticoagulant treatment. *Gut* 1994; **35**: 1004-1006 [PMID: 8063206 DOI: 10.1136/gut.35.7.1004]

31 **Darwish Murad S**, Valla DC, de Groen PC, Zeitoun G, Hopmans JA, Haagsma EB, van Hoek B, Hansen BE, Rosendaal FR, Janssen HL. Determinants of survival and the effect of portosystemic shunting in patients with Budd-Chiari syndrome. *Hepatology* 2004; **39**: 500-508 [PMID: 14768004]

32 **Janssen HL**, Garcia-Pagan JC, Elias E, Mentha G, Hadengue A, Valla DC. Budd-Chiari syndrome: a review by an expert panel. *J Hepatol* 2003; **38**: 364-371 [PMID: 12586305 DOI: 10.1016/S0168-8278(02)00434-8]

33 **DeLeve LD**, Valla DC, Garcia-Tsao G. Vascular disorders of the liver. *Hepatology* 2009; **49**: 1729-1764 [PMID: 19399912 DOI: 10.1002/hep.22772]

34 **Shrestha SM**, Joshi BL, Shrestha S, Maharajan KG. Cavographic study of an early stage of obstruction of the hepatic portion of the inferior vena cava. *J Gastroenterol Hepatol* 2000; **15**: 202-210 [PMID: 10735545]

35 **Kibel MA**, Marsden HB. Inferior vena caval and hepatic vein thrombosis: the Chiari syndrome in childhood. *Arch Dis Child* 1956; **31**: 225-228 [PMID: 13328163]

36 **Gentil-Kocher S**, Bernard O, Brunelle F, Hadchouel M, Maillard JN, Valayer J, Hay JM, Alagille D. Budd-Chiari syndrome in children: report of 22 cases. *J Pediatr* 1988; **113**: 30-38 [PMID: 3290415 DOI: 10.1016/S0022-3476(88)80524-9]

37 **Shrestha SM**, Shrestha S. Hepatic vena cava syndrome: a common cause of liver cirrhosis in children in Nepal. *Trop Gastroenterol* 2014; **35**: 85-95 [PMID: 25470870 DOI: 10.7869/tg.186]

38 **Shrestha SM**. Hepatic IVC disease: Non-surgical management. In: Sarin SK, Sharma BC, Kumar M. Hepatology: Postgraduate course and current review. New Delhi: CBS Publishers and Distributers, 2004: 43-49

39 **Khuroo MS**, Datta DV. Budd-Chiari syndrome following pregnancy. Report of 16 cases, with roentgenologic, hemodynamic and histologic studies of the hepatic outflow tract. *Am J Med* 1980; **68**: 113-121 [PMID: 7350798 DOI: 10.1016/0002-9343(80)90180-1]

40 **Okuda K**, Kage M, Shrestha SM. Proposal of a new nomenclature for Budd-Chiari syndrome: hepatic vein thrombosis versus thrombosis of the inferior vena cava at its hepatic portion. *Hepatology* 1998; **28**: 1191-1198 [PMID: 9794901]

41 **Kika G**. Statistical study of 110 cases with primary liver carcinoma at the department of Pathology, Tokyo University School of Medicine (in Japanese). *Gann* 1927; **23**: 341

42 **Okuda H**, Yamagata H, Obata H, Iwata H, Sasaki R, Imai F, Okudaira M, Ohbu M, Okuda K. Epidemiological and clinical features of Budd-Chiari syndrome in Japan. *J Hepatol* 1995; **22**: 1-9 [PMID: 7751574 DOI: 10.1016/0168-8278(95)80252-5]

43 **Wang ZG**. Need, genesis, and future of the International Society for the Budd-Chiari syndrome. 3rd International Congress on the Budd-Chiari syndrome. Chennai, India: Programme and Abstract, 1998: 22-23

44 **Shrestha S**, Shrestha S. Bacterial peritonitis in hepatic inferior vena cava disease: a hypothesis to explain the cause of infection in high protein ascites. *Hepatol Res* 2002; **24**: 42 [PMID: 12243791]

45 **Shrestha SM**. Liver cirrhosis and hepatocellular carcinoma in hepatic vena cava disease, a liver disease caused by obstruction of inferior vena cava. *Hepatol Int* 2009; **3**: 392-402 [PMID: 19669366 DOI: 10.1007/s12072-009-9122-5]

46 **Okuda K**. Membranous obstruction of the inferior vena cava: etiology and relation to hepatocellular carcinoma. *Gastroenterology* 1982; **82**: 376-379 [PMID: 6274733]

47 **Valla DC**. Hepatic venous outflow tract obstruction etiopathogenesis: Asia versus the West. *J Gastroenterol Hepatol* 2004; **19**: S204-S211 [DOI: 10.111/j.1400-1746.2004.03642.x]

48 **Ganguli SC**, Ramzan NN, McKusick MA, Andrews JC, Phyliky RL, Kamath PS. Budd-Chiari syndrome in patients with hematological disease: a therapeutic challenge. *Hepatology* 1998; **27**: 1157-1161 [PMID: 9537458]

49 **Hirooka M**. Membranous obstruction of the hepatic portion of the inferior vena cava. Presumtive theory based on developmental abnormality. *Acta Hepatologica Japonica* 1969; **6**: 566-577 [DOI: 10.2957/kanzo.10.566]

50 **Takaishi Y**, Asada M, Kimura K, Okuda K. Aetiology of membranous obstruction of the inferior vena cava: congenital or acquired. *Gastroenterology Int* 1990; **3**: 70-80

51 **Kage M**, Arakawa M, Kojiro M, Okuda K. Histopathology of membranous obstruction of the inferior vena cava in the Budd-Chiari syndrome. *Gastroenterology* 1992; **102**: 2081-2090 [PMID: 1587428]

52 **Parker RG**. Occlusion of the hepatic veins in man. *Medicine* (Baltimore) 1959; **38**: 369-402 [PMID: 14430507]

53 **Shrestha SM**. Hepatic venous outflow obstruction in Nepal. *Trop Gastroenterol* 1996; **17**: 165-171 [PMID: 8987408]

54 **Thompson T**, Turnbull HM. Primary occlusion of the ostia of the hepatic veins. *Q J Med* 1912; **5**: 277-295

55 **Rigdon RH**. On the relation between thrombophlebitis of inferior vena cava and occlusion of hepatic veins (Endophlebitis hepatic obliterans). *Bull Johns Hopkins Hosp* 1933; **53**: 162-171

56 **Bronte-Stewart B**, Goetz RH. Budd-Chiari syndrome: high inferior vena caval obstruction demonstrated by venography. *Angiology* 1952; **3**: 167-178 [DOI: 10.1177/000331975200300203]

57 **Osler W**. Obliteration of Vena Cava Inferior, with great Stenosis of Orifices of Hepatic Veins. *J Anat Physiol* 1879; **13**: 291-304.1 [PMID: 17231259]

58 **Mann JD**, Hall IW. Obstruction of inferior vena cava. *Edinburgh Med J* 1904; **16**: 56-62

59 **Shrestha SM**, Ghimire RK, Basnyat P, Pradhan V, Poudel V. Acute on chronic phenomenon in hepatic IVC obstruction: a case report. *Trop Gastroenterol* 1999; **20**: 182-184 [PMID: 10769609]

60 **Shrestha SM**. Pleural effusion in hepatic vena cava disease. *Kathmandu Univ Med J* (KUMJ) 2007; **5**: 218-224 [PMID: 18604023]

61 **Shrestha SM**, Shrestha S, Shrestha A, Tsuda F, Endo K, Takahashi M, Okamoto H. High prevalence of hepatitis B virus infection and inferior vena cava obstruction among patients with liver cirrhosis or hepatocellular carcinoma in Nepal. *J Gastroenterol Hepatol* 2007; **22**: 1921-1928 [PMID: 17914971 DOI: 10.1111/j.1440-1746.2006.04611.x]

62 **Wright TL**, Boyer TD. Diagnosis and management of cirrhotic ascites. In: Zakim D, Boyer TD. Hepatology. New York: WB Saunders Co, 1990: 616-634

63 **Arvaniti V**, D'Amico G, Fede G, Manousou P, Tsochatzis E, Pleguezuelo M, Burroughs AK. Infections in patients with cirrhosis increase mortality four-fold and should be used in determining prognosis. *Gastroenterology* 2010; **139**: 1246-1256, 1256.e1-5 [PMID: 20558165 DOI: 10.1053/j.gastro.2010.06.019]

64 **Bras G**, Brooks SE, Watler DC. Cirrhosis of the liver in Jamaica. *J Pathol Bacteriol* 1961; **82**: 503-512 [PMID: 13872533 DOI: 10.1002/path.1700820226]

65 **Nayak NC**. Indian childhood cirrhosis. In: Ahuja MMS. Progress in Clinical Medicine, Second series. India: Arnold-Heinnemann, 1978: 304-314

66 **Pandit A**. Indian childhood cirrhosis: a reappraisal. In: Tandon BN, Nayak NC, Nundy S. Advances in Liver Diseases. Delhi: Macmillan India Ltd, 1989: 69-74

67 **Vajro P**, Hadchouel P, Hadchouel M, Bernard O, Alagille D. Incidence of cirrhosis in children with chronic hepatitis. *J Pediatr* 1990; **117**: 392-396 [PMID: 2391593 DOI: 10.1016/S0022-3476(05)81078-9]

68 **Wanless IR**. Pathogenesis of cirrhosis. *J Gastroenterol Hepatol* 2004; **19**: S369-S371 [DOI: 10.1111/j.1400-1746.2004.03705.x]

69 **Wanless IR**, Shiota K. The pathogenesis of nonalcoholic steatohepatitis and other fatty liver diseases: a four-step model including the role of lipid release and hepatic venular obstruction in the progression to cirrhosis. *Semin Liver Dis* 2004; **24**: 99-106 [PMID: 15085490 DOI: 10.1055/s-2004-823104]

70 **Yoshizawa H**. Hepatocellular carcinoma associated with hepatitis C virus infection in Japan: projection to other countries in the foreseeable future. *Oncology* 2002; **62** Suppl 1: 8-17 [PMID: 11868791 DOI: 10.1159/000048270]

71 **Shrestha SM**. Membranous obstruction of the hepatic portion of the inferior vena cava: is this an underdiagnosed entity in developing countries? *Am J Gastroenterol* 1995; **90**: 303-306 [PMID: 7847306]

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MINIREVIEWS

Hepatitis C infection in hemodialysis patients: A review

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Abstract

Hepatitis C virus (HCV)-related liver disease is a significant cause of morbidity and mortality in patients with end-stage renal disease (ESRD) who is treated with dialysis or kidney transplantation (KT). The survival rate for HCV-infected renal transplant recipients is better than that for HCV-infected hemodialysis patients on transplant waiting lists. Early diagnosis and treatment HCV infection prior to KT prevents complications post-transplantation and reduces mortality. In addition to screening for anti-HCV antibodies and detecting HCV RNA, percutaneous liver biopsy is particularly valuable for assessing the stage of liver damage in HCV-infected patients, because the stage of fibrosis is important determining optimal treatment for HCV. Studies have been demonstrated that with conventional interferon (IFN) monotherapy or pegylated IFN monotherapy are similar efficacy and safety in HCV-infected hemodialysis patients. Sustained viral responses (SVRs) with these monotherapies have ranged approximately 30% to 40%. Limited reports support the use of IFN and ribavirin combination therapy as antiviral treatment for ESRD patients or patients on hemodialysis. Ribavirin can be started at low dose and careful monitoring for side effects. Patients that show SVR after treatment are strong candidates for KT. It is also generally accepted that ESRD patients with decompensated cirrhosis and portal hypertension should be referred to the liver transplant team for consideration of combined liver-KT.

Key words: Hepatitis C virus; Hemodialysis; End-stage renal disease; Kidney transplantation; Hepatitis C treatment

Core tip: Hepatitis C virus (HCV)-related liver disease is a significant cause of morbidity and mortality in patients with end-stage renal disease who are treated with dialysis or KT. Early diagnosis and treatment of HCV infection prior kidney transplantation (KT) prevent complications after transplantation and reduces mortality. Issues with current mode HCV therapy include lack of tolerability and suboptimal response rates.

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INTRODUCTION

Liver disease related to hepatitis C virus (HCV) infection

is a significant cause of morbidity and mortality in hemodialysis (HD) patients and kidney transplant recipients. In developed countries, the prevalence of anti-HCV seropositivity among patients on maintenance HD ranges between 5% and 60%. Patients on HD are at high risk for HCV, with frequency of infection several times higher than that in non-uremic patients[1]. The spread of HCV in HD units is declining, but the prevalence of HCV in HD patients remains high[1].

Several observational studies have demonstrated a significant and independent relationship between anti-HCV seropositive status and lower survival rate in patients with end-stage renal disease (ESRD). The two major complications of HCV-related chronic liver disease are cirrhosis and hepatocellular carcinoma, and these are suspected causes of higher mortality among HCV-positive patients.

Management of HCV-positive ESRD patients is complicated. There are unknowns related to prevention of HCV spread in dialysis units, anti-viral therapy in dialysis patients, and effects of HCV infection after renal transplantation. A wide range of studies have been published on HCV in ESRD patients, but clinical evidence is lacking with respect to most of the important issues, and most existing evidence is from uncontrolled clinical trials or retrospective surveys[2-4].

EPIDEMIOLOGY OF HCV INFECTION

Serologic testing has clearly demonstrated that HCV infection is highly prevalent among ESRD patients and is a serious cause of increased morbidity and mortality in this group. Failures of HCV screening, excessive exposure to blood and blood products, nosocomial transmission of HCV in HD units, and long dialysis duration are the main determinants of increased risk of HCV infection in the HD patient group[5]. The worldwide prevalence of HCV infection among HD patients varies widely, with estimates ranging from 5% to approximately 60% depending on geographic location[6-9]. In 2002, the prevalence of HCV infection across HD centers of the United States was approximately 8%, nearly five times greater than that of the general population in that country[10,11]. In some European dialysis centers, the yearly incidence of HCV infection reportedly ranges from 0.4% to 16.0%[12]. In 2011, the Turkish Society of Nephrology documented 7.9% anti-HCV seropositivity and 1.7% HCV-RNA seropositivity among Turkish HD patients[13]. Introduction of stricter blood bank screening rules, widespread use of erythropoiesis-stimulating agents instead of blood transfusions, and stronger adherence to infection control practices in dialysis units have reduced the prevalence of HCV infection in the HD patient group[14]. For example, Spain observed a decline from 24% in 1992 to 9.2% in 2002[15]. Anti-HCV prevalence among patients on chronic HD in the United States decreased from 10.4% in 1995 to 7.8% (*i.e.,* 164632 Americans) in 2002[2]. A recent multicenter survey revealed that prevalence of anti-HCV positivity for a Belgian cohort of HD patients (n = 1710) dropped steadily from 13.5% in 1991 to 6.8% in 2000, and the same survey revealed significant drops in many other countries including France (42% to 30%), Italy (27% to 16%), and Sweden (16% to 9%)[2].

IMPACT OF HCV ON SURVIVAL

One study that involved the database of more than 13000 HD patients in the United States revealed that HCV infection was more strongly associated with all causes of mortality than with HCV-negative status[16]. Lee *et al*[17] identified HCV infection as an independent risk factor for the transition from chronic kidney disease to ESRD. A meta-analysis by Fabrizi *et al*[18] revealed that HD can negatively modify the course of HCV infection. The authors found that the estimated relative risk of liver-related mortality in anti-HCV-positive patients on HD was 1.57 times (95%CI: 1.33-1.86; $P < 0.001$) than that observed for anti-HCV-negative counterparts. The authors concluded that, in HD patients, the presence of anti-HCV antibodies is an independent risk factor for death, because of increased risk of cirrhosis and hepatocellular carcinoma. Other research has shown that kidney transplantation (KT) improves the long-term survival of ESRD patients with HCV infection[19,20]. While there is considerable evidence that HCV infection threatens the success of KT, the survival of HCV-infected renal transplant recipients is better than that for HCV-infected HD patients who are on transplant waiting lists[21]. This survival advantage may reflect systemic effects of well-functioning renal allografts that is clearing uremic toxins, and may also reflect reduced inflammatory responses and oxidative stress. HCV-related deterioration of renal transplant recipients may be linked to the immunosuppressive treatment that is required after KT. This can result in flares of HCV infection and can increase liver- and kidney-related morbidity and mortality from conditions such as cirrhosis, hepatocellular carcinoma, transplant glomerulopathy, and graft dysfunction[22].

PREVENTION OF HCV TRANSMISSION

Health care procedures related to nosocomial infections, unsafe drug injection practices, and blood transfusions are key factors in HCV transmission. In HD facilities, the most common lapses of healthcare quality are contamination of dialysis systems, inadequate disinfection and cleaning of environmental surfaces, improper contact of health care staff with equipment and patients, and mishandling of parenteral medications[23,24]. The guidelines for preventing HCV infection in HD settings recommend fundamental infection control practices, and routine screening of HD patients for HCV. Isolating-HCV-infected patients or using dedicated machines for such patients are not advocated, except as necessary during local outbreaks[12,24].

DIAGNOSIS OF HCV INFECTION IN PATIENTS WITH ESRD

Infection with HCV normally leads to increased serum alanine aminotransferase (ALT), and laboratory blood testing for ALT is used to screen for liver disease in the general population. However, this test has weak diagnostic value in ESRD patients because ALT tends to be below reference range in this patient group. The potential causes of this are vitamin B6 deficiency, presence of uremic toxins, or presence of blood components that absorb ultraviolet light[25]. To detect HCV viremia in HD patients, new thresholds for serum ALT have been proposed that are less than half (approximately 0.4 to 0.45 times) the conventional threshold[26]. Serial testing of serum ALT level might be valuable for monitoring patients on HD with known HCV infection.

Enzyme-linked immunoassays (EIAs) are commonly used to detect HCV antibodies. Third-generation EIAs for anti-HCV antibody detection have high sensitivity and specificity because these tests are based on antigens in the core, non-structural 3-4-5 proteins of the virus. Screening for anti-HCV antibodies by EIA remains a simple method, but this type of test is only meaningful for ruling out HCV infection in ESRD patients in low-prevalence settings. In the ESRD patient group, the proposed interval for HCV screening *via* antibody test is 6 to 12 mo[27].

One disadvantage of this serologic test is false-negative result, which can present challenges for distinguishing acute from chronic HCV infection[2]. In a case where HCV infection is strongly suspected but the HCV antibody EIA is negative, blood testing for HCV RNA should be done directly using polymerase chain reaction technique[28]. Detection of HCV RNA indicates HCV replication. In populations with known higher prevalence of HCV infection, a negative EIA result does not rule out HCV infection, and testing for HCV RNA is appropriate in such cases to avoid missing HCV infections[27]. When EIA reveals that an ESRD patient is anti-HCV positive, the next step is quantitative determination of viral load. This helps confirm the antibody test result and is also useful for assessing the patient's prognostic risk stratification prior to antiviral treatment[28]. There are two main reasons why blood for HCV RNA testing should be drawn prior to a dialysis procedure: (1) presence of heparin in the blood sample can lead to false-positive PCR result for HCV; and (2) a patient's HCV RNA level can decrease during the HD session (though it will return to baseline within 48 h). Adsorption of HCV to dialysis membrane, destruction of HCV particles during the HD session, and, rarely, escape of HCV into the dialysate are other reasons why a patient's HCV RNA could be falsely low[29].

In addition to detecting HCV RNA, HCV genotyping is also required to predict response to treatment and to specify the duration and dosage of treatment. HCV genotypes 1, 4, 5, and 6 are more resistant to treatment and require longer courses of therapy. One study identified HCV genotype 1b as the most prevalent subtype in patients receiving HD or continuous ambulatory peritoneal dialysis in Turkey[1]. In study of Perez *et al*[30] reported findings that HCV genotype 1a was the most prevalent subtype in patients receiving HD, with genotype 1b the next most frequent, followed by genotype 3, and other less prevalent genotypes (genotypes 2, 4, and 5). This observation may reflect differences in the epidemiology of HCV infection, viral characteristics, and host factors in ESRD patients[31].

Percutaneous liver biopsy constitutes the most reliable tool for examining the effects of HCV infection (*i.e.,* stage of liver disease) and ruling out possible other concomitant liver diseases[31]. Neither HCV RNA viral load nor liver enzymes reflect the severity of liver injury decisively[32,33]. Liver enzyme activity and quantity of HCV RNA can fluctuate during HCV infection, whereas fibrosis is progressive and largely irreversible. There is strong evidence that the stage of liver fibrosis during HCV infection predicts survival for kidney transplant candidates and for renal grafts. In addition, determining stage of fibrosis is important for planning the treatment of HCV[34]. According to one report, up to 25% of HCV-infected patients exhibit bridging fibrosis or cirrhosis on liver biopsy[31]. Presence of advanced fibrosis does not exclude a candidate from KT, but it is inevitable that such patients will develop the comorbidities and complications of portal hypertension after transplantation[35].

Although liver biopsy is reliable tool, it has significant limitations including serious bleeding events, and sampling and interpretation errors. Coagulopathy, thrombocytopenia, platelet dysfunction, anticoagulation during HD and anti-platelet therapy all pose increased bleeding risk in ESRD patients or patients on HD. In these individuals with increased bleeding risk, a transjugular or transfemoral route for liver biopsy may be recommended. Note that even when an experienced physician performs the biopsy and an experienced pathologist interprets the findings, this gold-standard technique is associated with up to 20% error in staging liver disease[34,35]. Ahmad *et al*[36] compared the results for 46 HD patients with chronic liver disease who underwent transjugular liver biopsy and 32 HD patients who had undergone percutaneous liver biopsy at the same institution. The authors found that both techniques obtained adequate specimens for histological diagnosis in all patients; however, the complications differed. There were no major complications in the transjugular liver biopsy group, whereas 12% of the patients in the percutaneous liver biopsy developed bleeding complications[36]. Some authors have reported the progression of HCV-related liver disease in HD patients who are on transplantation waiting lists. One study concluded that patients whose liver biopsy show Metavir fibrosis score 1 or 2 should undergo a repeat liver biopsy every 5 years, whereas those with Metavir score 3 should be followed more intensely, with biopsies every 3 years[14].

There are also novel non-invasive techniques for

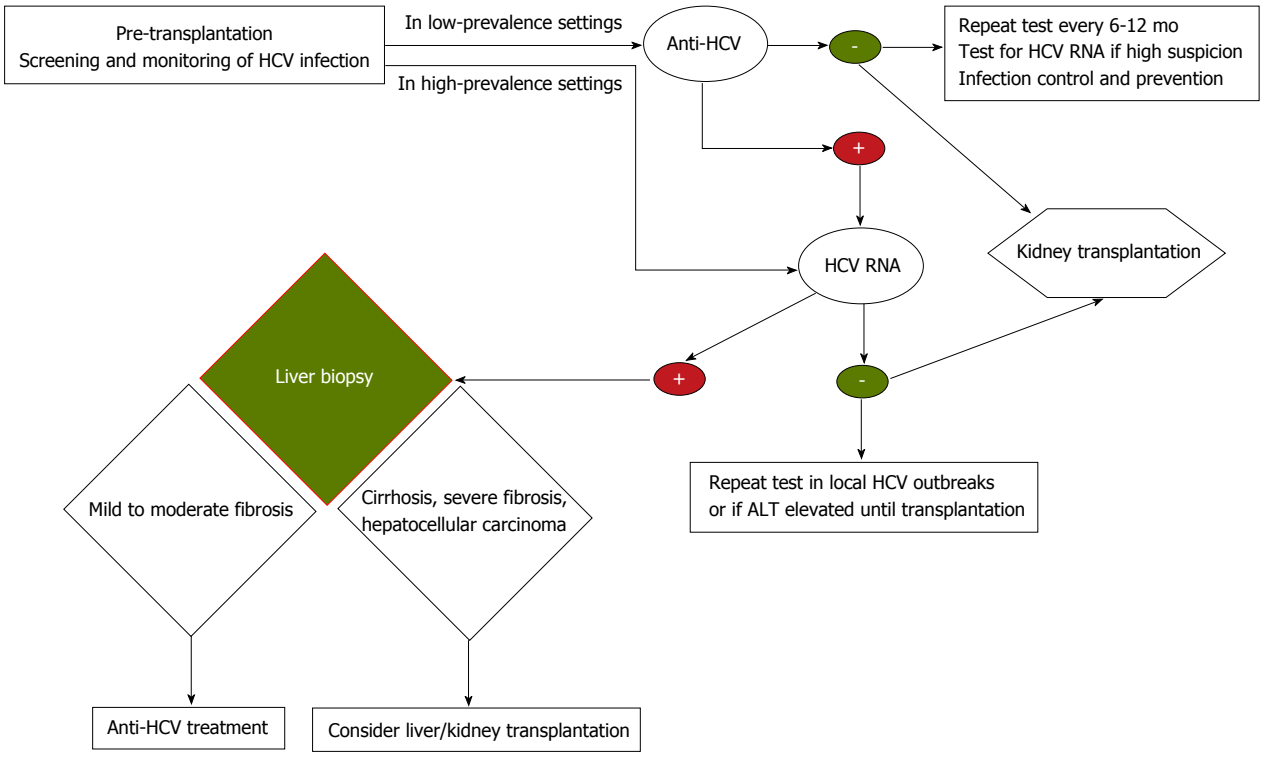

Figure 1 Decision tree for follow-up of hepatitis C virus-positive end-stage renal disease patients. HCV: Hepatitis C virus; ALT: Alanine aminotransferase.

assessing liver fibrosis in ESRD patients with HCV infection. Two of these are the aspartate aminotransferase to-platelet ratio index (APRI) and transient elastography (TE) which is performed with a Fibroscan® machine. Studies have demonstrated that APRI and TE are effective for evaluating hepatic fibrosis in HD patients with chronic HCV infection, but both methods have some limitations[37,38]. The predictive values and cut-off values for these methods as optimized for the healthy population may not be valid for ESRD patients[39]. As well, the HD procedure and the presence of uremic toxins may alter blood levels of apolipoprotein A1 and α-2 macroglobulin, which can influence Fibroscan® results[37]. An APRI score cannot precisely predict the histological severity of liver disease, particularly the intermediate fibrosis stage[38].

NATURAL COURSE OF HCV IN ESRD PATIENTS

In patients with ESRD, chronic HCV infection usually takes an insidious clinical course. Early diagnosis and identification of individuals at greater risk for fibrosis progression should be the clinician's main concern. Evidence clearly indicates that ESRD patients who undergo KT have more favorable results than those who do not, and that this also applies for ESRD patients with HCV infection. Every ESRD patient should be evaluated as a candidate for KT as soon as possible after diagnosis. This is important because during the interval between diagnosis and transplantation there is increased mortality risk due to higher incidence of hepatic and non-hepatic complications. It is essential that each dialysis center carefully follows every HCV-infected ESRD patient to determine viral load, do HCV genotyping, assess the extent of hepatic fibrosis, and establish optimal treatment strategies. A decision tree for the follow-up process is shown in Figure 1. Treating HCV infection prior to KT helps to prevent post-transplantation complications and reduce mortality[3,39-42].

There is general agreement that ESRD patients with decompensated cirrhosis and portal hypertension should be removed from the KT list and referred to the liver transplant team to be considered for combined liver-KT[43].

It is also generally accepted that every HCV-positive ESRD patient who is being evaluated for KT should undergo a liver biopsy to assess the stage of liver disease, unless there is clear radiological or clinical evidence of portal hypertension or decompensated cirrhosis[35]. Patients with Metavir fibrosis score $\leqslant$ 3 on histologic examination should be considered for antiviral treatment. Patients with successful sustained viral response (SVR) are definite candidates for KT, and those on the waiting list should be tested at least annually to confirm the durability of the SVR prior to transplantation[35,41,44]. Those who have ready living-related donors could be referred for KT immediately. There is some largely anecdotal evidence that inducing an SVR prior to KT reduces the risk of post-transplantation complications. For patients who have attained SVR, some authors consider the probability of HCV reactivation to be extremely low, however, this is controversial, especially when aggressive

immunosuppression is administered[45].

The growing number of ESRD patients with HCV-related compensated cirrhosis is a major problem. In this group, treatment is a dilemma because interferon and ribavirin carry potential risk of decompensation and serious adverse effects[3,45]. Some authors consider these patients "too healthy" for liver transplantation, and yet their limited liver reserve, poor nutritional status, and increased susceptibility to infections puts these individuals at risk for increased perioperative morbidity and mortality if they undergo KT[44]. These patients require comprehensive and detailed evaluation. Presence of portal hypertension should be carefully evaluated to decide whether KT alone or combined kidney and liver transplantation is appropriate[42,43]. It is recommended that patients with wedge hepatic vein pressure > 10 mmHg may be listed for combined kidney and liver transplantation[43]. Some transplantation centers use different criteria to make this decision, such as platelet count or presence of varices on endoscopy[44]. Making the correct treatment decision in these cases in this challenging. To improve decision-making in future, transplant centers need to report incremental experiences and long-term post-transplantation observations.

The other problematic patient group with respect to treatment decisions is ESRD patients with HCV who do not respond to antiviral treatment or who have been assessed as ineligible for antiviral treatment. As HCV replication progresses, there is greater risk of accelerated liver disease, including hepatic failure and hepatocellular carcinoma[46]. KT is not contraindicated for these individuals, but each such candidate must be informed of all difficulties (*i.e.,* those related to progression of HCV and restricted antiviral treatment) after transplantation[47]. It should also be kept in mind that extending waiting time for KT will likely result in poorer liver status for these individuals[46,47]. However, any ESRD patient with HCV who remains viremic while on a KT waiting list should be placed on hold status. These patients should be evaluated carefully and frequently to assess HCV disease status and their suitability for KT as time on the waiting list extended[44].

HCV infection after KT

The natural history of HCV infection after non-liver solid organ transplantation is still not fully understood. One study revealed that HCV-positive kidney transplant recipients had lower survival than HCV-negative patients[42]. As noted previously, KT is associated with better long-term survival for ESRD patients, even those diagnosed with HCV infection. In other words, anti-HCV positivity must not preclude HD patients from KT[48].

After KT: Progression and treatment of HCV-related liver disease

Patients with HCV infection who undergo KT can experience progression of liver disease after the operation. This has been identified as the fourth most frequent cause of mortality (reported range, 8% to 28%) in long-term survivors of KT. Increased risk of cardiovascular disease, post-transplantation diabetes mellitus, and sepsis are considered the primary causes of death after KT[49].

A patient's HCV viral load will increase in the setting of immunosuppression, which usually develops within the first months after KT[50], but there is no evidence that progression of liver disease is correlated with HCV RNA load.

Kamar *et al*[47] investigated natural course of HCV-related liver fibrosis after KT. They observed that liver biopsy samples from HCV-positive patients after KT showed progression of fibrosis in 21 patients, stable phase in 21 patients, and regression of fibrosis in 10 patients. The risk factor most strongly associated with progression of hepatic fibrosis was severity of liver disease prior to KT[47].

In HD patients with HCV who undergo KT, antiviral treatment is mandatory after renal transplantation only if the individual develops advanced fibrosis or severe cholestatic hepatitis. Both these conditions are associated with high mortality, and antiviral therapy can be life-saving[46,51]. For such patients, the decision regarding when to provide antiviral therapy should be made based on a risk-benefit assessment for each individual case[51]. The main concern about interferon (IFN) treatment is that it can trigger acute graft rejection[45,46]. The optimal approach for antiviral treatment of HCV after KT is unclear. A recent meta-analysis by Fabrizi *et al*[52] evaluated 12 clinical trials. In three of these, a combination of IFN and ribavirin was used, and the estimated rates of SVR and dropout were 18% (95%CI: 7.0%-29%) and 35% (95%CI: 20%-50%), respectively. The most frequent reason for discontinuing the combination treatment was graft dysfunction (71.7%). There is no evidence that the IFN-ribavirin combination is superior to IFN monotherapy[52]. Overall, Fabrizi *et al*[52] agree that treating these patients with anti-viral therapy before KT is safer and more effective than administering this treatment post-transplantation.

The risk for acute rejection in HCV-infected patients is higher during the first year after KT. In cases where anti-viral therapy is necessary after KT, some authors recommend waiting at least 1 year after the surgery to initiate this treatment[51]. In contrast, other research has shown that anti-viral treatment may be more effective during the first year after transplantation if the patient has stable renal function and no acute rejection occurs[53].

Renal transplant recipients with HCV who have stable renal and liver function should be carefully monitored, as should those receiving anti-viral therapy. In all cases liver function tests should be done every 3 mo and viral load should be measured every 6 mo. Liver biopsy should be repeated every 3 years[54].

After KT: Immunosuppression in patients with HCV-related liver disease

In patients with HCV who undergo KT, immunosuppressive therapy is required to prevent rejection of

the renal graft, but this may cause a more rapid and aggressive course of HCV infection[55]. In addition to the severity and duration of HCV infection, at the time of KT, the choice of immunosuppressive drug combination and doses can significantly affect the course of HCV infection after KT. There is still controversy regarding what constitutes the most appropriate immunosuppressive combination.

Induction immunosuppressive therapies for kidney transplant recipients typically contain T-cell-depleting antibodies (OKT3, anti-thymocyte globulin). Induction therapies with non-depleting antibodies (*i.e.,* antibodies that block interleukin-2) have also been debated. There are conflicting reported outcomes regarding the hazardous or beneficial effects of these induction immunosuppressive drugs on the course of HCV infection and on survival of the renal graft. Earlier studies suggested that OKT3 was not a good choice for induction[56], whereas a recent report by Roth *et al*[21] indicated that patients who received OKT3 had better liver fibrosis scores than those who received daclizumab (an agent that binds the interleukin-2 receptors of T-cells). At minimum, HCV-infected patients who have undergone KT should receive a short course of induction therapy.

Glucocorticosteroids are given in wide range of doses to prevent rejection after KT. This treatment is associated with increased HCV replication[55]; however, Luan *et al*[57] demonstrated no significant difference in mortality between patients who received steroids and those who did not. Akalin *et al*[58] pointed out that rapid discontinuation of steroid treatment was not associated with worse outcome in HCV-positive renal transplant recipients.

Cyclosporin might inhibit HCV replication through mediating a blockage of interaction between cyclophilins and non-structural protein 5B (HCV-RNA polymerase)[59,60]. Kahraman *et al*[61] investigated kidney recipients with HCV, and observed no significant differences between a group that received tacrolimus and a group that received cyclosporin with respect to viral replication and development of hepatic fibrosis.

Mycophenolate mofetil is known to have anti-viral effects in HCV patients, and acts by inducing the expression of anti-viral IFN-related genes[59,62]. In, there is no evidence of a specific destructive effect on either the renal graft or the HCV infection[55].

There are scarce data on the influence of sirolimus and everolimus [both inhibitors of the mammalian target of rapamycin (mTOR)] in renal transplant recipients with HCV. Luan *et al*[57] found that mTOR inhibitors were associated with 13% increased risk of mortality in this patient group. These drugs are not yet recommended as standard regimen for renal transplant recipients with HCV[55].

After KT: Infections

Several studies have confirmed that HCV-positive kidney recipients are at increased risk for infections of the central nervous system, respiratory system, urinary tract and bloodstream[41,63]. Research has identified a significant relationship between development of tuberculosis and presence of HCV infection in renal transplant recipients[64], but it is not clear why HCV-positive recipients are more susceptible to this infection. Immunosuppression and diabetes are two possible explanations[63].

After KT: HCV-related glomerular disease

HCV infection has been directly linked to glomerular disease in both native and transplanted kidneys[65]. The most common renal diseases associated with HCV infection are membranoproliferative glomerulonephritis with or without cryoglobulinemia and membranous glomerulonephritis[66]. Meyers *et al*[65] hypothesized that these relationships are explained by increased HCV viral load and decreased immunoglobulin synthesis in the setting of immunosuppression, and an imbalance of antigen and antibody complex status and deposition of these complexes in the allograft. Kamar *et al*[67] proposed that these diseases were explained by higher cytokine production rather than direct cytotoxic effects of HCV on kidney cells.

After KT: HCV-related new-onset diabetes mellitus

HCV-related new-onset diabetes after KT in patients with chronic HCV is an interesting and relatively frequent complication[35]. Reported prevalences of this in HCV-positive and HCV-negative kidney transplant recipients are 39.4% and 9.8% respectively[68]. Unfortunately, it has been shown that new onset of diabetes after KT impairs graft function[69]. The mechanisms proposed for HCV-related diabetes mellitus include increased insulin resistance, direct cytopathic action of HCV on beta cells, and side effects of immunosuppressive drugs[70,71].

After KT: HCV-related extrahepatic neoplasia

The role of HCV in the pathogenesis of post-transplantation hematologic malignancies is obvious[72]. Post-transplant lymphoproliferative disorder was found to be significantly more frequent in HCV-positive kidney recipients than in HCV-negative kidney recipients[73].

TREATMENT OF HCV INFECTION IN PATIENTS WITH ESRD

Carefully treating HCV and achieving SVR prior to KT should be primary goals to reduce the likelihood of HCV-related complications in the liver and other organs/systems[74]. Another reason it is important to attain SVR before KT relates to the concern that anti-viral therapy administered post-transplantation is associated with high risk of graft rejection[75].

The document entitled Improving Global Outcomes recommended that HCV-infected HD patients awaiting KT should be treated for HCV, and that attending clinicians should decide whether to treat other HCV-infected patients (*i.e.,* those not on the KT waiting list)

on case-by-case basis. However, HD patients with HCV infection rarely receive antiviral therapy[76].

Monotherapy with standard interferon or pegylated interferon

Several forms of IFN are available for therapeutic use, including α-2a, α-2b, α-n1. In ESRD patients with HCV, the recommended administration of IFN ranges from 1-6 mU as a/daily dose or up to three times weekly. A long-acting IFN α, namely pegylated IFN (pegIFN), has been used safely and effectively for more than a decade. Peg IFN α-2a administered at 135 μg weekly and peg IFN α-2b administered at 0.5-1 μg/kg are currently approved for HCV treatment and are administered weekly in stage 3-5 ESRD. Treatment duration is 24 wk for HCV genotypes 2 or -3 and 48 wk for HCV genotypes 1-4[77,78].

Three recently published meta-analyses have indicated that SVR, side effects and withdrawal rates in patients with ESRD vary according to treatment with IFN and pegIFN.

The meta-analysis by Fabrizi *et al*[79] evaluated results from 645 patients the overall SVR rate was 40%; in the subset with HCV genotype 1, the SVR rate was 33% and dropout rates were 19% in the group that received IFN and 27% in the group that received pegIFN. A typical flu-like syndrome was the most common side effect. This occurred in 41% of patients and required withdrawal of anti-viral treatment in 11%. However, the meta-analysis by Fabrizi *et al*[79] was criticized because the studies examined were somewhat heterogeneous with regard to viral response and dropout rates.

A meta-analysis by Gordon *et al*[80] in 2008, involved 546 chronic HD patients with HCV infection who were either treated with IFN or pegIFN, with or without ribavirin. Only 49 individuals received pegIFN and ribavirin. The overall SVR rates were 41% for the IFN group (95%CI: 33%-49%) and 37% for the pegIFN group (95%CI: 9%-77%). The frequencies of treatment discontinuation were 26% for the IFN group (95%CI: 20%-34%) and 28% for the pegIFN group (95%CI: 12%-53%). The main side effects were fatigue/weakness and loss of appetite. The authors also found that higher dose of IFN, lower HCV RNA load prior to treatment, early stage of hepatic fibrosis, and HCV genotype other than genotype 1 were associated with higher SVR rates[80].

A more recent meta-analysis evaluated data from 770 HD patients with chronic HCV infection, 491 of whom received IFN-alfa2a or IFN-alfa2b and 279 of whom received pegIFN-alfa2a or PegIFN-alfa2b. The corresponding SVR rates for these two groups were 39.1% (95%CI: 32.1%-46.1%) and 39.3% (95%CI: 26.5%-52.1%), and the corresponding dropout rates were 22.6% (95%CI: 10.4%-34.8%) and 29.7% (95%CI: 21.7%-37.7%). Age younger than 40 years was significantly associated with SVR (OR = 2.17; 95%CI: 1.03-4.50)[81].

Although the above three meta-analyses suggest that conventional IFN treatment and pegIFN therapy have similar efficacy and safety, many studies have shown that pegIFN is superior[27]. For example, one study revealed that patients with renal dysfunction who were treated with pegIFN had a higher HCV eradication rate than HCV patients with normal kidney function who received this treatment. This can be attributed to decreased renal clearance of pegIFN in the setting of ESRD. In practice once weekly dosing of pegIFN is more convenient for the patients with renal dysfunction[82].

Combination therapies: Interferon or pegIFN with ribavirin

The combination of pegIFN and ribavirin is considered the gold standard therapy for patients with chronic HCV who have normal renal function[77]. Some physicians are reluctant to use ribavirin in patients with ESRD or in those who are on HD due to fear of hemolytic anemia which can be exacerbated in the presence of kidney dysfunction. Because ribavirin is filtered by the kidneys, its clearance is impaired in patients with ESRD, and this agent is not removed by dialysis. Despite the fact that ribavirin is contraindicated in the setting of renal failure, this drug can be used at markedly reduced daily doses and with careful monitoring for anemia. Patients can be started on a low dose of ribavirin, and doses can be increased gradually as long as side effects are manageable[82-84].

Only a few reports support the combined use of pegIFN and ribavirin in ESRD patients or patients on HD (Table 1). Rendina *et al*[88] published the largest series to date on the combined use of pegIFN α-2a (135 μg/wk) plus ribavirin (200 mg daily to every other day) for 48 wk in 35 HD patients. They observed an SVR rate of 97% (34 of the/35 patients) and a dropout rate of 14%. Only one patient developed severe anemia and had to be weaned off treatment.

The dose of ribavirin should be adjusted based on target plasma level which has been identified as 10-15 mcmol/L in patients with normal kidney function. For patients with ESRD, the average dose of ribavirin can be 200 mg daily, but some individuals can only tolerate 200 mg every other day[46]. Assays for monitoring plasma ribavirin levels are not routinely available. Even when therapeutic ribavirin levels are maintained the potential for anemia in HD patients cannot be ruled out. Recombinant human erythropoietin or blood transfusions can be while maintaining the desired ribavirin dosage, for these patients, as these measures can correct anemia and improve quality of life during treatment[83].

Direct-acting anti-viral agents

Direct-acting anti-viral agents (DAA) have yielded exciting results in ESRD patients. When combined with IFN and ribavirin, DAA increase SVR rates in patients with intact kidney function. However, anemia is an important potential side effect of DAA even in patients with normal glomerular filtration rate. Further information and experience are needed with respect to using DAA-based therapy in patients with ESRD[89]. More aggressive

Table 1 Results from trials: Interferon-ribavirin combined treatments in patients with end-stage renal disease and hepatitis C virus infection

Ref.	Year	No. of patients	Proportion with HCV genotype 1 (%)	Treatment	SVR (%)	Dropout (%)
Tan *et al*[85]	2001	5	NA	IFN-3.0 MU/d to 3 times/wk + RBV 200 mg/d, 3 times/wk for 40 wk	NA	40
Mousa *et al*[86]	2004	20	60-66	IFN-3.0 MU/d to 3 times/wk + RBV 200 mg/d, 3 times/wk		
				For 24 wk	66	0
				For 48 wk	55	0
Bruchfeld *et al*[87]	2006	6	66	PegIFN - 50 μg/wk + RBV 200-400 mg/d for 24-48 wk (depending on genotype)	50	50
Rendina *et al*[88]	2007	35	45.7	PegIFN-α2a 135 μg/wk + RBV 200 mg/d for 24-48 wk (depending on genotype)	97	15

IFN: İnterferon; NA: Not available; PegIFN: Pegylated IFN; RBV: Ribavirin; SVR: Sustained virological response; HCV: Hepatitis C virus.

therapy maybe considered for HD patients who are eligible for KT, as HCV eradication prior to transplantation can improve outcomes[90].

Two new HCV drugs were approved in 2011: telaprevir and boceprevir. Both these are first-wave, first-generation NS3-4A protease inhibitors. Two other drug were approved in 2013/2014: simeprevir, a second-wave, first-generation NS3-4A protease inhibitor, and sofosbuvir, a nucleotide analogue inhibitor of viral polymerase[91].

Triple therapy for HCV infection currently may have several opportunities including a better therapeutic schedule in patients with renal failure, which apparently do not require dose adjustments to the kidney function, yet the detailed data regarding the new drugs in these patients are not available[92].

Sofosbuvir and simeprevir are not recommended for patients with ESRD or patients who require HD. While no dose reduction is necessary when sofosbuvir or simeprevir are administered to patients mild to moderate HCV infection, a lower dose is needed when this drug is administered to patients with severe HCV infection. Renal insufficiency has no impact on the pharmacokinetic profile of asunaprevir[93].

The development of IFN-free or IFN-sparing regimens represents a breakthrough in the history of anti-HCV treatment. It is expected that treatment scenarios for chronic HCV patients will change radically in the next few years, as safe and potent therapies become more accessible. This will simplify the mangement of these cases, and will open possibilities to include patient populations for which pegIFN is currently contraindicated[94].

In conclusion, HCV infection remains a major health problem that can cause substantial liver-related morbidity and mortality in patients with ESRD. Various forms of IFN with or without ribavirin can be used to treat ESRD patients with HCV infection prior to KT; however, only approximately one-third of these patients will achieve SVR. After an HCV-infected patient has undergone KT, IFN-based treatments are generally not recommended owing to the high risk of graft rejection. Recently introduced IFN- free treatment options are promising, but data are lacking regarding their use in HD patients with HCV infection. Well-designed prospective studies are needed to evaluate the efficacy and safety of the new IFN- free regimes in this patient group.

REFERENCES

1 **Selcuk H**, Kanbay M, Korkmaz M, Gur G, Akcay A, Arslan H, Ozdemir N, Yilmaz U, Boyacioglu S. Distribution of HCV genotypes in patients with end-stage renal disease according to type of dialysis treatment. *Dig Dis Sci* 2006; **51**: 1420-1425 [PMID: 16868830 DOI: 10.1007/s10620-005-9025-9]

2 **Mangia A**, Burra P, Ciancio A, Fagiuoli S, Guido M, Picciotto A, Fabrizi F. Hepatitis C infection in patients with chronic kidney disease. *Int J Artif Organs* 2008; **31**: 15-33 [PMID: 18286451]

3 **Perico N**, Cattaneo D, Bikbov B, Remuzzi G. Hepatitis C infection and chronic renal diseases. *Clin J Am Soc Nephrol* 2009; **4**: 207-220 [PMID: 19129320 DOI: 10.2215/CJN.03710708]

4 **Aoufi Rabih S**, García Agudo R. Management of HCV infection in chronic kidney disease. *Nefrologia* 2011; **31**: 260-267 [PMID: 21407274 DOI: 10.3265/Nefrologia.pre2011.Jan.10768]

5 **Goodkin DA**, Bragg-Gresham JL, Koenig KG, Wolfe RA, Akiba T, Andreucci VE, Saito A, Rayner HC, Kurokawa K, Port FK, Held PJ, Young EW. Association of comorbid conditions and mortality in hemodialysis patients in Europe, Japan, and the United States: the Dialysis Outcomes and Practice Patterns Study (DOPPS). *J Am Soc Nephrol* 2003; **14**: 3270-3277 [PMID: 14638926 DOI: 10.1097/01.ASN.0000100127.54107.57]

6 **Fissell RB**, Bragg-Gresham JL, Woods JD, Jadoul M, Gillespie B, Hedderwick SA, Rayner HC, Greenwood RN, Akiba T, Young EW. Patterns of hepatitis C prevalence and seroconversion in hemodialysis units from three continents: the DOPPS. *Kidney Int* 2004; **65**: 2335-2342 [PMID: 15149347 DOI: 10.1111/j.1523-1755.2004.00649.x]

7 **Di Napoli A**, Pezzotti P, Di Lallo D, Petrosillo N, Trivelloni C, Di Giulio S. Epidemiology of hepatitis C virus among long-term dialysis patients: a 9-year study in an Italian region. *Am J Kidney Dis* 2006; **48**: 629-637 [PMID: 16997059 DOI: 10.1053/j.ajkd.2006.07.004]

8 **Sun J**, Yu R, Zhu B, Wu J, Larsen S, Zhao W. Hepatitis C infection and related factors in hemodialysis patients in china: systematic review and meta-analysis. *Ren Fail* 2009; **31**: 610-620 [PMID: 19839861 DOI: 10.1080/08860220903003446]

9 **Alavian SM**, Kabir A, Ahmadi AB, Lankarani KB, Shahbabaie MA, Ahmadzad-Asl M. Hepatitis C infection in hemodialysis patients in Iran: a systematic review. *Hemodial Int* 2010; **14**: 253-262 [PMID: 20491973 DOI: 10.1111/j.1542-4758.2010.00437.x]

10 **Finelli L**, Miller JT, Tokars JI, Alter MJ, Arduino MJ. National

surveillance of dialysis-associated diseases in the United States, 2002. *Semin Dial* 2002; **18**: 52-61 [PMID: 15663766 DOI: 10.1111/j.1525-139X.2005.18108.x]

11 **Patel PR**, Thompson ND, Kallen AJ, Arduino MJ. Epidemiology, surveillance, and prevention of hepatitis C virus infections in hemodialysis patients. *Am J Kidney Dis* 2010; **56**: 371-378 [PMID: 20570422 DOI: 10.1053/j.ajkd.2010.01.025]

12 **Zampieron A**, Jayasekera H, Elseviers M, Lindley E, DeVos JY, Visser R, Harrington M. European study on epidemiology and management of hepatitis C virus (HCV) infection in the haemodialysis population. Part 3: prevalence and incidence. *EDTNA ERCA J* 2006; **32**: 42-44 [PMID: 16700168 DOI: 10.1111/j.1755-6686.2006.tb00445.x]

13 **Süleymanlar G,** Altıparmak MR, Seyahi N, Trabulus S. National hemodialysis, transplantation and nephrology registry report of Turkey. İstanbul: Turkish Society of Nephrology, 2012

14 **Gordon CE**, Balk EM, Becker BN, Crooks PA, Jaber BL, Johnson CA, Michael MA, Pereira BJ, Uhlig K, Levin A. KDOQI US commentary on the KDIGO clinical practice guideline for the prevention, diagnosis, evaluation, and treatment of hepatitis C in CKD. *Am J Kidney Dis* 2008; **52**: 811-825 [PMID: 18971009 DOI: 10.1053/j.ajkd.2008.08.005]

15 **Espinosa M**, Martn-Malo A, Ojeda R, Santamara R, Soriano S, Aguera M, Aljama P. Marked reduction in the prevalence of hepatitis C virus infection in hemodialysis patients: causes and consequences. *Am J Kidney Dis* 2004; **43**: 685-689 [PMID: 15042545 DOI: 10.1053/j.ajkd.2003.12.030]

16 **Kalantar-Zadeh K**, Kilpatrick RD, McAllister CJ, Miller LG, Daar ES, Gjertson DW, Kopple JD, Greenland S. Hepatitis C virus and death risk in hemodialysis patients. *J Am Soc Nephrol* 2007; **18**: 1584-1593 [PMID: 17429053 DOI: 10.1681/ASN.2006070736]

17 **Lee JJ**, Lin MY, Chang JS, Hung CC, Chang JM, Chen HC, Yu ML, Hwang SJ. Hepatitis C virus infection increases risk of developing end-stage renal disease using competing risk analysis. *PLoS One* 2014; **9**: e100790 [PMID: 24971499 DOI: 10.1371/journal.pone.0100790]

18 **Fabrizi F**, Martin P, Dixit V, Bunnapradist S, Dulai G. Meta-analysis: Effect of hepatitis C virus infection on mortality in dialysis. *Aliment Pharmacol Ther* 2004; **20**: 1271-1277 [PMID: 15606388 DOI: 10.1111/j.1365-2036.2004.02290.x]

19 **Sezer S**, Ozdemir FN, Akcay A, Arat Z, Boyacioglu S, Haberal M. Renal transplantation offers a better survival in HCV-infected ESRD patients. *Clin Transplant* 2004; **18**: 619-623 [PMID: 15344970 DOI: 10.1111/j.1399-0012.2004.00252.x]

20 **Ruhı Ç**, Süleymanlar İ, Koçak H, Yilmaz VT, Çolak D, Dınçkan A, Gürkan A, Ersoy F, Yakupoğlu G, Süleymanlar G. The impact of hepatitis C virus infection on long-term outcome in renal transplant patients. *Turk J Gastroenterol* 2011; **22**: 165-170 [PMID: 21796553]

21 **Roth D**, Gaynor JJ, Reddy KR, Ciancio G, Sageshima J, Kupin W, Guerra G, Chen L, Burke GW. Effect of kidney transplantation on outcomes among patients with hepatitis C. *J Am Soc Nephrol* 2011; **22**: 1152-1160 [PMID: 21546575 DOI: 10.1681/ASN.2010060668]

22 **Ingsathit A**, Kamanamool N, Thakkinstian A, Sumethkul V. Survival advantage of kidney transplantation over dialysis in patients with hepatitis C: a systematic review and meta-analysis. *Transplantation* 2013; **95**: 943-948 [PMID: 23425817 DOI: 10.1097/TP.0b013e3182848de2]

23 Recommendations for preventing transmission of infections among chronic hemodialysis patients. *MMWR Recomm Rep* 2001; **50**: 1-43 [PMID: 11349873]

24 **Bianco A**, Bova F, Nobile CG, Pileggi C, Pavia M. Healthcare workers and prevention of hepatitis C virus transmission: exploring knowledge, attitudes and evidence-based practices in hemodialysis units in Italy. *BMC Infect Dis* 2013; **13**: 76 [PMID: 23391009 DOI: 10.1186/1471-2334-13-76]

25 **Tang S**, Lai KN. Chronic viral hepatitis in hemodialysis patients. *Hemodial Int* 2005; **9**: 169-179 [PMID: 16191066 DOI: 10.1111/j.1492-7535.2005.01129.x]

26 **Lopes EP**, Gouveia EC, Albuquerque AC, Sette LH, Mello LA, Moreira RC, Coelho MR. Determination of the cut-off value of serum alanine aminotransferase in patients undergoing hemodialysis, to identify biochemical activity in patients with hepatitis C viremia. *J Clin Virol* 2006; **35**: 298-302 [PMID: 16290052 DOI: 10.1016/j.jcv.2005.09.010]

27 **Liu CH**, Kao JH. Treatment of hepatitis C virus infection in patients with end-stage renal disease. *J Gastroenterol Hepatol* 2011; **26**: 228-239 [PMID: 21261711 DOI: 10.1111/j.1440-1746.2010.06488.x]

28 **European Association for the Study of the Liver**. EASL Clinical Practice Guidelines: management of hepatitis C virus infection. *J Hepatol* 2011; **55**: 245-264 [PMID: 21371579 DOI: 10.1016/j.jhep.2011.02.023]

29 **Kaiser T**, Damerow HC, Tenckhoff S, Finger A, Böttcher I, Hafer C, Schwarz A, Lüth JB, Schmidt Gürtler H, Colucci G, Manns MP, Wedemeyer H, Tillmann HL. Kinetics of hepatitis C viral RNA and HCV-antigen during dialysis sessions: evidence for differential viral load reduction on dialysis. *J Med Virol* 2008; **80**: 1195-1201 [PMID: 18461613 DOI: 10.1002/jmv.21190]

30 **Perez RM**, Ferraz ML, Figueiredo MS, Contado D, Koide S, Ferreira AP, Cendoroglo Neto M, Medina Pestana JO, Silva AE. Unexpected distribution of hepatitis C virus genotypes in patients on hemodialysis and kidney transplant recipients. *J Med Virol* 2003; **69**: 489-494 [PMID: 12601756 DOI: 10.1002/jmv.10336]

31 **Martin P**, Carter D, Fabrizi F, Dixit V, Conrad AJ, Artinian L, Peacock V, Han S, Wilkinson A, Lassman CR, Danovitch G. Histopathological features of hepatitis C in renal transplant candidates [see comment]. *Transplantation* 2000; **69**: 1479-1484 [PMID: 10798774 DOI: 10.1097/00007890-200004150-00045]

32 **Boyacioğlu S**, Gür G, Yilmaz U, Korkmaz M, Demirhan B, Bilezikçi B, Ozdemir N. Investigation of possible clinical and laboratory predictors of liver fibrosis in hemodialysis patients infected with hepatitis C virus. *Transplant Proc* 2004; **36**: 50-52 [PMID: 15013298 DOI: 10.1016/j.transproceed.2003.11.066]

33 **Canbakan M**, Senturk H, Canbakan B, Toptas T, Tabak O, Ozaras R, Tabak F, Balcı H, Sut N, Ozbay G. Validation of biochemical markers for the prediction of liver fibrosis and necroinflammatory activity in hemodialysis patients with chronic hepatitis C. *Nephron Clin Pract* 2011; **117**: c289-c295 [PMID: 20847572 DOI: 10.1159/000320751]

34 **Gürsoy M**, Bilezikci B, Colak T, Köksal R, Demirhan B, Karavelioğlu D, Boyacioğlu S, Bilgin N, Arslan G. Histologic outcome of hepatitis C virus infection in renal transplant recipients and the effect of pretransplantation interferon treatment. *Transplant Proc* 2000; **32**: 558-560 [PMID: 10812112 DOI: 10.1016/S0041-1345(00)00889-7]

35 **Tang IY**, Walzer N, Aggarwal N, Tzvetanov I, Cotler S, Benedetti E. Management of the kidney transplant patient with chronic hepatitis C infection. *Int J Nephrol* 2011; **2011**: 245823 [PMID: 21603155 DOI: 10.4061/2011/245823]

36 **Ahmad A**, Hasan F, Abdeen S, Sheikh M, Kodaj J, Nampoory MR, Johny KV, Asker H, Siddique I, Thalib L, Al-Nakib B. Transjugular liver biopsy in patients with end-stage renal disease. *J Vasc Interv Radiol* 2004; **15**: 257-260 [PMID: 15028810]

37 **Liu CH**, Liang CC, Huang KW, Liu CJ, Chen SI, Lin JW, Hung PH, Tsai HB, Lai MY, Chen PJ, Chen JH, Chen DS, Kao JH. Transient elastography to assess hepatic fibrosis in hemodialysis chronic hepatitis C patients. *Clin J Am Soc Nephrol* 2011; **6**: 1057-1065 [PMID: 21393486 DOI: 10.2215/CJN.04320510]

38 **Liu CH**, Liang CC, Liu CJ, Hsu SJ, Lin JW, Chen SI, Hung PH, Tsai HB, Lai MY, Chen PJ, Chen JH, Chen DS, Kao JH. The ratio of aminotransferase to platelets is a useful index for predicting hepatic fibrosis in hemodialysis patients with chronic hepatitis C. *Kidney Int* 2010; **78**: 103-109 [PMID: 20357753 DOI: 10.1038/ki.2010.74]

39 **Kellner P**, Anadol E, Hüneburg R, Hundt F, Bös D, Klein B, Woitas RP, Spengler U, Sauerbruch T, Trebicka J. The effect of hemodialysis on liver stiffness measurement: a single-center series. *Eur J Gastroenterol Hepatol* 2013; **25**: 368-372 [PMID: 23114792 DOI: 10.1097/MEG.0b013e32835ad180]

40 **Botero RC**. Should patients with chronic hepatitis C infection

be transplanted? *Transplant Proc* 2004; **36**: 1449-1454 [PMID: 15251356 DOI: 10.1016/j.transproceed.2004.06.003]
41 **Morales JM**, Bloom R, Roth D. Kidney transplantation in the patient with hepatitis C virus infection. *Contrib Nephrol* 2012; **176**: 77-86 [PMID: 22310783 DOI: 10.1159/000332385]
42 **Carbone M**, Cockwell P, Neuberger J. Hepatitis C and kidney transplantation. *Int J Nephrol* 2011; **2011**: 593291 [PMID: 21755059 DOI: 10.4061/2011/593291]
43 **Eason JD**, Gonwa TA, Davis CL, Sung RS, Gerber D, Bloom RD. Proceedings of Consensus Conference on Simultaneous Liver Kidney Transplantation (SLK). *Am J Transplant* 2008; **8**: 2243-2251 [PMID: 18808402 DOI: 10.1111/j.1600-6143.2008.02416.x]
44 **Roth D**, Bloom R. Selection and management of hepatitis C virus-infected patients for the kidney transplant waiting list. *Contrib Nephrol* 2012; **176**: 66-76 [PMID: 22310782 DOI: 10.1159/000333774]
45 **Kim E**, Ko HH, Yoshida EM. Treatment issues surrounding hepatitis C in renal transplantation: a review. *Ann Hepatol* 2011; **10**: 5-14 [PMID: 21301003]
46 **Terrault NA**, Adey DB. The kidney transplant recipient with hepatitis C infection: pre- and posttransplantation treatment. *Clin J Am Soc Nephrol* 2007; **2**: 563-575 [PMID: 17699464 DOI: 10.2215/CJN.02930806]
47 **Kamar N**, Rostaing L, Selves J, Sandres-Saune K, Alric L, Durand D, Izopet J. Natural history of hepatitis C virus-related liver fibrosis after renal transplantation. *Am J Transplant* 2005; **5**: 1704-1712 [PMID: 15943629 DOI: 10.1111/j.1600-6143.2005.00918.x]
48 **Morales JM**, Aguado JM. Hepatitis C and renal transplantation. *Curr Opin Organ Transplant* 2012; **17**: 609-615 [PMID: 23111646 DOI: 10.1097/MOT.0b013e32835a2bac]
49 **Pereira BJ**, Levey AS. Hepatitis C virus infection in dialysis and renal transplantation. *Kidney Int* 1997; **51**: 981-999 [PMID: 9083262 DOI: 10.1038/ki.1997.139]
50 **Izopet J**, Rostaing L, Sandres K, Cisterne JM, Pasquier C, Rumeau JL, Duffaut M, Durand D, Puel J. Longitudinal analysis of hepatitis C virus replication and liver fibrosis progression in renal transplant recipients. *J Infect Dis* 2000; **181**: 852-858 [PMID: 10720504 DOI: 10.1086/315355]
51 **Du LY**, Tang H. Treatment of HCV patients before and after renal transplantation. *Hepat Mon* 2011; **11**: 880-886 [PMID: 22308151 DOI: 10.5812/kowsar.1735143X.712]
52 **Fabrizi F**, Lunghi G, Dixit V, Martin P. Meta-analysis: anti-viral therapy of hepatitis C virus-related liver disease in renal transplant patients. *Aliment Pharmacol Ther* 2006; **24**: 1413-1422 [PMID: 17081162 DOI: 10.1111/j.1365-2036.2006.03151.x]
53 **Kamar N**, Sandres-Saune K, Selves J, Ribes D, Cointault O, Durand D, Izopet J, Rostaing L. Long-term ribavirin therapy in hepatitis C virus-positive renal transplant patients: effects on renal function and liver histology. *Am J Kidney Dis* 2003; **42**: 184-192 [PMID: 12830471 DOI: 10.1016/S0272-6386(03)00422-0]
54 **Sharma RK**, Bansal SB, Gupta A, Gulati S, Kumar A, Prasad N. Chronic hepatitis C virus infection in renal transplant: treatment and outcome. *Clin Transplant* 2006; **20**: 677-683 [PMID: 17100715 DOI: 10.1111/j.1399-0012.2006.00534.x]
55 **Manuel O**, Baid-Agrawal S, Moradpour D, Pascual M. Immunosuppression in hepatitis C virus-infected patients after kidney transplantation. *Contrib Nephrol* 2012; **176**: 97-107 [PMID: 22310785 DOI: 10.1159/000332387]
56 **Rosen HR**, Shackleton CR, Higa L, Gralnek IM, Farmer DA, McDiarmid SV, Holt C, Lewin KJ, Busuttil RW, Martin P. Use of OKT3 is associated with early and severe recurrence of hepatitis C after liver transplantation. *Am J Gastroenterol* 1997; **92**: 1453-1457 [PMID: 9317061]
57 **Luan FL**, Schaubel DE, Zhang H, Jia X, Pelletier SJ, Port FK, Magee JC, Sung RS. Impact of immunosuppressive regimen on survival of kidney transplant recipients with hepatitis C. *Transplantation* 2008; **85**: 1601-1606 [PMID: 18551066 DOI: 10.1097/TP.0b013e3181722f3a]
58 **Akalin E**, Murphy B, Sehgal V, Ames S, Daly L, Bromberg JS. Rapid steroid withdrawal in hepatitis C virus-positive kidney transplant recipients. *Clin Transplant* 2004; **18**: 384-389 [PMID: 15233814 DOI: 10.1111/j.1399-0012.2004.00177.x]
59 **Hsu SH**, Yeh ML, Wang SN. New insights in recurrent HCV infection after liver transplantation. *Clin Dev Immunol* 2013; **2013**: 890517 [PMID: 23710205 DOI: 10.1155/2013/890517]
60 **Nakagawa M**, Sakamoto N, Tanabe Y, Koyama T, Itsui Y, Takeda Y, Chen CH, Kakinuma S, Oooka S, Maekawa S, Enomoto N, Watanabe M. Suppression of hepatitis C virus replication by cyclosporin a is mediated by blockade of cyclophilins. *Gastroenterology* 2005; **129**: 1031-1041 [PMID: 16143140 DOI: 10.1053/j.gastro.2005.06.031]
61 **Kahraman A**, Witzke O, Scherag A, Pütter C, Miller M, Dechêne A, Ross SR, Gerken G, Hilgard P. Impact of immunosuppressive therapy on hepatitis C infection after renal transplantation. *Clin Nephrol* 2011; **75**: 16-25 [PMID: 21176747]
62 **Pan Q**, de Ruiter PE, Metselaar HJ, Kwekkeboom J, de Jonge J, Tilanus HW, Janssen HL, van der Laan LJ. Mycophenolic acid augments interferon-stimulated gene expression and inhibits hepatitis C Virus infection in vitro and in vivo. *Hepatology* 2012; **55**: 1673-1683 [PMID: 22213147 DOI: 10.1002/hep.25562]
63 **López-Medrano F**, Fernández-Ruiz M, Morales JM, San-Juan R, Cervera C, Carratalá J, Torre-Cisneros J, Gavaldá J, Muñoz P, Len O, Martín-Dávila P, Ramos A, Montejo M, Lumbreras C, Moreno A, Aguado JM. Impact of hepatitis C virus infection on the risk of infectious complications after kidney transplantation: data from the RESITRA/REIPI cohort. *Transplantation* 2011; **92**: 543-549 [PMID: 21869745 DOI: 10.1097/TP.0b013e318225dbae]
64 **Torres J**, Aguado JM, San Juan R, Andrés A, Sierra P, López-Medrano F, Morales JM. Hepatitis C virus, an important risk factor for tuberculosis in immunocompromised: experience with kidney transplantation. *Transpl Int* 2008; **21**: 873-878 [PMID: 18482173 DOI: 10.1111/j.1432-2277.2008.00694.x]
65 **Meyers CM**, Seeff LB, Stehman-Breen CO, Hoofnagle JH. Hepatitis C and renal disease: an update. *Am J Kidney Dis* 2003; **42**: 631-657 [PMID: 14520615 DOI: 10.1016/S0272-6386(03)00828-X]
66 **Sandri AM**, Elewa U, Poterucha JJ, Fervenza FC. Treatment of hepatitis C-mediated glomerular disease. *Nephron Clin Pract* 2011; **119**: c121-129; discussion c129-130 [PMID: 21757949 DOI: 10.1159/000325220]
67 **Kamar N**, Rostaing L, Boulestin A, Sandres K, Dubois M, Ribes D, Modesto A, Durand D, Izopet J. Evolution of hepatitis C virus quasispecies in renal transplant patients with de novo glomerulonephritis. *J Med Virol* 2003; **69**: 482-488 [PMID: 12601755 DOI: 10.1002/jmv.10335]
68 **Fabrizi F**, Martin P, Dixit V, Bunnapradist S, Kanwal F, Dulai G. Post-transplant diabetes mellitus and HCV seropositive status after renal transplantation: meta-analysis of clinical studies. *Am J Transplant* 2005; **5**: 2433-2440 [PMID: 16162192 DOI: 10.1111/j.1600-6143.2005.01040.x]
69 **Bloom RD**, Lake JR. Emerging issues in hepatitis C virus-positive liver and kidney transplant recipients. *Am J Transplant* 2006; **6**: 2232-2237 [PMID: 16869798 DOI: 10.1111/j.1600-6143.2006.01457.x]
70 **Bloom RD**, Rao V, Weng F, Grossman RA, Cohen D, Mange KC. Association of hepatitis C with posttransplant diabetes in renal transplant patients on tacrolimus. *J Am Soc Nephrol* 2002; **13**: 1374-1380 [PMID: 11961026 DOI: 10.1097/01.ASN.0000012382.97168.E0]
71 **Kasiske BL**, Snyder JJ, Gilbertson D, Matas AJ. Diabetes mellitus after kidney transplantation in the United States. *Am J Transplant* 2003; **3**: 178-185 [PMID: 12603213 DOI: 10.1034/j.1600-6143.2003.00010.x]
72 **Zignego AL**, Giannini C, Gragnani L. HCV and lymphoproliferation. *Clin Dev Immunol* 2012; **2012**: 980942 [PMID: 22852020 DOI: 10.1155/2012/980942]
73 **Burra P**, Buda A, Livi U, Rigotti P, Zanus G, Calabrese F, Caforio A, Menin C, Canova D, Farinati F, Luciana Aversa SM. Occurrence of post-transplant lymphoproliferative disorders among over thousand adult recipients: any role for hepatitis C infection? *Eur J Gastroenterol Hepatol* 2006; **18**: 1065-1070 [PMID: 16957512 DOI: 10.1097/01.meg.0000231752.50587.ae]

74 **Esforzado N**, Campistol JM. Treatment of hepatitis C in dialysis patients. *Contrib Nephrol* 2012; **176**: 54-65 [PMID: 22310781 DOI: 10.1159/000332383]

75 **Rostaing L**, Weclawiak H, Izopet J, Kamar N. Treatment of hepatitis C virus infection after kidney transplantation. *Contrib Nephrol* 2012; **176**: 87-96 [PMID: 22310784 DOI: 10.1159/000333775]

76 **Goodkin DA**, Bieber B, Gillespie B, Robinson BM, Jadoul M. Hepatitis C infection is very rarely treated among hemodialysis patients. *Am J Nephrol* 2013; **38**: 405-412 [PMID: 24192505 DOI: 10.1159/000355615]

77 **Weclawiak H**, Kamar N, Ould-Mohamed A, Cardeau-Desangles I, Izopet J, Rostaing L. Treatment of chronic hepatitis C virus infection in dialysis patients: an update. *Hepat Res Treat* 2010; **2010**: 267412 [PMID: 21152180 DOI: 10.1155/2010/267412]

78 **Ghany MG**, Strader DB, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C: an update. *Hepatology* 2009; **49**: 1335-1374 [PMID: 19330875 DOI: 10.1002/hep.22759]

79 **Fabrizi F**, Ganeshan SV, Lunghi G, Messa P, Martin P. Antiviral therapy of hepatitis C in chronic kidney diseases: meta-analysis of controlled clinical trials. *J Viral Hepat* 2008; **15**: 600-606 [PMID: 18444984 DOI: 10.1111/j.1365-2893.2008.00990.x]

80 **Gordon CE**, Uhlig K, Lau J, Schmid CH, Levey AS, Wong JB. Interferon treatment in hemodialysis patients with chronic hepatitis C virus infection: a systematic review of the literature and meta-analysis of treatment efficacy and harms. *Am J Kidney Dis* 2008; **51**: 263-277 [PMID: 18215704 DOI: 10.1053/j.ajkd.2007.11.003]

81 **Alavian SM**, Tabatabaei SV. Meta-analysis of factors associated with sustained viral response in patients on hemodialysis treated with standard or pegylated interferon for hepatitis C infection. *Iran J Kidney Dis* 2010; **4**: 181-194 [PMID: 20622305]

82 **Okoh EJ**, Bucci JR, Simon JF, Harrison SA. HCV in patients with end-stage renal disease. *Am J Gastroenterol* 2008; **103**: 2123-2134 [PMID: 18796105 DOI: 10.1111/j.1572-0241.2008.01981.x]

83 **Carrion AF**, Fabrizi F, Martin P. Should ribavirin be used to treat hepatitis C in dialysis patients? *Semin Dial* 2011; **24**: 272-274 [PMID: 21480995 DOI: 10.1111/j.1525-139X.2011.00851.x]

84 **Liu CH**, Huang CF, Liu CJ, Dai CY, Liang CC, Huang JF, Hung PH, Tsai HB, Tsai MK, Chen SI, Lin JW, Yang SS, Su TH, Yang HC, Chen PJ, Chen DS, Chuang WL, Yu ML, Kao JH. Pegylated interferon-α2a with or without low-dose ribavirin for treatment-naive patients with hepatitis C virus genotype 1 receiving hemodialysis: a randomized trial. *Ann Intern Med* 2013; **159**: 729-738 [PMID: 24297189 DOI: 10.7326/0003-4819-159-11-201312030-00005]

85 **Tan AC**, Brouwer JT, Glue P, van Leusen R, Kauffmann RH, Schalm SW, de Vries RA, Vroom B. Safety of interferon and ribavirin therapy in haemodialysis patients with chronic hepatitis C: results of a pilot study. *Nephrol Dial Transplant* 2001; **16**:193-195 [PMID: 11209032]

86 **Mousa DH**, Abdalla AH, Al-Shoail G, Al-Sulaiman MH, Al-Hawas FA, Al-Khader AA. Alpha-interferon with ribavirin in the treatment of hemodialysis patients with hepatitis C. *Transplant Proc* 2004; **36**: 1831-1834 [PMID: 15350490]

87 **Bruchfeld A**, Lindahl K, Reichard O, Carlsson T, Schvarcz R. Pegylated interferon and ribavirin treatment for hepatitis C in haemodialysis patients. *J Viral Hepat* 2006; **13**: 316-321 [PMID: 16637862]

88 **Rendina M**, Schena A, Castellaneta NM, Losito F, Amoruso AC, Stallone G, Schena FP, Di Leo A, Francavilla A. The treatment of chronic hepatitis C with peginterferon alfa-2a (40 kDa) plus ribavirin in haemodialysed patients awaiting renal transplant. *J Hepatol* 2007; **46**: 768-774 [PMID: 17383045 DOI: 10.1016/j.jhep.2006.12.016]

89 **Lee LY**, Tong CY, Wong T, Wilkinson M. New therapies for chronic hepatitis C infection: a systematic review of evidence from clinical trials. *Int J Clin Pract* 2012; **66**: 342-355 [PMID: 22420497 DOI: 10.1111/j.1742-1241.2012.02895.x]

90 **Kawaoka T**, Aikata H, Miyaki D, Murakami E, Azakami T, Takaki S, Nagaoki Y, Hashimoto Y, Katamura Y, Hiramatsu A, Waki K, Hiraga N, Miki D, Tsuge M, Imamura M, Kawakami Y, Takahashi S, Ochi H, Tashiro H, Ohdan H, Chayama K. Eradication of hepatitis C virus genotype 1 after liver transplantation by interferon therapy before surgery: Report of three patients with analysis of interleukin-28 polymorphism, hepatitis C virus core region and interferon-sensitivity determining region. *Hepatol Res* 2011; **41**: 1126-1131 [PMID: 22032680 DOI: 10.1111/j.1872-034X.2011.00853.x]

91 **Pawlotsky JM**. New hepatitis C virus (HCV) drugs and the hope for a cure: concepts in anti-HCV drug development. *Semin Liver Dis* 2014; **34**: 22-29 [PMID: 24782255 DOI: 10.1055/s-0034-1371007]

92 **Fabrizi F**, Penatti A, Messa P, Martin P. Treatment of hepatitis C after kidney transplant: a pooled analysis of observational studies. *J Med Virol* 2014; **86**: 933-940 [PMID: 24610278 DOI: 10.1002/jmv.23919]

93 **Zimner-Rapuch S**, Janus N, Deray G, Launay-Vacher V. New therapies for hepatitis C: considerations in patients with renal impairment. *Drugs* 2014; **74**: 1307-1313 [PMID: 25060981 DOI: 10.1007/s40265-014-0268-7]

94 **Degasperi E**, Aghemo A. Sofosbuvir for the treatment of chronic hepatitis C: between current evidence and future perspectives. *Hepat Med* 2014; **6**: 25-33 [PMID: 24822024 DOI: 10.2147/HMER.S44375]

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Lamivudine resistance in children with chronic hepatitis B

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Abstract

Currently, although lamivudine (LAM) has a low genetic barrier, only interferon-alpha and LAM are available as a first-line treatment in children with chronic hepatitis B (CHB). LAM is a potent inhibitor of hepatitis B virus-deoxyribonucleic acid (HBV-DNA) polymerase replication by termination of the proviral HBV-DNA chain. LAM has a good safety and tolerability profile in CHB patients with hepatic decompensation. However, the main disadvantages of this HBV reverse transcriptase inhibitor are: (1) pre-existing covalently closed circular DNA cannot be eradicated by LAM, thus relapse after therapy withdrawal is frequent; and (2) although the longer LAM treatment induced the higher seroconversion rate, the risk of viral resistance increased through the selection of YMDD (tyrosine, methionine, aspartate, aspartate) motif. Insufficient suppression of viral replication leads to the emergence of resistant strains that could result in virological breakthrough which is usually followed by biochemical breakthrough. Mutant strains affects additional resistance and cross resistance, leading to drug resistance in a significant number of CHB patients. In this case, efficacy of more powerful anti-viral agents with higher genetic barrier against development of resistance is diminished. Furthermore, strains that are resistant to LAM could bring about vaccine escape mutants, decreasing the efficacy of HBV vaccine. A more potent drug with a high genetic barrier to resistance needs to be approved as the first-line treatment option for CHB in children.

Key words: Children; Chronic hepatitis B; Lamivudine; Lamivudine-resistant mutants; *YMDD* mutation

Core tip: In present day, antiviral drugs with higher genotypic barrier to resistance cannot be used for children with chronic hepatitis B since these drugs are not covered by the general health insurance in many countries. Therefore, lamivudine (LAM) which is not used for adults due to its many drawbacks has been used as a first-line of treatment for children out of necessity. Even though long term treatment results with LAM appear to be good, long term treatment increases the possibility of occurrence of resistant strains. These strains which are resistant to LAM could develop cross resistance to other anti-viral agents.

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INTRODUCTION

Approximately 400 million human are globally affected by chronic hepatitis B virus (HBV) infection. There is a high risk of developing serious complications such as cirrhosis and liver cancer in these people. Despite the development of new therapies using antiviral agents fighting chronic hepatitis B (CHB) remains to be a major clinical challenge. Interferon-alpha (IFN-α), lamivudine (LAM), adefovir, entecavir and lately tenofovir are all amongst the approved drugs for medical care of children affected by CHB. IFN-α for 12 mo and older children; LAM initiating at 3 years of age; adefovir and tenofovir in children 12 years and older; and entecavir initiating from 16 years of age are used[1]. Even though LAM is the primary antiviral drug officially accepted in present day for children with CHB less than 12 years old, use of antiviral drugs with a high genetic barrier against the emergence of resistance (such as entecavir and tenofovir) are not practiced for children with HBV because these drugs are not covered by the general health insurance in many countries.

CLINICAL ASPECTS OF LAM RESISTANCE IN CHILDREN

LAM, a nucleoside analogue has been officially accepted for treatment of CHB infection by United States Food and Drug Administration in 1998. LAM is found to be effective in suppression of HBV-DNA, normalizing aminotransferase values and improving histologic activity index. However, hepatitis B e antigen (HBeAg) seroconversion is not always resulted from LAM treatment[1,2]. Choe *et al*[3] found that HBeAg seroconversion developed in 34% of children with CHB within one year after initiation of LAM treatment. The probability of response to LAM treatment increases with high aminotransferase levels and high histologic activity index at baseline. Hom *et al*[4] found out that there is no significance of age, gender, previous IFN therapy, baseline weight, HBV-DNA, and body mass index in prediction of response to LAM treatment in children with CHB. However, Hong *et al*[5] showed that high aminotransferase levels affect the HBeAg seroconversion as well as younger age in children with long-term LAM treatment. Figlerowicz *et al*[6] reported that pretreatment serum HBV-DNA level is related to seroconversion of HBeAg and sustained viral response rate. Although LAM is a potent antiviral drug in the treatment of HBV, it does not help to purify liver from covalently closed circular DNA integrated into the cell nuclei. Covalently closed circular DNA brings about continued presence of HBV in liver cells[1,2]. Therefore, after stopping LAM treatment HBV replication may return to pretreatment levels. In fact, it has been reported that relapse rates varied from 19% to 62% after cessation of treatment with LAM[7]. Kansu *et al*[8] reported that relapse rates of 6.8% in children treated with combined IFN-α2a and LAM. Jonas *et al*[9] determined a relapse rate of 17.5% in a placebo controlled LAM trial in children. Hagmann *et al*[10] found relapse rate of 25% after cessation of LAM treatment. It is likely that duration of LAM treatment would be a culprit for the variations in relapse rates. Choe *et al*[3] reported long term LAM treatment increased HBeAg seroconversion rates more than IFN treatment. This is especially seen in pre-school children. High relapse rates have been observed when LAM treatment is discontinued before and right after HBeAg seroconversion. Because of this, treatment should continue possibly 12 mo after HBeAg seroconversion is observed. Nevertheless, major limitation of prolonged LAM therapy is formation of resistant mutants[1,5]. It is recognized that resistance to LAM develops as a result of emerging mutations which are formed in catalytic part of the reverse transcriptase YMDD [(Y) tyrosine, (M) methionine, (D) aspartate, (D) aspartate]. In *YMDD* mutation formations, methionine is replaced with valine (rtM204V), isoleucine (rtM204I) or rarely serine (rtM204S). In these mutations, rtM204V is always together with rtL180M which is a compensating mutation. This mutation partially restores replication fitness of HBV. However, it has been shown that rtM204I differentiation is independent from rtL180M. In addition, rtV173L differentiation which is found in some samples resistant to LAM, increased replication capacity of HBV[11]. Resistance to LAM causes absence of HBV-DNA suppression and eventually advancement of liver disease. However, replication capacity of *YMDD* mutants is less than the wild virus. Because of this, lower aminotransferase and HBV-DNA levels can be found in YMDD mutant virus infections[12]. Hartman *et al*[13] showed that 54% of the *YMDD* mutants were maintained normal aminotransferase values. After development of LAM resistance, usually serum HBV-DNA becomes positive (virologic breakthrough) and then serum alanine aminotransferase level increases (biochemical breakthrough). Mutant strains generally emerge after 6 mo of therapy with LAM. Resistance rates of 38%, 49% and 65% have been reported at 2, 3 and 5 years of therapy with LAM[14]. In a multicenter trial carried out by Jonas *et al*[9], the *YMDD* mutation was detected in 19% of children who had undergone LAM therapy for 52 wk. No LAM resistance mutations were identified in the placebo group during the first year of this study. Sokal *et al*[15] found YMDD mutation rates of 49% and 64% in second and third year of treatment, respectively. Hartman *et al*[16] found LAM resistant mutants in 11 of 17 (65%) children at the end of the first year of LAM treatment. Interestingly, *YMDD* mutation rate of this study was extremely higher than other studies. Hagmann *et al*[10] reported development of clinical resistance to LAM in 3 children (19%) in the first year of therapy. Furthermore, in this study, frequency of drug resistance is found to be low in children with high HBV-DNA suppression level. Hong *et al*[5] reported breakthrough in 25.9% (21 out of 81) of patients treated with LAM. These patients were followed up for more than 1 year. Lee *et al*[17] reported viral breakthrough in 12 children (27%) during the therapy and documented

YMDD mutation in 11 children (25%). In this study, average time for development of mutation was 22.7 mo. Ni *et al*[18] found mutant strains in 34% of the children after 12 mo of therapy with LAM. In this study, higher resistance rates were found compared to other studies. Akman *et al*[19] reported YMDD mutants in 58.4% of the total 24 children treated with LAM for 30 ± 10 mo. Choe *et al*[3] found viral breakthrough developed 10% in the first year and 23% in the second year of LAM treatment. In this study, *YMDD* mutation was found in 9 of 11 patients who have developed breakthrough. Liberek *et al*[20] determined mild and temporary aminotransferase increase in 4 out of 59 children with CHB and 2 children with *YMDD* mutation between third and twelfth months of LAM treatment. Koh *et al*[21] reported breakthrough and relapse rates in 10% and 3.3% of children with CHB after 52 mo with LAM therapy. In this study, although the exact reason of lower breakthrough and relapse rates are not known, clinical characteristics of patients and differences in treatment schedule could be reasons for this phenomenon.

Resistance to LAM increases with longer treatment periods. Therefore, LAM therapy should be discontinued 6 mo after HBeAg seroconversion or appearance of *YMMD* mutations[1]. On the other hand, higher proportion of LAM resistance is associated with higher viral load after first 6 mo of therapy[22]. It has been shown that complete virologic response reduces the risk of resistance to LAM. Yuen *et al*[23] established a relationship between high HBV-DNA level and alanine aminotransferase level at beginning with the emergence of YMDD mutations. Paik *et al*[24] determined a significant relationship between the *YMDD* mutations emerging at three months with viral breakthrough. In another study, after 12 mo of LAM treatment, Yuen *et al*[25] showed no significant differences exist in virologic response and *YMDD* mutant rates between patients with genotypes B and C. Contrary to this study, Kobayashi *et al*[26] showed development of *YMDD* mutants was influenced by HBV genotypes in patients with CHB. Numerous studies have been performed to determine whether a combination regimen with LAM and IFN-α prevents or delays the emergence of *YMDD* mutants. There are conflicting results in literature regarding this matter. In accordance with a study conducted by Chan *et al*[27], a lower LAM resistance was found in combination treatment with pegylated-IFN and LAM (21%) compared with LAM monotherapy (40%). However, Marrone *et al*[28] showed risk for emergence of LAM resistance was not reduced with IFN and LAM combination treatment. It is possible that older patients and moderately high aminotransferase levels prior to treatment in the study of Chan *et al*[27] could have caused the differences between these two studies. Furthermore, results may have been affected in favor of the combination therapy since Chan *et al*[27] conducted combination of LAM with IFN eight weeks longer than monotherapy with LAM. Ozgenç *et al*[29] determined high breakthrough incidence in children with partial response to long-term LAM therapy. In this study, reported breakthrough rates of LAM were 13.3%, 69.4%, and 82.4% in 1, 2, and 3 years, respectively. Kansu *et al*[8] reported breakthrough rates of 17.9% in simultaneous therapy group and 24.6% in sequential therapy group. Yilmaz *et al*[30] did not find breakthrough in any patient that could suggest *YMDD* mutation. Selimoglu *et al*[31] reported breakthrough in 11 (23.4%) children treated with IFN-α and LAM combination therapy. In another combination treatment of IFN-ɑ and LAM, Dikici *et al*[32] demonstrated no viral breakthrough with the exception of one patient during the follow-up period after the treatment. The viral breakthrough for this child was accepted as an *YMDD* mutation. Kuloğlu *et al*[33] reported breakthrough and *YMDD* mutant rates of 65.8% and 55.2% respectively with combined IFN-α and long term LAM therapy. Saltik-Temizel *et al*[34] provided no information about viral breakthrough rates in their article on combination therapy with LAM and high-dose IFN-α. Results from different treatment regimens are presented in Table 1.

In accordance with the results of these studies, avoiding unnecessary use of antiviral drugs can help to reduce resistance. Therefore, LAM should be prescribed only for patients with good predictors of response. If there is no finding for resistance to LAM, children should be treated for one year. However, there may be a need for longer treatment[35]. Although the optimal duration of therapy is not well-established, patients should be treated for at least six more months after HBeAg seroconversion[36]. Treatment may be discontinued in those who have HBV-DNA replication or mutant strains[37]. High HBV-DNA load before treatment was shown to be an important factor causing virologic breakthrough. Early suppression of viral replication plays a key role for prevention of LAM resistance. Insufficient response to LAM therapy with persistence of viremia can increase the resistance[38]. On the other hand, an elevated pretreatment alanine aminotransferase level (more than twofold the upper normal limit) is a key factor reducing the LAM resistance[39]. Patients who have not achieved a complete virologic response (partial response) to LAM at week 24, switching to a more potent antiviral agent or add-on another antiviral agent without cross-resistance profile is the only useful treatment approach[40]. Treatment guidelines for children have not been established yet. However, in case of failure with LAM therapy, addition of adefovir or switching to either adefovir or entecavir therapies should be considered in older children.

LAM RESISTANCE IN PREVIOUSLY UNTREATED PATIENTS WITH CHB

Because HBV polymerase lacks of proofreading mechanism, spontaneous polymerase mutations occur naturally[1,2]. Therefore, YMDD motif variants can develop not only as secondary to LAM usage, but also

Table 1 Outcomes of different therapeutic regimens in children with chronic hepatitis B

Ref.	Therapeutic regimen	Duration of treatment	HBeAg seroconversion rate (%)	Relapse rate (%)	Breakthrough rate (%)
Jonas *et al*[9]	LAM	52 wk	26	18	19
Hagmann *et al*[10]	LAM	12[6] mo	50	25	19
Sokal *et al*[15]	LAM	24 mo	25	11	49
		36 mo	35	0	64
Hartman *et al*[16]	LAM	12[6] mo	18	0	65
Hong *et al*[5]	LAM	12 mo	60.5	NA	25.9
Lee *et al*[17]	LAM	12 mo	60	0	27
Ni *et al*[18]	LAM	12 mo	38	0	34
Akman *et al*[19]	LAM	32.3 ± 8.3 mo	20.8	NA	58.4
Choe *et al*[3]	LAM	12[6] mo	65	4	10[3]
					23[4]
Liberek *et al*[20]	LAM	12 mo	27.1	NA	3.38
Koh *et al*[21]	LAM	12[6] mo	42	3.3	10
Kansu *et al*[8]	LAM + IFN	6 m IFN	60.2[1]	6.8[1]	17.9[1]
		24 mo LAM	39.4[2]	0[2]	24.6[2]
Ozgenç *et al*[29]	LAM + IFN	6 mo IFN	15.6[3]	6.8[3]	13.3[3]
		12-36 mo	5.6[4]		69.4[4]
		LAM	0[5]		82.4[5]
Dikici *et al*[32]	LAM + IFN	6 mo IFN	37	3.7	3.3
		12 mo LAM			
Kuloğlu *et al*[33]	LAM + IFN	6 mo IFN	34.2	NA	65.8
		12[6] mo LAM			
Saltik-Temizel *et al*[34]	LAM + IFN	6 mo IFN	60	NA	NA
		12 mo LAM			

[1]Simultaneous therapy; [2]Sequential therapies; [3]First year; [4]Second year; [5]Third year; [6]Until HBeAg seroconversion or evidence of resistance. NA: Information was not given in the study; IFN: Interferon; LAM: Lamivudine; HBeAg: Hepatitis B e antigen.

it can naturally occur with a relatively high incidence in previously untreated patients with CHB[41]. Recently, the incidence of *YMDD* mutants in previously untreated patients from eight countries was found to be 12.2%[42]. It is important to investigate these mutations in primary LAM-nonresponsive patients. Although some correlation between virologic breakthrough during LAM therapy and previously presence of LAM-resistant mutants in untreated patients has been found, its clinical significance during LAM therapy is still unknown. However, there is a small possibility for these mutants to be dominant during HBV infection and CHB can effectively be treated with LAM. Lee *et al*[43] indicated that previously presence of LAM resistant mutants was rapidly cleared with LAM therapy in untreated CHB patients. Further researches are necessary to evaluate the influence of LAM-resistant mutants in previously untreated patients with CHB.

CROSS-RESISTANCE

Presently, medications such as adefovir, entecavir and, recently, tenofovir have been used for the treatment of adolescents with CHB[1]. However, only IFN-α and LAM are still available as a first-line treatment especially in young children at this time. In patients with LAM-resistance, sufficient suppression of HBV-DNA is not obtained and the incidence of resistance to adefovir is increased. It has been observed that adding adefovir to continued LAM therapy is found to be linked with lower adefovir resistance rates. Because only one additional substitution at T184, S202, and/or M250 is enough to emergence of entecavir resistance, the development of entecavir resistance occurs more easily in LAM-resistant patients than treatment-naïve patients[11]. After two years therapy, the resistance rates of entecavir have been increased (8%) in LAM-resistant patients[1]. Tenney *et al*[44] reported a low rate of entecavir resistance (0.8%) and a high rate of entecavir resistance (43%) in LAM-resistant patients after five years of therapy.

TREATMENT OF LAM-RESISTANT CHB IN CHILDREN

In case of virologic breakthrough, to avoid the emergence of cross resistance, a second antiviral agent without cross-resistance is added to LAM[1]. There have been no beneficial effects of using adefovir in children between 2 and 12 years of age. Therefore, adefovir was licensed for use in adolescents. Jonas *et al*[45] reported that early virologic response was a good predictor for emergence of resistance against adefovir. Both the combination of LAM with adefovir and entecavir monotherapy were found to be more effective by Chu *et al*[46] in suppressing HBV replication compared to adefovir monotherapy in LAM-resistant children. Ryu *et al*[47] reported that high baseline viral load was rapidly declined with entecavir monotherapy in LAM refractory children. However combination of LAM with adefovir was more effective in suppressing the viral load than entecavir.

LAM-ASSOCIATED VACCINE-ESCAPE MUTATIONS

Currently, there are two types of LAM-associated HBV mutants with antigenically modified HBsAg. In the genome organization of HBV, surface and polymerase genes overlap; and changes in the polymerase reverse transcriptase which involve LAM resistance substitutions may cause mutations [first type hepatitis B surface antigen (*HBsAg*) mutant] in the surface gene of HBV. A triple substitution pattern (V173L + L180M + M204V) of LAM resistance is associated with the changes (sE164D + sI195M) in the overlapping surface gene[48]. These mutants may act as a vaccine escape mutants (sG145R). As a result, those viruses which have mutated cannot be recognized and eliminated by existing monoclonal antibodies (anti-HBs). Because of the prolonged viral suppression with LAM treatment, the second type *HBsAg* mutants are emerged from the selection of surface antigen escape mutants[49]. The development of LAM resistant and HBsAg escape mutants is associated with decreased attachment of anti-HBs antibodies to HBsAg[50]. LAM-resistant *HBV* mutants with the capability to escape from anti-hepatitis B surface antibodies have the ability to infect individuals both vaccinated and unvaccinated for HBV. Therefore, it is imperative that physician weigh up the possible benefits and harms of treatment with LAM carefully.

CONCLUSION

Currently, LAM monotherapy is not used in adults because of very high recalcitrance rates. Similarly, most potent antiviral agents with optimal resistance profile should be used as first-line therapy in children. It is important to monitor early detection of virologic breakthrough and determine genotypic resistance to decide the optimal intervention. Monitoring the levels of HBV-DNA and determination of types of resistant strains would be necessary to establish therapeutic strategies. Because the LAM resistant viruses appear to be more prevalent in population, these mutants may become a potential serious public health problem.

In conclusion, there is a need to conduct further studies and new arrangements in general health insurance policies for use of the antiviral drugs which have strong antiviral effects and low resistance rates as first-line treatment in children with CHB.

REFERENCES

1 **Williams JN**. A window on the future: can computerization help your practice? *Ky Dent J* 1990; **42**: 8-9 [PMID: 2370736 DOI: 10.1016/j.jhep.2013.05.016.7]

2 **Palumbo E**. Lamivudine for chronic hepatitis B in children. *Infect Dis Clin Prac* 2008; **16**: 13-15 [DOI: 10.1097/ipc.obo13e31815aa2dd]

3 **Choe BH**, Lee JH, Jang YC, Jang CH, Oh KW, Kwon S, Hyun MC, Ko CW, Lee KS, Lee WK. Long-term therapeutic efficacy of lamivudine compared with interferon-alpha in children with chronic hepatitis B: the younger the better. *J Pediatr Gastroenterol Nutr* 2007; **44**: 92-98 [PMID: 17204960 DOI: 10.1097/01.mpg.0000243439.47334.4e]

4 **Hom X**, Little NR, Gardner SD, Jonas MM. Predictors of virologic response to Lamivudine treatment in children with chronic hepatitis B infection. *Pediatr Infect Dis J* 2004; **23**: 441-445 [PMID: 15131468 DOI: 10.1097/01.inf.0000126412.93562.f5]

5 **Hong SJ**, Kim YH, Choe BH, Park HJ, Tak WY, Kweon YO. Current role of Lamivudine regarding therapeutic response and resistance in children with chronic hepatitis B. *Pediatr Gastroenterol Hepatol Nutr* 2013; **16**: 80-88 [PMID: 24010111 DOI: 10.5223/pghn.2013.16.2.80]

6 **Figlerowicz M**, Kowala-Piaskowska A, Filipowicz M, Bujnowska A, Mozer-Lisewska I, Służewski W. Efficacy of lamivudine in the treatment of children with chronic hepatitis B. *Hepatol Res* 2005; **31**: 217-222 [PMID: 15799860 DOI: 10.1016/j.hepres.2005.02.003]

7 **Lok AS**, McMahon BJ. Chronic hepatitis B. *Hepatology* 2001; **34**: 1225-1241 [PMID: 11732013 DOI: 10.1053/jhep.2001.29401]

8 **Kansu A**, Doğanci T, Akman SA, Artan R, Kuyucu N, Kalayci AG, Dikici B, Dalgiç B, Selimoğlu A, Kasirga E, Ozkan TB, Kuloğlu Z, Aydoğdu S, Boşnak M, Ertekin V, Tanir G, Haspolat K, Girgin N, Yağci RV. Comparison of two different regimens of combined interferon-alpha2a and lamivudine therapy in children with chronic hepatitis B infection. *Antivir Ther* 2006; **11**: 255-261 [PMID: 16640106]

9 **Jonas MM**, Mizerski J, Badia IB, Areias JA, Schwarz KB, Little NR, Greensmith MJ, Gardner SD, Bell MS, Sokal EM. Clinical trial of lamivudine in children with chronic hepatitis B. *N Engl J Med* 2002; **346**: 1706-1713 [PMID: 12037150 DOI: 10.1056/NEJMoa012452]

10 **Hagmann S**, Chung M, Rochford G, Jani M, Trinh-Shevrin C, Sitnitskaya Y, Neumann AU, Pollack H. Response to lamivudine treatment in children with chronic hepatitis B virus infection. *Clin Infect Dis* 2003; **37**: 1434-1440 [PMID: 14614664 DOI: 10.1086/378739]

11 **Zoulim F**, Locarnini S. Management of treatment failure in chronic hepatitis B. *J Hepatol* 2012; **56** Suppl 1: S112-S122 [PMID: 22300461 DOI: 10.1016/S0168-8278(12)60012-9]

12 **Lai CL**, Dienstag J, Schiff E, Leung NW, Atkins M, Hunt C, Brown N, Woessner M, Boehme R, Condreay L. Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. *Clin Infect Dis* 2003; **36**: 687-696 [PMID: 12627352 DOI: 10.1086/368083]

13 **Hartman C**, Berkowitz D, Shouval D, Eshach-Adiv O, Hino B, Rimon N, Satinger I, Kra-Oz T, Daudi N, Shamir R. Lamivudine treatment for chronic hepatitis B infection in children unresponsive to interferon. *Pediatr Infect Dis J* 2003; **22**: 224-229 [PMID: 12634582 DOI: 10.1097/01.inf.0000055062.64965.2e]

14 **Lok AS**, Lai CL, Leung N, Yao GB, Cui ZY, Schiff ER, Dienstag JL, Heathcote EJ, Little NR, Griffiths DA, Gardner SD, Castiglia M. Long-term safety of lamivudine treatment in patients with chronic hepatitis B. *Gastroenterology* 2003; **125**: 1714-1722 [PMID: 14724824 DOI: 10.1053/j.gastro.2003.09.033]

15 **Sokal EM**, Kelly DA, Mizerski J, Badia IB, Areias JA, Schwarz KB, Vegnente A, Little NR, Gardener SD, Jonas MM. Long-term lamivudine therapy for children with HBeAg-positive chronic hepatitis B. *Hepatology* 2006; **43**: 225-232 [PMID: 16440364 DOI: 10.1002/hep.21020]

16 **Hartman C**, Berkowitz D, Eshach-Adiv O, Hino B, Rimon N, Satinger I, Kra-Oz T, Shamir R. Long-term lamivudine therapy for chronic hepatitis B infection in children unresponsive to interferon. *J Pediatr Gastroenterol Nutr* 2006; **43**: 494-498 [PMID: 17033525 DOI: 10.1097/01.mpg.0000235982.34323.67]

17 **Lee EH**, Jang JY, Kim KM. Efficacy of lamivudine therapy for chronic hepatitis B in children. *Korean J Pediatr Gastroenterol Nutr* 2008; **11**: 130-136

18 **Ni YH**, Huang FC, Wu TC, Kong MS, Jeng YM, Chen PJ, Tsuei DJ, Chen HL, Hsu HY, Chang MH. Lamivudine treatment in maternally transmitted chronic hepatitis B virus infection patients. *Pediatr Int* 2005; **47**: 372-377 [PMID: 16091071 DOI: 10.1111/

j.1442-200x.2005.02101.x]
19 **Akman SA**, Kose S, Halicioglu O. Lamivudine and adefovir resistance in children and young adults with chronic hepatitis B. *Int J Infect Dis* 2010; **14**: e236-e239 [PMID: 19665408 DOI: 10.1016/j.ijid.2009.04.002]
20 **Liberek A**, Szaflarska-Popławska A, Korzon M, Łuczak G, Góra-Gebka M, Łoś-Rycharska E, Bako W, Czerwionka-Szaflarska M. Lamivudine therapy for children with chronic hepatitis B. *World J Gastroenterol* 2006; **12**: 2412-2416 [PMID: 16688835 DOI: 10.3748/wjg.v12.i15.2412]
21 **Koh H**, Baek SY, Chung KS. Lamivudine therapy for korean children with chronic hepatitis B. *Yonsei Med J* 2007; **48**: 927-933 [PMID: 18159582 DOI: 10.3349/ymj.2007.48.6.927]
22 **Hong SJ**, Choe BH. Strategy to overcome drug resistance that develops during treatment of chronic hepatitis b in children. *Pediatr Gastroenterol Hepatol Nutr* 2012; **15**: 63-73 [DOI: 10.5223/pghn.2012.15.2.63]
23 **Yuen MF**, Sablon E, Hui CK, Yuan HJ, Decraemer H, Lai CL. Factors associated with hepatitis B virus DNA breakthrough in patients receiving prolonged lamivudine therapy. *Hepatology* 2001; **34**: 785-791 [PMID: 11584376 DOI: 10.1053/jhep.2001.27563]
24 **Paik YH**, Han KH, Hong SP, Lee HW, Lee KS, Kim SO, Shin JE, Ahn SH, Chon CY, Moon YM. The clinical impact of early detection of the YMDD mutant on the outcomes of long-term lamivudine therapy in patients with chronic hepatitis B. *Antivir Ther* 2006; **11**: 447-455 [PMID: 16856618]
25 **Yuen MF**, Wong DK, Sablon E, Yuan HJ, Sum SM, Hui CK, Chan AO, Wang BC, Lai CL. Hepatitis B virus genotypes B and C do not affect the antiviral response to lamivudine. *Antivir Ther* 2003; **8**: 531-534 [PMID: 14760886]
26 **Kobayashi M**, Suzuki F, Akuta N, Suzuki Y, Arase Y, Ikeda K, Hosaka T, Sezaki H, Kobayashi M, Iwasaki S, Sato J, Watahiki S, Miyakawa Y, Kumada H. Response to long-term lamivudine treatment in patients infected with hepatitis B virus genotypes A, B, and C. *J Med Virol* 2006; **78**: 1276-1283 [PMID: 16927289 DOI: 10.1002/jmv.20701]
27 **Chan HL**, Leung NW, Hui AY, Wong VW, Liew CT, Chim AM, Chan FK, Hung LC, Lee YT, Tam JS, Lam CW, Sung JJ. A randomized, controlled trial of combination therapy for chronic hepatitis B: comparing pegylated interferon-alpha2b and lamivudine with lamivudine alone. *Ann Intern Med* 2005; **142**: 240-250 [PMID: 15710957 DOI: 10.7326/0003-4819-142-4-200502150-00006]
28 **Marrone A**, Zampino R, Portella G, Grimaldi M, Durante-Mangoni E, Santarpia L, Ruggiero G, Utili R. Three-phase sequential combined treatment with lamivudine and interferon in young patients with chronic hepatitis B. *J Viral Hepat* 2005; **12**: 186-191 [PMID: 15720534 DOI: 10.1111/j.1365-2893.2005.00619.x]
29 **Ozgenç F**, Arikan C, Sertoz RY, Nart D, Aydogdu S, Yagci RV. Effect of long-term lamivudine in chronic hepatitis B virus-infected children. *Antivir Ther* 2004; **9**: 729-732 [PMID: 15535410]
30 **Yilmaz A**, Akcam M, Gelen T, Artan R. Lamivudine and high-dose interferon alpha 2a combination treatment in naïve HBeAg-positive immunoactive chronic hepatitis B in children: an East Mediterranean center's experience. *Eur J Pediatr* 2007; **166**: 195-199 [PMID: 16944240 DOI: 10.1007/s00431-006-0220-2]
31 **Selimoglu MA**, Aydogdu S, Unal F, Zeytinoglu A, Yüce G, Yagci RV. Alpha interferon and lamivudine combination therapy for chronic hepatitis B in children. *Pediatr Int* 2002; **44**: 404-408 [PMID: 12139566 DOI: 10.1046/j.1442-200X.2002.01589.x]
32 **Dikici B**, Bosnak M, Kara IH, Dogru O, Dagli A, Gürkan F, Haspolat K. Lamivudine and interferon-alpha combination treatment of childhood patients with chronic hepatitis B infection. *Pediatr Infect Dis J* 2001; **20**: 988-992 [PMID: 11642634 DOI: 10.1097/00006454-200110000-00013]
33 **Kuloğlu Z**, Kansu A, Erden E, Girgin N. Efficacy of combined interferon alpha and long-term lamivudine therapy in children with chronic hepatitis B. *Turk J Pediatr* 2010; **52**: 457-463 [PMID: 21434529]
34 **Saltik-Temizel IN**, Koçak N, Demir H. Lamivudine and high-dose interferon-alpha combination therapy for naive children with chronic hepatitis B infection. *J Clin Gastroenterol* 2005; **39**: 68-70 [PMID: 15599215]
35 **Chen EQ**, Tang H. Optimization therapy for the treatment of chronic hepatitis B. *World J Gastroenterol* 2014; **20**: 5730-5736 [PMID: 24914334 DOI: 10.3748/wjg.v20.i19.5730]
36 **Paganelli M**, Stephenne X, Sokal EM. Chronic hepatitis B in children and adolescents. *J Hepatol* 2012; **57**: 885-896 [PMID: 22634122 DOI: 10.1016/j.jhep.2012.03.036]
37 **Jonas MM**, Block JM, Haber BA, Karpen SJ, London WT, Murray KF, Narkewicz MR, Rosenthal P, Schwarz KB, McMahon BJ. Treatment of children with chronic hepatitis B virus infection in the United States: patient selection and therapeutic options. *Hepatology* 2010; **52**: 2192-2205 [PMID: 20890947 DOI: 10.1002/hep.23934]
38 **Della Corte C**, Nobili V, Comparcola D, Cainelli F, Vento S. Management of chronic hepatitis B in children: an unresolved issue. *J Gastroenterol Hepatol* 2014; **29**: 912-919 [PMID: 24863185 DOI: 10.1111/jgh.12550]
39 **Choe HJ**, Choe BH. What physicians should know about the management of chronic hepatitis B in children: East side story. *World J Gastroenterol* 2014; **20**: 3582-3589 [PMID: 24707141 DOI: 10.3748/wjg.v20.i13.3582]
40 **El Sherbini A**, Omar A. Treatment of children with HBeAg-positive chronic hepatitis B: a systematic review and meta-analysis. *Dig Liver Dis* 2014; **46**: 1103-1110 [PMID: 25195086 DOI: 10.1016/j.dld.2014.08.032]
41 **Akarsu M**, Sengonul A, Tankurt E, Sayiner AA, Topalak O, Akpinar H, Abacioglu YH. YMDD motif variants in inactive hepatitis B carriers detected by Inno-Lipa HBV DR assay. *J Gastroenterol Hepatol* 2006; **21**: 1783-1788 [PMID: 17074014 DOI: 10.1111/j.1440-1746.2006.04567.x]
42 **Tan Y**, Ding K, Su J, Trinh X, Peng Z, Gong Y, Chen L, Cui Q, Lei N, Chen X, Yu R. The naturally occurring YMDD mutation among patients chronically infected HBV and untreated with lamivudine: a systematic review and meta-analysis. *PLoS One* 2012; **7**: e32789 [PMID: 22479339 DOI: 10.1371/journal.pone.0032789]
43 **Lee SH**, Kim HS, Byun IS, Jeong SW, Kim SG, Jang JY, Kim YS, Kim BS. Pre-existing YMDD mutants in treatment-naïve patients with chronic hepatitis B are not selected during lamivudine therapy. *J Med Virol* 2012; **84**: 217-222 [PMID: 22170540 DOI: 10.1002/jmv.23191]
44 **Tenney DJ**, Rose RE, Baldick CJ, Pokornowski KA, Eggers BJ, Fang J, Wichroski MJ, Xu D, Yang J, Wilber RB, Colonno RJ. Long-term monitoring shows hepatitis B virus resistance to entecavir in nucleoside-naïve patients is rare through 5 years of therapy. *Hepatology* 2009; **49**: 1503-1514 [PMID: 19280622 DOI: 10.1002/hep.22841]
45 **Jonas MM**, Kelly D, Pollack H, Mizerski J, Sorbel J, Frederick D, Mondou E, Rousseau F, Sokal E. Safety, efficacy, and pharmacokinetics of adefovir dipivoxil in children and adolescents (age 2 to & lt; 18 years) with chronic hepatitis B. *Hepatology* 2008; **47**: 1863-1871 [PMID: 18433023 DOI: 10.1002/hep.22250]
46 **Chu M**, Cho SM, Choe BH, Cho MH, Kwon S, Lee WK. Virologic responses to add-on adefovir dipivoxil treatment versus entecavir monotherapy in children with lamivudine-resistant chronic hepatitis B. *J Pediatr Gastroenterol Nutr* 2012; **55**: 648-652 [PMID: 22688509 DOI: 10.1097/MPG.0b013e318262a737]
47 **Ryu HJ**, Lee JM, Ahn SH, Kim do Y, Lee MH, Han KH, Chon CY, Park JY. Efficacy of adefovir add-on lamivudine rescue therapy compared with switching to entecavir monotherapy in patients with lamivudine-resistant chronic hepatitis B. *J Med Virol* 2010; **82**: 1835-1842 [PMID: 20872709 DOI: 10.1002/jmv.21898]
48 **Sheldon J**, Soriano V. Hepatitis B virus escape mutants induced by antiviral therapy. *J Antimicrob Chemother* 2008; **61**: 766-768 [PMID: 18218641 DOI: 10.1093/jac/dkn014]
49 **Yeh CT**. Development of HBV S gene mutants in chronic hepatitis B patients receiving nucleotide/nucleoside analogue therapy.

Antivir Ther 2010; **15**: 471-475 [PMID: 20516567 DOI: 10.3851/IMP1552]

50 **Sayan M**, Akhan SC. Antiviral drug-associated potential vaccine-escape hepatitis B virus mutants in Turkish patients with chronic hepatitis B. *Int J Infect Dis* 2011; **15**: e722-e726 [PMID: 21784687 DOI: 10.1016/j.ijid.2011.05.019]

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ORIGINAL ARTICLE

Prospective Study

Aetiological factors of Budd-Chiari syndrome in Algeria

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Abstract

AIM: To study the clinical presentation of Budd-Chiari syndrome (BCS) and identify the aetiologies of this disease in Algeria.

METHODS: Patients with BCS, hospitalised in our unit from January 2004 until June 2010 were included and the aetiological factors were assessed. Patients presenting a BCS in the setting of advanced-stage cirrhosis or a liver transplantation were excluded from the study. The diagnosis was established when an obstruction of hepatic venous outflow (thrombosis, stenosis or compression) was demonstrated. We diagnosed myeloproliferative disease (MPD) by bone marrow biopsy and *V617F JAK2* mutation. Anti-phospholipid syndrome (APLS) was detected by the presence of anticardiolipin antibodies, anti-β2 glycoprotein antibodies and Lupus anticoagulant. We also detected paroxysmal nocturnal haemoglobinuria (PNH) by flow cytometry. Celiac disease and Behçet disease were systematically investigated in our patients. Hereditary anticoagulant protein deficiencies were also assessed. We tested our patients for the G20210A mutation at Beaujon Hospital. Imaging procedures were performed to determine a local cause of BCS, such as a hydatid cyst or a liver tumour.

RESULTS: One hundred and fifteen patients were included. Mean follow up: 32.12 mo. Mean age: 34.41 years, M/F = 0.64. Chronic presentation was frequent:

63.5%. The revealing symptoms for the BCS were ascites (74.8%) and abdominal pain (42.6%). The most common site of thrombosis was the hepatic veins (72.2%). Involvement of the inferior vena cava alone was observed in 3 patients. According to the radiological investigations, BCS was primary in 94.7% of the cases (*n* = 109) and secondary in 5.2% (*n* = 6). An aetiology was identified in 77.4% of the patients (*n* = 89); it was multifactorial in 27% (*n* = 31). The predominant aetiology of BCS in our patients was a myeloproliferative disease, observed in 34.6% of cases. APLS was found in 21.7% and celiac disease in 11.4%. Other acquired conditions were: PNH (*n* = 4), systemic disease (*n* = 6) and inflammatory bowel disease (*n* = 5). Anticoagulant protein deficiency was diagnosed in 28% of the patients (*n* = 18), dominated by protein C deficiency (*n* = 13). Secondary BCS was caused by a compressing hydatic cyst (*n* = 5) and hepatocellular carcinoma (*n* = 1).

CONCLUSION: The main aetiologic factor of BCS in Algeria is MPD. The frequency of celiac disease justifies its consideration when BCS is diagnosed in our region.

Key words: Algeria; Etiologie; Celiac disease; Budd-Chiari; Thrombosis

Core tip: Budd-Chiari syndrome (BCS) is a rare disease, but it seems quite common in our country and in North Africa in general. However, we do not know the etiological features of this disease in our region. We collected 115 cases of BCS in 6 years. A fairly complete etiologic assessment was achieved. We identified the cause of BCS in 77%. It was multifactorial in 27%. The etiologies were dominated by the myeloproliferative disease 34%, followed by antiphospholipid syndrome in 21%. Finally, the etiological distribution in our patients does not differ too much from what is reported in Western countries.

Afredj N, Guessab N, Nani A, Faraoun SA, Ouled Cheikh I, Kerbouche R, Hannoun D, Amir ZC, Ait Kaci H, Bentabak K, Plessier A, Valla DC, Cazals-Hatem V, Denninger MH, Boucekkine T, Debzi N. Aetiological factors of Budd-Chiari syndrome in Algeria. *World J Hepatol* 2015; 7(6): 903-909 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i6/903.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i6.903

INTRODUCTION

Budd-Chiari syndrome (BCS) has been characterised by a panel of European experts as a group of disorders that results from an obstruction of hepatic venous outflow at any level from the small hepatic veins (HV) to the junction of the inferior vena cava (IVC) and the right atrium. BCS may be secondary to a non-thrombotic obstruction of hepatic venous drainage pathways, but in most cases, it is related to a venous thrombosis. This is referred to as primary BCS. Heart failure, constrictive pericarditis and veno-occlusive disease are excluded from this definition[1].

BCS is a rare disease, with an estimated prevalence of 2 per 100000 inhabitants and an annual incidence of approximately 0.2 cases per million inhabitants[2].

The clinical presentation of BCS depends on the extent and the acute or progressive occurrence of venous thrombosis. We distinguish asymptomatic forms of BCS, which are discovered fortuitously, and symptomatic forms, which can be acute or chronic. The acute form is characterised by abdominal pain, ascites and hepatomegaly, without evidence of portal hypertension. This form can be complicated by fulminant hepatitis. The chronic form is difficult to distinguish from cirrhosis regardless of the aetiology. The subacute form is characterised by features of acute BCS with portal hypertension. It reflects an extension of a previous occurrence of thrombosis in the HV.

Primary BCS is the clinical expression of an underlying thrombotic condition that should be identified by an exhaustive aetiological investigation.

The causal mechanism of venous thrombosis, which is frequently multifactorial, often involves a myeloproliferative disorder (MPD)[1,2]. The purpose of this work was to identify causal factors of BCS in Algeria. We also sought to study the anatomic aspects and the clinical presentation of BCS in our patients.

MATERIALS AND METHODS

This was a prospective study that included consecutive patients, over the age of 16 years with BCS, who were hospitalised in our unit from January 2004 until June 2010. Patients who presented with BCS complicating advanced-stage cirrhosis and transplantation were excluded from the study. At inclusion, Doppler ultrasound exploration, triphasic computed tomography (CT) and/or magnetic resonance (MR) angiography were performed in all patients, to confirm the diagnosis.

The diagnosis was established when a thrombosis, a stenosis or a compression of hepatic venous outflow (HV and/or suprahepatic IVC) was demonstrated. We also looked for indirect signs of BCS, such as HV dilatation upstream of a stenosis, spiderweb collateral venous circulation between HV, enlarged segment I of the liver or patchy enhancement of hepatic parenchyma.

For the aetiological work-up, we looked for acquired causes of thrombosis, especially MPD, with a bone marrow biopsy (BMB) and a test for *V617F JAK2* mutation by real-time polymerase chain reaction (RQ-PCR) (JAK2 MutaQuant kit, Ipsogen). In doubtful cases, a second examination of the BMB was performed in the pathology unit of Beaujon Hospital in Paris. The diagnosis of MPD was established using the World Health Organization 2008 revised criteria[3]. MPD was considered latent if BMB was abnormal and/or if a *JAK2* mutation was

Table 1 Revealing symptoms

Symptoms	*n*	%
Ascites	86	74.8
Abdominal pain	49	42.6
Jaundice	16	13.9
Bleeding	14	12.2
Hepatic encephalopathy	6	5.2
Fever	18	15.7
Lower limb edema	15	13

found while the blood count was normal.

Tests were conducted to detect anticardiolipin (ACL) IgG and IgM antibodies, anti-β2 glycoprotein antibodies and lupus anticoagulant to determine the presence of anti-phospholipid syndrome (APLS). Patients were considered positive when the results from two successive tests performed within a 2 to 3 mo interval exceeded the cutoff levels of 20 U/L for ACL antibodies and 10 U/mL for A2βGP antibodies. Autoantibody tests were also performed to distinguish primary and secondary APLS that may be associated with Lupus.

The classical diagnostic criteria were used to identify Behçet's disease[4]; genotyping for the Human Major Histocompatibility Complex (HLA) B51 was performed in doubtful cases. To identify paroxysmal nocturnal haemoglobinuria (PNH) in patients with haemolytic anemia and elevated lactate dehydrogenase (LDH) levels, flow cytometry was performed to detect CD55/CD59 deficient peripheral blood cells.

The systematic assessment for celiac disease included tests for anti-endomysium and/or anti-transglutaminase antibodies and duodenal biopsies in patients without major coagulation disorders. PCR for HLA class Ⅰ and class Ⅱ antigens was performed in seven patients with both celiac disease and BCS. The work-up included an examination of other acquired prothombotic disorders including hyperhomocysteinaemia, inflammatory bowel disease and tuberculosis.

We also looked for an inherited cause of thrombosis (*i.e.,* proteins C, S or antithrombin Ⅲ deficiency). When a family survey was not available, the deficiency in anticoagulant proteins was considered to be a primary disorder in the absence of severe liver failure (prothrombine time > 60%), nephrotic syndrome, acute thrombosis or anticoagulant treatment. Activated protein C resistance was used to diagnose a factor V mutation, because genetic testing (Leiden mutation) was not available. The prothrombin gene was sequenced in 21 patients in the molecular biology unit of Beaujon Hospital, to detect the *G20210A* mutation.

We also looked for oral contraceptive use or the occurrence of BCS during or after pregnancy. Appropriate imaging procedures were performed to search for a local cause of BCS, notably a hydatid cyst or a liver tumour.

The statistical analysis was performed with SPSS 13.0 (SPSS Inc., Chicago, IL). Estimated variables are reported with the 95% confidence interval. $P < 0.05$ was considered statistically significant. Fisher's exact test and the χ^2 test were used for the comparison of qualitative variables and the Fisher-Snedecor test was used for the comparison of quantitative and qualitative variables.

RESULTS

A total of 115 patients were included in this study. The mean time from the onset of symptoms to the diagnosis was 13.03 ± 4.5 mo (range: 4 d-10 years). The mean patient age was 34 years; the M/F sex-ratio was 0.64. The majority of patients were aged from 20 to 29 years at the time of diagnosis. The chronic form of BCS predominated, which was observed in 63.5% of the patients ($n = 73$). In contrast, the acute form was found in 8.7% of the patients ($n = 10$) and the fulminant form was observed in 3 patients.

BCS was latent and was discovered fortuitously in 9.6% of the patients ($n = 11$). In the majority of cases, BCS was revealed by ascites and abdominal pain, as observed in 74.8% and 42.6% of patients respectively. Revealing symptoms are reported in Table 1.

Ascites and gastrointestinal bleeding were more frequent in patients with the chronic form of BCS, however, as might be expected, pain, fever and an elevated transaminase level were predominantly observed in the acute and sub-acute forms of the disease. This difference was highly significant ($P < 0.001$). Demographic, clinical and laboratory features are reported in Table 2.

The Child-Pugh classification scores at admission were: B ($n = 62$, 53.9%); A ($n = 34$, 29.6%); C ($n = 19$, 16.5%).

Doppler ultrasound was performed in 90% of patients ($n = 104$) and established the diagnosis in 87%. For the remaining cases, the diagnosis was based on the CT-scan. MR-angiography completed the work-up in 46% of patients ($n = 53$). According to the radiological investigations, BCS was secondary in 5.3% ($n = 6$) and primary in 94.7% ($n = 109$). Secondary BCS was caused by a hydatic liver cyst ($n = 5$) or hepatocellular carcinoma ($n = 1$). Isolated involvement of the HV was observed in 72% of patients and both the HV and IVC were involved in 25.2%. Involvement of the IVC alone was observed in 3 patients. Thrombosis in other venous areas was observed in 32.7% of the cases (Table 3). Thoraco-abdominal collateral venous circulation (CVC) was predominant in patients with an isolated HV thrombosis ($P = 0.05$), whereas lumbar CVC was prevalent in patients with combined HV and IVC thrombosis ($P = 0.007$). A dysmorphic liver was found in 64.3% of patients ($n = 74$). Hypertrophy of the caudate lobe was noted in 86.1% of the cases ($n = 99$). Intrahepatic collateral veins typical of BCS were found in 81.7% ($n = 94$) and hypervascular regenerative macronodules were observed in 27.8% of the patients ($n = 32$).

The aetiologies of BCS are reported in Table 4. At least one cause of BCS was found in 77.4% of the cases ($n = 89$) and a combination of several prothrombotic

Table 2 Clinical and laboratory characteristics at diagnosis

Demographic parameters	
Age (yr)	34 (16-72)
M/F (*n*)	45/70
Distribution by age, *n* (%)	
< 20 yr	8 (9)
20-40 yr	63 (72)
> 40 yr	29 (34)
Clinical parameters *n* (%)	
Disease onset	
Acute	8.7 (10)
Chronic	63.5 (73)
Subacute	27.8 (32)
Ascites	62.6 (72)
Hepatomegaly	62.6 (72)
Splenomegaly	42.6 (49)
Thoraco-abdominal venous collaterals	43.5 (50)
Lumber venous collaterals	11.3 (13)
Hepatic encephalopathy	3.5 (4)
Jaundice	40 (46)
Lower limb oedema	24.3 (28)
Biological parameters	
ALT (ULN)	2.5 (1-60)
Bilirubin (mg/dL)	20.58 (3-265)
Prothrombin time (%)	57 (14-98)
Haemoglobin (g/dL)	11.7 (3-17)
RBC (10^6/mm^3)	4.5 (1.7-7.9)
WBC (10^3/mm^3)	7.7 (1.3-21.5)
Platelet count (/mm^3)	263212 (29000-695000)
Albumin (g/L)	29.4 (16.7-45)
Thrombocytosis *n* (%)	20 (23)
Erythrocytosis *n* (%)	11.3 (13)
Hyperleucocytosis *n* (%)	25.2 (29)
Cholestasis *n* (%)	73.9 (85)
Hyperbilirubinaemia *n* (%)	41.7 (48)
Elevated liver enzymes *n* (%)	49.6 (57)
Liver failure *n* (%)	61.7 (71)
Renal failure *n* (%)	9.5 (11)

ALT: Alanine aminotransferase; ULN: Upper limit of normal; M/F: Male/female; RBC: Red blood cells; WBC: White blood cells.

Table 3 Radiological features

Thrombosis site	*n*	%
Hepatic veins	83	72.2
IVC	3	2.6
IVC and HV	29	25.2
Associated thrombosis	37/113	32.2
Retrohepatic IVC	7	6.0
Portal vein	26	22.6
Mesenteric vein/splenic vein	4	3.4
Renal veins	4	3.4
Iliac veins	3	2.6

IVC: Inferior vena cava; HV: Hepatic veins.

Table 4 Budd-Chiari syndrome aetiologies

Aetiologies	Tested patients	*n*	% (*n*/tested *n*)
MPD	104	36	34.6
Patent		19	
Latent		17	
APL syndrome	92	20	21.7
Protein C deficiency	67	13	19.4
Protein S deficiency	59	5	8.5
Antithrombin deficiency	68	0	0
APCR	68	7	10.3
Celiac disease	88	10	11.4
Hyperhomocysteinaemia	42	5	11.9
PNH	11	4	
Systemic disease[1]	106	6	5.6
Inflammatory bowel disease[2]	60	5	8.3
Gene Ⅱ mutation	21	1	4.7
Liver hydatic cyst	115	5	4.3
Hepatocellular carcinoma	115	1	0.8
Hormonal factors	70	25	35.7
Oral contraception	70	24	34.3
Pregnancy	70	3	4.3
Hormonal treatment	70	1	1.4
Unknown aetiology		24	20.9

[1]Systemic lupus erythematosus (*n* = 1), granulomatosis (*n* = 1), sarcoidosis (*n* = 1), Behçet disease (*n* = 3); [2]Ulcerative colitis (*n* = 1), Crohn's disease (*n* = 4). PNH: Paroxysmal nocturnal haemoglobinuria; APL: Anti-phospholipid syndrome; APCR: Activated protein C resistance; MPD: Myeloproliferative disease.

conditions was noted in 27% (*n* = 31). The most common aetiological association was MPD and APLS. Secondary BCS was often associated with an underlying thrombophilia.

MPD was the predominant causal factor of BCS and was observed in 34% (*n* = 36) of the tested patients. A BMB was performed in 95 cases (83%) and testing for the *JAK2* mutation was performed in 53 patients (46%). The MPD was patent in 19 cases (polyglobulia *n* = 5; essential thrombocytaemia *n* = 9; myelofibrosis *n* = 5) and latent in 17 cases. Fourteen patients were positive for the *JAK2* mutation and 30 patients had signs of MPD on BMB. Among patients with *JAK2* mutation, 8 had histological features of MPD. The BMB was not contributive otherwise (*n* = 6). Patients with and without MPD were comparable; no statistically significant differences were observed between the groups with respect to the mean age, sex ratio, disease onset or site of thrombosis.

ACL antibodies were detected in 21% of the tested patients (*n* = 20), and were associated with systemic lupus in one patient. A second cause of thrombophilia was associated with APLS in 79% of these patients.

Celiac disease was found in 10 patients and was diagnosed during the work-up for BCS in most cases (*n* = 6). In these patients, an underlying thrombophilia, apart from celiac disease was noted in only 40% of cases. The immunogenetic study failed to disclose any HLA class Ⅰ specificity. With respect to the HLA class Ⅱ antigens, the patients were positive for DQβ1*02 and/or DQβ1*03, two alleles strongly linked with celiac disease and for alleles DRβ1*04 and/or DRβ1*07 which are known to be in linkage disequilibrium with DQβ1*03 and DQβ1*02.

PNH was found in 4 patients and had been undetected prior to the BCS work-up in 3 of them. Severe anaemia with an elevated LDH level was found in 3 patients; the flow cytometry results were positive in all of these patients (*n* = 4). An associated splanchnic vein

thrombosis was also found in 100% of cases (*n* = 4).

Other acquired causes of thrombosis were: systemic disease (*n* = 6) and inflammatory bowel disease (*n* = 5).

Anticoagulant protein deficiency was observed in 28% of patients (*n* = 18), dominated by protein C deficiency (*n* = 13). Only 5 patients demonstrated a protein S deficiency, one had a mutation in the prothrombin gene and no patient demonstrated an antithrombin deficiency.

In 3 cases, BCS occurred during pregnancy or during the post-partum period. Nevertheless, a general cause of thrombosis was found in the majority of these patients.

DISCUSSION

This series collected 115 cases of BCS over a period of seven years in a single centre, which corresponds to approximately 18 cases annually. This is comparable with the data provided by the French national BCS observatory (20 new cases/year)[5]. The present series is the largest reported to date in North Africa.

Except for a few series in Asia, where the mean age was less than 30 years[6,7] and the M/F sex-ratio > 1[8], BCS has generally been observed in young female patients[9-11]. This was the case in our series where the M/F ratio was 0.6, and the mean age was 34 years.

The diagnosis of BCS depends on the results of non-invasive imaging techniques. Though the results of Doppler-ultrasound are operator-dependent, its estimated diagnostic yield is 90%, according to recent studies[12-14], or even greater with advanced technical devices such as contrast ultrasound[15]. The diagnostic yield was estimated to be 87% in our series. MR-angiography should be proposed if the diagnosis by Doppler ultrasound is doubtful, because it avoids the radiation exposure associated with CT-angiography. In our work, MR-angiography was performed in only 46.1% of the patients because it was not available before October 2007.

Isolated HV involvement occurred in 72% of our patients. This same pattern is generally reported in European series[11,16], but not in Asian series, particularly those from Japan, where IVC obstruction is frequent[5,17]. Splanchnic thrombosis, which worsens the prognosis of patients with BCS, was observed in 23% in our series, which contrast with the literature, where this association is noted in less than 14%[8,9,11]. The number of aetiological factors has been significantly related to the extension of the thrombosis into the splanchnic territory[18]. Indeed, in our patients, multifactorial thrombophilia was more frequently observed among patients with combined BCS and portal vein involvement (34% *vs* 24% for isolated BCS). An earlier and well-conducted anticoagulant regimen would most likely reduce the rate of this complication which compromises the efficacy of the therapeutic options for BCS.

Our work-up, although incomplete, enabled an etiological diagnosis in 77.4% of cases.

In the literature, the cause of thrombophilia was found in 87%, 72% and 84% of the cases presented in recent studies from India[8], Turkey[19] and Europe[9] respectively. The causes encountered in our work were similar to those observed in Europe, with a clear predominance of MPD. In Southeast Asian countries, bacterial and parasitic infections have been suggested to be risk factors for IVC thrombosis[8,20,21]. In recent studies from India, the aetiological profile of SBC has changed. Prothrombotic states are now found in more than 60% of the cases in those series[22,23], which is similar to the rates reported in Western series. This could be linked to the widespread availability of diagnostic tests, including the essays to detect the *JAK2* mutation.

In our series, the rate of MPD-related BCS did not reach 50% as reported in the Western literature, but was most likely underestimated because only 46% of our patients were tested for *JAK2* mutation. The concordance of the *JAK2* mutation with BMB was not strong in our study: among the 14 patients with a positive mutation, only 8 (57%) had histological features of MPD on BMB. This finding is most likely related to the poor sample quality of the BMB.

In accordance with the data from the literature[2,9], the second leading cause of BCS in our patients after MPD was APLS. ACL antibodies were detected in 21.7% of the tested patients. An associated thrombophilia was observed in 75% of these patients. This result is comparable with data published by Espinoza from a series of 43 patients with APLS-associated BCS, where ACL antibodies were often associated with another thrombophilic disorder[24].

The rate of factor V Leiden mutation, the most common inherited cause of thrombophilia, was unusually low in our study. However, the only test available for the diagnosis of this condition at our centre was the activated protein C resistance test, which lacks diagnostic reliability, and most likely underestimates the frequency of this condition.

A high rate of celiac disease was also noted in our patients with BCS. This association seems to be frequent in North Africa. We found 16 published cases in the literature until 2012[25-35], and noted that 12 of them were originated from Algeria or Tunisia. Environmental factors, particularly geophagia or special diets might be involved, although they were not demonstrated. These factors were excluded in our patients. The immunogenetic study that was performed did not show any association with a specific major histocompatibility complex antigen in these patients[36]. Further information from a broader genetic study might be useful.

The frequency of celiac disease in Maghreb might also explain the high number of the association of celiac disease with BCS. An epidemiological study conducted in Oran (west Algeria) gave an estimated rate of 2.34 ± 1.3 celiac disease cases per 1000 live births[37]. In our setting, we thus propose a systematic search for celiac disease in the aetiological work-up for BCS.

All of patients with PNH died. The management of these patients was challenging in our setting because

oral anticoagulants were often discontinued when haemolytic events occurred. This led to an extension of the thrombosis to the splanchnic veins. It should be noted, however, that Eculizumab was not yet available when this study was conducted. This treatment has significantly improved the prognosis of patients with PNH by reducing the rate of thromboembolic events[38]. Furthermore, in specialised centres, patients with PNH-BCS currently have the same prognosis as other patients with BCS[39].

As mentioned above, the aetiological workup was not exhaustive. The lack of molecular biolgy techniques constitutes the main limitation of our work. Tests for mutations in *JAK2* and prothrombine genes were not performed in all patients, whereas tests for factor V Leiden mutation were simply unavailables. We used indirect methods for the diagnosis of this last condition, which may be insufficient. An improvement in the diagnostic tools should most likely enhance the results of the aetiological investigation in our patients.

In conclusion, this study demonstrates that BCS is not rare in Algeria and that the predominant cause of thrombophilia is MPD, as reported in Western countries. There is however one particular aspect that concerns the presence of celiac disease and hydatid cysts of the liver that should be systematically included in the aetiological work-up of BCS in our geographic location.

COMMENTS

Background

The relevance of this article is that Budd-Chiari syndrome (BCS) has never been explored in the authors' region. Practitioners often associated BCS with Behçet's disease; the authors provided an update on this pathology, imperfectly known, and they proved that the predominant etiology was not Behçet's disease but the myeloproliferative syndrome.

Research frontiers

This is a research work because the authors identified the etiologies of this disease in Algeria, which were not previously known. The results can serve as reference for future works on this field. The authors achieved an etiological assessment including molecular biology, like *JAK2* mutation and prothrombin gene mutation. Some of these tests have required moving abroad to achieve them. For all this, they can consider that this is research work.

Innovations and breakthroughs

Some studies have been made in the same field in Algeria and other Maghreb countries but it was a low sample studies or case reports. The authors work will allow a breakthrough in understanding the etiologies of BCS in their region.

Applications

The work has practical applications. Thus, in any patient with Budd-Chiari originated from Algeria or even from Maghreb, practitioners must first search for a myeloproliferative syndrome or systemic autoimmune disease. Celiac disease should also be sought systematically, given the frequency of the association of these two diseases in their study.

Terminology

The authors ensured that the words used in the text are easily understood by all scientists' readers. Abbreviations are always enclosed in parentheses, next to the full name at the first appearance in the text.

Peer-review

The authors have performed a good study, the manuscript is interesting.

REFERENCES

1 **Janssen HL**, Garcia-Pagan JC, Elias E, Mentha G, Hadengue A, Valla DC; European Group for the Study of Vascular Disorders of the Liver. Budd-Chiari syndrome: a review by an expert panel. *J Hepatol* 2003; **38**: 364-371 [PMID: 12586305]

2 **Valla DC**. Primary Budd-Chiari syndrome. *J Hepatol* 2009; **50**: 195-203 [PMID: 19012988 DOI: 10.1016/j.jhep.2008.10.007]

3 **Tefferi A**, Vardiman JW. Classification and diagnosis of myeloproliferative neoplasms: the 2008 World Health Organization criteria and point-of-care diagnostic algorithms. *Leukemia* 2008; **22**: 14-22 [PMID: 17882280 DOI: 10.1038/sj.leu.2404955]

4 Criteria for diagnosis of Behçet's disease. International Study Group for Behçet's Disease. *Lancet* 1990; **335**: 1078-1080 [PMID: 1970380]

5 **Valla DC**. Hepatic venous outflow tract etiopathogenesis. Asia versus the west. *J Gastroen Hepatol* 2004; **19**: S204-S211 [DOI: 10.111/J.1400-1746.03642.x]

6 **Shrestha SM**, Okuda K, Uchida T, Maharjan KG, Shrestha S, Joshi BL, Larsson S, Vaidya Y. Endemicity and clinical picture of liver disease due to obstruction of the hepatic portion of the inferior vena cava in Nepal. *J Gastroenterol Hepatol* 1996; **11**: 170-179 [PMID: 8672764 DOI: 10.1111/j.1440-1746.1996.tb00056.x]

7 **Singh V**, Sinha SK, Nain CK, Bambery P, Kaur U, Verma S, Chawla YK, Singh K. Budd-Chiari syndrome: our experience of 71 patients. *J Gastroenterol Hepatol* 2000; **15**: 550-554 [PMID: 10847443 DOI: 10.1046/j.1440-1746.2000.02157.x]

8 **Amarapurkar DN**, Punamiya SJ, Patel ND. Changing spectrum of Budd-Chiari syndrome in India with special reference to non-surgical treatment. *World J Gastroenterol* 2008; **14**: 278-285 [PMID: 18186568 DOI: 10.3748/wjg.14.278]

9 **Darwish Murad S**, Plessier A, Hernandez-Guerra M, Fabris F, Eapen CE, Bahr MJ, Trebicka J, Morard I, Lasser L, Heller J, Hadengue A, Langlet P, Miranda H, Primignani M, Elias E, Leebeek FW, Rosendaal FR, Garcia-Pagan JC, Valla DC, Janssen HL. Etiology, management, and outcome of the Budd-Chiari syndrome. *Ann Intern Med* 2009; **151**: 167-175 [PMID: 19652186 DOI: 10.7326/0003-4819-151-3-200908040-00004]

10 **Zeitoun G**, Escolano S, Hadengue A, Azar N, El Younsi M, Mallet A, Boudet MJ, Hay JM, Erlinger S, Benhamou JP, Belghiti J, Valla D. Outcome of Budd-Chiari syndrome: a multivariate analysis of factors related to survival including surgical portosystemic shunting. *Hepatology* 1999; **30**: 84-89 [PMID: 10385643 DOI: 10.1002/hep.510300125]

11 **Darwish Murad S**, Valla DC, de Groen PC, Zeitoun G, Hopmans JA, Haagsma EB, van Hoek B, Hansen BE, Rosendaal FR, Janssen HL. Determinants of survival and the effect of portosystemic shunting in patients with Budd-Chiari syndrome. *Hepatology* 2004; **39**: 500-508 [PMID: 14768004 DOI: 10.1002/hep.20064]

12 **Buckley O**, O' Brien J, Snow A, Stunell H, Lyburn I, Munk PL, Torreggiani WC. Imaging of Budd-Chiari syndrome. *Eur Radiol* 2007; **17**: 2071-2078 [PMID: 17206425 DOI: 10.1007/s00330-006-0537-2]

13 **Erden A**. Budd-Chiari syndrome: a review of imaging findings. *Eur J Radiol* 2007; **61**: 44-56 [PMID: 17123764 DOI: 10.1016/j.ejrad.2006.11.004]

14 **Bolondi L**, Gaiani S, Li Bassi S, Zironi G, Bonino F, Brunetto M, Barbara L. Diagnosis of Budd-Chiari syndrome by pulsed Doppler ultrasound. *Gastroenterology* 1991; **100**: 1324-1331 [PMID: 2013376]

15 **Rossi S**, Rosa L, Ravetta V, Cascina A, Quaretti P, Azzaretti A, Scagnelli P, Tinelli C, Dionigi P, Calliada F. Contrast-enhanced versus conventional and color Doppler sonography for the detection of thrombosis of the portal and hepatic venous systems. *AJR Am J Roentgenol* 2006; **186**: 763-773 [PMID: 16498104 DOI: 10.2214/AJR.04.1218]

16 **Mahmoud AE**, Mendoza A, Meshikhes AN, Olliff S, West R, Neuberger J, Buckels J, Wilde J, Elias E. Clinical spectrum, investigations and treatment of Budd-Chiari syndrome. *QJM* 1996; **89**: 37-43 [PMID: 8730341 DOI: 10.1093/oxfordjournals.qjmed.a030135]

17 **Okuda H**, Yamagata H, Obata H, Iwata H, Sasaki R, Imai F, Okudaira M, Ohbu M, Okuda K. Epidemiological and clinical features of Budd-Chiari syndrome in Japan. *J Hepatol* 1995; **22**: 1-9 [PMID: 7751574 DOI: 10.1016/0168-8278(95)80252-5]

18 **Darwish Murad S**, Valla DC, de Groen PC, Zeitoun G, Haagsma EB, Kuipers EJ, Janssen HL. Pathogenesis and treatment of Budd-Chiari syndrome combined with portal vein thrombosis. *Am J Gastroenterol* 2006; **101**: 83-90 [PMID: 16405538 DOI: 10.1111/j.1572-0241.2006.00353.x]

19 **Uskudar O**, Akdogan M, Sasmaz N, Yilmaz S, Tola M, Sahin B. Etiology and portal vein thrombosis in Budd-Chiari syndrome. *World J Gastroenterol* 2008; **14**: 2858-2862 [PMID: 18473410 DOI: 10.3748/wjg.14.2858]

20 **Victor S**, Jayanthi V, Panchanadam M, Chitra S, Vijayalakshmi CS, Madanagopalan N. Budd Chiari syndrome and pericaval filariasis. *Trop Gastroenterol* 1994; **15**: 161-168 [PMID: 7863553]

21 **Shrestha SM**, Shrestha S. Hepatic vena cava disease: Etiologic relation to bacterial infection. *Hepatol Res* 2007; **37**: 196-204 [PMID: 17362302 DOI: 10.1111/j.1872-034X.2007.00012.x]

22 **Mohanty D**, Shetty S, Ghosh K, Pawar A, Abraham P. Hereditary thrombophilia as a cause of Budd-Chiari syndrome: a study from Western India. *Hepatology* 2001; **34**: 666-670 [PMID: 11584361 DOI: 10.1053/jhep.2001.27948]

23 **Pati HP**, Dayal S, Srivastava A, Pande GK, Acharya SK. Spectrum of hemostatic derangements, in Budd-Chiari syndrome. *Indian J Gastroenterol* 2003; **22**: 59-60 [PMID: 12696825]

24 **Espinosa G**, Font J, García-Pagan JC, Tàssies D, Reverter JC, Gaig C, Cervantes F, Cervera R, Bosch J, Ingelmo M. Budd-Chiari syndrome secondary to antiphospholipid syndrome: clinical and immunologic characteristics of 43 patients. *Medicine* (Baltimore) 2001; **80**: 345-354 [PMID: 11704712 DOI: 10.1097/00005792-200111000-00001]

25 **Gelsi E**, Ruitord F, Saint-Paul MC, Filippi J, Arab K, Hébuterne X. [Association of Budd-Chiari syndrome with a coeliac disease in patient native from North Africa]. *Gastroenterol Clin Biol* 2004; **28**: 903-905 [PMID: 15523228 DOI: 10.1016/S0399-8320(04)95155-X]

26 **Martínez F**, Berenguer M, Prieto M, Montes H, Rayón M, Berenguer J. Budd-Chiari syndrome caused by membranous obstruction of the inferior vena cava associated with coeliac disease. *Dig Liver Dis* 2004; **36**: 157-162 [PMID: 15002826 DOI: 10.1016/j.dld.2003.06.006]

27 **El Younsi S**, Nassif T, Kuoch V, Boytchev I, Pelletier G, Buffet C. [Association of Budd-Chiari syndrome and celiac disease]. *Gastroenterol Clin Biol* 2003; **27**: 236-237 [PMID: 12658136]

28 **Manzano ML**, Garfia C, Manzanares J, Urruzuno P, Yela C, Castellano G, Solís JA. [Celiac disease and Bud-Chiari syndrome: an uncommon association]. *Gastroenterol Hepatol* 2002; **25**: 159-161 [PMID: 11864539]

29 **Aguirrebarrena G**, Pulcinelli S, Giovannoni AG, Gidekel L. [Celiac disease and Budd-Chiari syndrome: infrequent association]. *Rev Esp Enferm Dig* 2001; **93**: 611-612 [PMID: 11767441]

30 **Marteau P**, Cadranel JF, Messing B, Gargot D, Valla D, Rambaud JC. Association of hepatic vein obstruction and coeliac disease in North African subjects. *J Hepatol* 1994; **20**: 650-653 [PMID: 8071543 DOI: 10.1016/S0168-8278(05)80355-1]

31 **Hamdi A**, Ayachi R, Saad H, Gargouri R, Zouari K, Chebbah MS. [Hemiplegia revealing Budd-Chiari syndrome associated with celiac disease in an infant]. *Presse Med* 1990; **19**: 1011-1012 [PMID: 2141148]

32 **Boudhina T**, Ghram N, Ben Becher S, Ayach R, Ben Ghachem K, Yedes A, Hamza M. [Budd-Chiari syndrome and total villous atrophy in children: apropos of 3 case reports]. *Tunis Med* 1990; **68**: 59-62 [PMID: 2309343]

33 **Ouakaa-Kchaou A**, Ennaifer R, Belhadj N, Gargouri D, Elloumi H, Romani M, Kochlef A, Kilani A, Kharrat J, Ghorbel A. [Celiac disease associated with Budd-Chiari syndrome]. *Presse Med* 2008; **37**: 239-241 [PMID: 18096356 DOI: 10.1016/j.lpm.2007.05.037]

34 **Ben Hriz F**, Habbassi H, Maamouri N, Azaiez O, Chaabouni H, Chouaib S, Mnif E, Ben Mami N. [Budd-Chiari syndrome associated with celiac disease]. *Rev Med Interne* 2010; **31**: 160-162 [PMID: 20045226 DOI: 10.1016/j.revmed.2008.12.016]

35 **Hmami F**, Chaouki S, Souilmi FZ, Abourazzak S, Idrissi M, Atmani S, Hida M. [Association of celiac disease and Budd-Chiari syndrome: a case report]. *Arch Pediatr* 2011; **18**: 102 [PMID: 21130629 DOI: 10.1016/j.arcped.2010.10.019]

36 **Afredj N**, Metatla S, Faraoun SA, Nani A, Guessab N, Benhalima M, Bendib SE, Debzi N, Layaida K, Gamar L, Baiod N, Balamane M, Kaddache N, Bounab N, Kecili L, Boucekkine T. Association of Budd-Chiari syndrome and celiac disease. *Gastroenterol Clin Biol* 2010; **34**: 621-624 [PMID: 20934289]

37 **Bessahraoui M**, Bouziane Nedjadi K, Naceur M, Niar S, Bouchetara A, Bouhamidi M. Eur Acad Paediatr. Nice (France) Poster, 2008: 181

38 **Hillmen P**, Muus P, Dührsen U, Risitano AM, Schubert J, Luzzatto L, Schrezenmeier H, Szer J, Brodsky RA, Hill A, Socié G, Bessler M, Rollins SA, Bell L, Rother RP, Young NS. Effect of the complement inhibitor eculizumab on thromboembolism in patients with paroxysmal nocturnal hemoglobinuria. *Blood* 2007; **110**: 4123-4128 [PMID: 17702897 DOI: 10.1182/blood-2007-06-095646]

39 **Hoekstra J**, Leebeek FW, Plessier A, Raffa S, Darwish Murad S, Heller J, Hadengue A, Chagneau C, Elias E, Primignani M, Garcia-Pagan JC, Valla DC, Janssen HL. Paroxysmal nocturnal hemoglobinuria in Budd-Chiari syndrome: findings from a cohort study. *J Hepatol* 2009; **51**: 696-706 [PMID: 19664836 DOI: 10.1016/j.jhep.2009.06.019]

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CASE REPORT

Disease control with sunitinib in advanced intrahepatic cholangiocarcinoma resistant to gemcitabine-oxaliplatin chemotherapy

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Author contributions: Dreyer C, Belghiti J, Raymond E and Faivre S conceived and designed the study; Dreyer C, Dokmak S, Belghiti J, Guedj N, Paradis V and Faivre S provided study materials of patients; Dreyer C, Sablin MP, Bouattour M and Faivre S collected and assembled data; Dreyer C, Sablin MP, Bouattour M, Raymond E and Faivre S analyzed and interpreted data; Ronot M and Faivre S participated in the acquisition and interpretation of radiological figures; all authors participated in manuscript written; all authors give final approval of manuscript.
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Ethics approval: The Ethics approval is not mandatory in France for individual compassionate use of a drug that has been approved in another indication.
Informed consent: An individual consent has been obtained from each patient regarding the off-label use of sunitinib in cholangiocarcinoma.
Conflict-of-interest: Eric Raymond and Sandrine Faivre have received honoraria from Pfizer. All other authors have no disclosures to declare.

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Abstract

Advanced cholangiocarcinoma is associated with poor prognostic survival and has limited therapeutic options available at present. The importance of angiogenesis and expression of pro-angiogenic factors in intrahepatic forms of cholangiocarcinoma suggest that therapies targeting angiogenesis might be useful for the treatment of this disease. Here we report three cases of patients with advanced intrahepatic cholangiocarcinoma progressive after standard chemotherapy and treated with sunitinib 50 mg/d in 6-wk cycles of 4 wk on treatment followed by 2 wk off treatment (Schedule 4/2). In all three patients, sunitinib treatment was associated with a sustained disease control superior to 4 mo, patients achieving either a partial response or stable disease. A reduction in tumor size and density was observed in all cases, suggesting tumor necrosis as a result of sunitinib treatment in these patients. In addition, sunitinib was generally well tolerated and the occurrence of side effects was managed with standard medical interventions, as required. Our results suggest that sunitinib therapy may

be associated with favorable outcomes and tolerability in patients with advanced cholangiocarcinoma. Those observations contributed to launch a prospective phase Ⅱ multicenter trial investigating sunitinib in advanced intrahepatic cholangiocarcinoma (SUN-CK study; NCT01718327).

Key words: Biliary tract tumors; Antiangiogenic therapy; Hypodensity; Tumor response; Vascular endothelial growth factor receptor inhibitors; Chemoresistance

Core tip: No systemic therapy after progression on platinum-based chemotherapy is currently approved. Based on imaging hypervascular pattern and molecular expression of vascular endothelial growth factor, we evaluated sunitinib, a multikinase inhibitor as second line treatment in patients with advanced intrahepatic cholangiocarcinoma. We report 3 cases of disease control lasting 4-16 mo that provide the rational for developing prospective clinical trials with sunitinib in second line for advanced intrahepatic cholangiocarcinoma.

Dreyer C, Sablin MP, Bouattour M, Neuzillet C, Ronot M, Dokmak S, Belghiti J, Guedj N, Paradis V, Raymond E, Faivre S. Disease control with sunitinib in advanced intrahepatic cholangiocarcinoma resistant to gemcitabine-oxaliplatin chemotherapy. *World J Hepatol* 2015; 7(6): 910-915 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i6/910.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i6.910

INTRODUCTION

While cholangiocarcinoma is the second-most common primary hepatic tumor after hepatocellular carcinoma (HCC), it is a rare disease for which there are few therapeutic options[1]. The only curative treatment is surgical resection; however, this is only viable for localized disease. Resectable cholangiocarcinoma is associated with frequent recurrences and a five-year survival rate of 20%-40% following surgery[1]. Treatment with cisplatin plus gemcitabine is associated with moderate efficacy when disease recurs[2]; however, for patients presenting with disease progression following first-line therapy, there is currently no consensus on the best treatment option.

Angiogenesis and the expression of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), play an important role in the pathogenesis of biliary tract cancers, including cholangiocarcinoma[3-5]. In addition, an association between microvessel density and inferior curative resection rate and local recurrence has also been observed[6]. In addition, our pathology team and other have showed that intrahepatic forms of cholangiocarcinoma are associated with high VEGF expression in comparison to hilar cholangiocarcinoma and that VEGF expression level correlates with poor prognosis[7,8]. Anti-angiogenic agents, such as the humanized anti-VEGF receptor (VEGFR) monoclonal antibody, bevacizumab, in combination with the receptor tyrosine kinase inhibitor erlotinib, have demonstrated activity in biliary tract carcinomas. In one study, among 49 patients with unresectable biliary tract cancer treated with bevacizumab 5 mg/kg intravenously on days 1 and 15 and erlotinib 150 mg by continuous daily dosing, time to progression was 4.4 mo and 6 patients demonstrated a partial response (PR)[9]. Targeted agents, such as sorafenib, are now commonly used in HCC, another disease in which angiogenesis plays an integral role, yielding prolonged survival with acceptable toxicity[10]. Recent advances in the knowledge of molecular alterations underlying cholangiocarcinoma support the need of better patient selection for appropriate medical therapy in this disease[11]. Together, those data suggest that anti-angiogenic therapy may confer benefits in the treatment of particular subtypes of cholangiocarcinoma.

We report here three cases of patients with recurrent intrahepatic cholangiocarcinoma who showed promising results under therapy with sunitinib, in the absence of other validated therapeutic options.

CASE REPORT

Case 1: A 39-year-old woman

The patient, referred to our center for an intrahepatic tumor in the right lobe of the liver revealed by abdominal pain and was treated with surgical resection. Pathological examination showed a 10 cm cholangiocarcinoma, with multiple satellite nodules and vascular, perineural, and regional lymph node involvement. Given the presence of multiple risk factors for recurrence, including large tumor size, multifocal nature, and lymph node involvement, adjuvant combination chemotherapy with 6 mo of gemcitabine 1000 mg/m² and oxaliplatin 85 mg/m² every two weeks was initiated after multidisciplinary consultation and patient consent. Four months after completion of adjuvant chemotherapy, intrahepatic recurrence in liver right lobe was diagnosed on computed tomography (CT) scan, and second-line chemotherapy with irinotecan plus 5-fluorouracil (5-FU) and leucovorin (FOLFIRI) (irinotecan 180 mg/m², 5-FU 400 mg/m² bolus, leucovorin 400 mg/m² then 5-FU 2400 mg/m² as a 47-h infusion every two weeks) was initiated. After three months of treatment, the patient exhibited stable disease (SD) and therapy was continued for an additional three months. At the time of second evaluation (following 6 mo of FOLFIRI therapy; 18 mo after initial diagnosis), a CT scan showed tumor progression with peripheral rim enhancement suggesting hypervascular lesions (Figure 1A). As the patient was young with excellent performance status (PS) and with vascularized lesions on CT scans, treatment with sunitinib 50 mg/d on

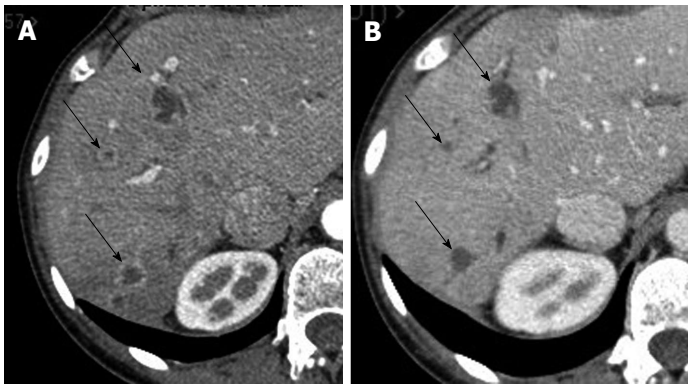

Figure 1 Computed tomography scans (A) prior to and (B) 4 mo after initiating sunitinib treatment, demonstrating density variation of three target lesions (arrows) in the liver. Baseline computed tomography (CT) shows heterogeneous lesions with contrast-enhanced peripheral ring, while post-treatment CT shows disappearance of the hypervascular aspect of the three lesions.

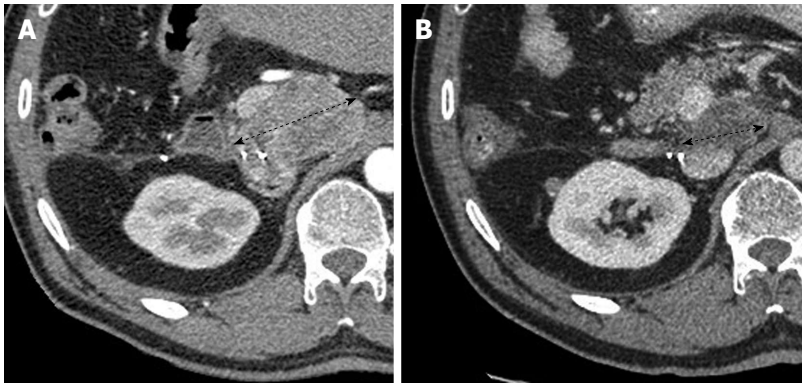

Figure 2 Computed tomography scans (A) prior to and (B) 4 mo after initiating sunitinib treatment, showing a partial response according to the response evaluation criteria in solid tumors criteria of a retroperitoneal lymphadenopathy (arrow), and a decrease in tumor density.

schedule 4/2 (4 wk on treatment followed by two weeks off treatment) was initiated. An early CT scan was performed after one month of treatment and showed the lesions to be hypodense, suggesting decrease tumor vasculature induced by anti-angiogenic treatment. After two months of treatment, the dose of sunitinib was reduced to 37.5 mg/d due to treatment-related grade 3 thrombocytopenia, asthenia and peripheral edema. Following four months of treatment, the patient had SD with disappearance of the hypervascular aspect of the tumor lesions (Figure 1B). In the context of persistent tumor stabilization in a young patient with excellent, sustained PS (0), surgical resection of the recurrent tumor was performed, consisting of repeated right hepatectomy associated with resection of segment Ⅳ. Sunitinib was stopped 3 wk before surgery. Pathological examination confirmed the diagnosis of a multifocal cholangiocarcinoma, consisting of tumor nodules with a fibro-hyaline center with multifocal vascular involvement. Sunitinib treatment was resumed three weeks after surgery at a dose of 37.5 mg/d but had to be stopped two months later due to a biliary fistula. Since the performance status of the patient further deteriorated, she was transferred to a palliative care unit in a primary center care close her home; the last news being available eight months after the second surgery.

Case 2: A 64-year-old man

The patient complained for 6 mo of intermittent abdominal pain; imaging including CT-scan and MRI revealed a 6 cm tumor of the segment 6 of liver. A biopsy was performed and showed a low-differentiated carcinoma proliferation but immunohistochemistry staining was inconclusive. A right hepatectomy was performed. Final pathological examination and immunoassaying of the surgical specimen confirmed the diagnosis of an 8 cm intrahepatic cholangiocarcinoma with satellite nodules, vascular embolism and perineural involvement. Given the risk factors of recurrence, 6 mo of adjuvant combination chemotherapy with gemcitabine 1000 mg/m^2 and oxaliplatin 85 mg/m^2 (GEMOX) every 15 d was administered. One month after completion of adjuvant chemotherapy, a 6 cm retroperitoneal lymphadenopathy was detected by CT scan (Figure 2A). As this was a unique recurrence in a patient with excellent PS (0), lymph node dissection was scheduled. However during the explorative laparotomy, the lesion was deemed unresectable due to regional tumor adherences. After multidisciplinary discussion and patient consent, systemic therapy with sunitinib 50 mg/d on schedule 4/2 was

initiated. After one month of treatment, CT scan showed extensive hypodensity of the lesion suggesting tumor necrosis, although sunitinib dose reduction to 37.5 mg/d was required due to treatment-related grade 3 thrombocytopenia. No further major toxicity was observed following dose reduction of sunitinib. After three months of treatment, CT scan showed a 25% reduction in tumor size, and sunitinib was recommenced at 37.5 mg/d with good tolerability. After four months of treatment, a PR, according to the response evaluation criteria in solid tumors (RECIST) criteria, was observed with increased necrosis of the tumor (Figure 2B). Sunitinib treatment was maintained for a total of 18 mo before disease progression was observed. The patient further developed jaundiced due to biliary compression requiring biliary stents. Gradually his general condition deteriorated and he died 7 mo after sunitinib interruption.

Case 3: A 36-year-old woman

The third patient was diagnosed with an intrahepatic cholangiocarcinoma, associated with epigastric pain. Ultrasound examination revealed a 6 cm tumor in the left lobe of the liver, and a 9 mm satellite nodule in the right lobe. The patient was treated with six cycles of GEMOX (gemcitabine 1000 mg/m², oxaliplatin 85 mg/m²), which was well tolerated. CT scan demonstrated stable disease in the left lobe of the liver but the occurrence of new lesions in the right lobe. Because of this tumor progression, treatment was changed to a combination of cisplatin and epirubicin. After nine treatment cycles, a further CT scan revealed again tumor progression. As the patient had a good PS (0), treatment was initiated with sunitinib 50 mg/d on Schedule 4/2 after multidisciplinary discussion and patient consent. A CT scan performed three weeks after initiating sunitinib treatment demonstrated tumor hypodensity and a 15% reduction in lesion size. Following three months of treatment, the lesions were stable and the patient experienced moderate toxicity with grade 1 asthenia, grade 1 hypertension, and grade 3 thrombocytopenia. Subsequently, sunitinib treatment was continued at a dose of 37.5 mg/d. After a total of eight months of sunitinib treatment with good tolerability, progression of the right liver lesions was observed, and the treatment was interrupted. The patient general condition further deteriorated, therefore she returned to her original country for palliative care management; the last news being available at the time of the end of sunitinib treatment.

DISCUSSION

Here we report the cases of three patients treated with sunitinib for intrahepatic cholangiocarcinoma with progressive disease following surgery (two patients) and chemotherapy. Sunitinib-associated toxicity was acceptable after dose reduction from 50 mg/d to 37.5 mg/d. Treatment duration was prolonged (6-12 mo), and was associated with a PR in one patient, and SD with tumor hypodensity on CT scans in two patients. These results are promising in a setting where few therapeutic options are available. Our results support the investigation of sunitinib in clinical trials in patients with good PS who are not responsive to chemotherapy, and especially in those with an intrahepatic type of cholangiocarcinoma displaying hypervascular features.

Cholangiocarcinoma represents a tumor type with many unmet medical needs[12]. Recent data have demonstrated the efficacy of chemotherapy in other related tumors for which limited treatment options are available. As a reference for first-line treatment, the ABC-02 randomized trial of 410 patients with locally advanced or metastatic cholangiocarcinoma, gallbladder cancer, or ampullary cancer, showed that cisplatin combined with gemcitabine was associated with a significant survival advantage, *vs* gemcitabine alone, for up to 24 wk [median overall survival (OS) 11.7 mo *vs* 8.1 mo, respectively; hazard ratio = 0.64; 95%CI: 0.52-0.80; $P < 0.001$][2].

Angiogenesis plays an important role in tumor growth and survival. The negative prognostic value of angiogenesis has been shown in cholangiocarcinoma, although vascular density is lower in this disease than in HCC[13]. In a study in 22 patients undergoing surgical resection for intrahepatic cholangiocarcinoma, increased microvessel density was associated with both poor prognosis and the presence of intra-hepatic metastases[14]. Moreover, there was a correlation between microvessel density and the levels of both VEGF and angiopoietin-2[13]. In addition, VEGF expression is especially important in intrahepatic cholangiocarcinoma, suggesting a potential benefit of anti-angiogenic agents in this particular subtype of tumors[7].

Thrombospondin 1 (TSP1) is also implicated in angiogenesis, although its role remains controversial. TSP1 was found to be overexpressed in tumor cells when compared with normal cells[11]. However, the risk of intrahepatic metastasis was found to be higher in cholangiocarcinomas with low levels of TSP1[15]. Moreover, a correlation was shown between levels of VEGF and lymph node involvement in a series of 36 intrahepatic cholangiocarcinomas[16].

Sunitinib is an oral multi-tyrosine kinases inhibitor targeting VEGFR, PDGF receptor, stem-cell factor receptor and fetal liver tyrosine kinase receptor 3. Sunitinib has shown potent antitumor and antiangiogenic activities with acceptable safety profile in patients with advanced solid tumors[17,18]. Adverse events related to sunitinib are generally manageable, the most common side effects including asthenia, hand-foot syndrome, and hematological toxicities. Taken together, there is a strong rational to evaluate antiangiogenics, including sunitinib, in patients with intrahepatic forms of cholangiocarcinoma.

Three previous phase Ⅱ trials in unselected subtypes of cholangiocarcinoma report disparate results. In the first study, where 31 eligible patients were treated in the first-line setting with sorafenib[19], median OS was nine months and median PFS was 3 mo. Two patients achieved an unconfirmed PR and ten patients

demonstrated SD. In another trial of 46 patients treated with sorafenib, one patient achieved PR and nine exhibited SD[20]. The third study investigated combination therapy with bevacizumab and erlotinib as first-line therapy[6]. Of 20 evaluable patients, four achieved PR and seven achieved SD lasting more than four months.

Of note, evaluation of tumor response is difficult in patients treated with anti-angiogenic agents for primary liver tumors. The most common evaluation criteria (RECIST) are based exclusively on tumor size. Other composite criteria, such as the Choi criteria, provide an alternative as they consider both tumor size and tumor density, particularly as anti-angiogenic agents may render a tumor hypodense without size modification[21,22]. However, these criteria require validation in large patient cohorts.

Based on the rationale to target VEGF pathways and those encouraging results in selected cases, our team has launched a prospective multicenter phase Ⅱ trial to investigate the activity of sunitinib in second line for patients with advanced intrahepatic cholangiocarcinoma previously treated with chemotherapy (SUN-CK trial; NCT01718327).

COMMENTS

Case characteristics

Three cases of recurrent/advanced intrahepatic cholangiocarcinoma; all cases presented with no specific symptoms including abdominal pain.

Clinical diagnosis

The initial clinical presentation of cases had low clinical relevance; in all cases positive diagnosis was confirmed histologically.

Differential diagnosis

Hepatocellular carcinoma was the main differential diagnosis. Imaging features were not typical of hepatocellular carcinoma but suggested cholangiocarcinoma imaging diagnosis. Differential diagnosis was excluded by histological examination in all 3 patients.

Imaging diagnosis

Computed tomography scan showed lesions in the liver (cases 1 and 3) or lymph nodes (case 2) with contrast update at the arterial phase suggesting hypervascularisation of tumor lesions. Upon sunitinib treatment all target lesions showed decrease in density compatible with sunitinib antiangiogenic effects.

Pathological diagnosis

For all cases, histological examination showed phenotypical features of cholangiocarcinoma; immunohistochemical staining confirmed the diagnosis with positivity for CK7 and CK20 but negativity for anti-hepatocyte and glypican.

Terminology

All patients received sunitinib at the time of progression after conventional chemotherapy. Two cases received sunitinib as a third-line option after progression on platinum-based chemotherapy and one case as second-line treatment after progression on GEMOX regimen.

Related reports

In the literature, many preclinical and clinical data have shown the rational to block angiogenesis pathways in cholangiocarcinoma. However, few data are available evaluating antiangiogenic agents in the specific subtype of intrahepatic cholangiocarcinoma.

Term explanation

Cholangiocarcinoma is a group of heterogeneous tumors including intrahepatic, perihilar and distal cholangiocarcinomas. It is the second most frequent primitive liver malignancy after hepatocellular carcinoma and the incidence of intrahepatic cholangiocarcinoma is increasing worldwide.

Experiences and lessons

Those cases reports showed promising results of sunitinib in chemotherapy-pretreated patients with advanced cholangiocarcinoma. These results support the concept to target deregulated signaling pathways in this disease, here vascular endothelial growth factor receptor signaling pathway in the intrahepatic subtype of cholangiocarcinoma. Based on these observations, a prospective phase Ⅱ study has been launched in France evaluating sunitinb as second-line treatment in patients progressive and/or intolerant to chemotherapy.

Peer-review

The manuscript addresses the question of second-line treatment where no standard of care is available for advanced cholangiocarcinoma. Those results support the investigation of targeted agents blocking angiogenesis in advanced intrahepatic forms of cholangiocarcinoma.

REFERENCES

1 **Razumilava N**, Gores GJ. Cholangiocarcinoma. *Lancet* 2014; **383**: 2168-2179 [PMID: 24581682 DOI: 10.1016/S0140-6736(13)61903-0]

2 **Valle J**, Wasan H, Palmer DH, Cunningham D, Anthoney A, Maraveyas A, Madhusudan S, Iveson T, Hughes S, Pereira SP, Roughton M, Bridgewater J; ABC-02 Trial Investigators. Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer. *N Engl J Med* 2010; **362**: 1273-1281 [PMID: 20375404 DOI: 10.1056/NEJMoa0908721]

3 **Fava G**, Demorrow S, Gaudio E, Franchitto A, Onori P, Carpino G, Glaser S, Francis H, Coufal M, Marucci L, Alvaro D, Marzioni M, Horst T, Mancinelli R, Benedetti A, Alpini G. Endothelin inhibits cholangiocarcinoma growth by a decrease in the vascular endothelial growth factor expression. *Liver Int* 2009; **29**: 1031-1042 [PMID: 19291182 DOI: 10.1111/j.1478-3231.2009.01997]

4 **Glaser SS**, Gaudio E, Alpini G. Vascular factors, angiogenesis and biliary tract disease. *Curr Opin Gastroenterol* 2010; **26**: 246-250 [PMID: 20061944 DOI: 10.1097/MOG.0b013e3283369d19]

5 **Möbius C**, Demuth C, Aigner T, Wiedmann M, Wittekind C, Mössner J, Hauss J, Witzigmann H. Evaluation of VEGF A expression and microvascular density as prognostic factors in extrahepatic cholangiocarcinoma. *Eur J Surg Oncol* 2007; **33**: 1025-1029 [PMID: 17400419]

6 **Thelen A**, Scholz A, Weichert W, Wiedenmann B, Neuhaus P, Gessner R, Benckert C, Jonas S. Tumor-associated angiogenesis and lymphangiogenesis correlate with progression of intrahepatic cholangiocarcinoma. *Am J Gastroenterol* 2010; **105**: 1123-1132 [PMID: 19997097 DOI: 10.1038/ajg.2009.674]

7 **Guedj N**, Zhan Q, Perigny M, Rautou PE, Degos F, Belghiti J, Farges O, Bedossa P, Paradis V. Comparative protein expression profiles of hilar and peripheral hepatic cholangiocarcinomas. *J Hepatol* 2009; **51**: 93-101 [PMID: 19446907 DOI: 10.1016/j.jhep.2009.03.017]

8 **Yoshikawa D**, Ojima H, Iwasaki M, Hiraoka N, Kosuge T, Kasai S, Hirohashi S, Shibata T. Clinicopathological and prognostic significance of EGFR, VEGF, and HER2 expression in cholangiocarcinoma. *Br J Cancer* 2008; **98**: 418-425 [PMID: 18087285]

9 **Lubner SJ**, Mahoney MR, Kolesar JL, Loconte NK, Kim GP, Pitot HC, Philip PA, Picus J, Yong WP, Horvath L, Van Hazel G, Erlichman CE, Holen KD. Report of a multicenter phase II trial testing a combination of biweekly bevacizumab and daily erlotinib in patients with unresectable biliary cancer: a phase II Consortium study. *J Clin Oncol* 2010; **28**: 3491-3497 [PMID: 20530271 DOI: 10.1200/JCO.2010.28.4075]

10 **Villanueva A**, Hernandez-Gea V, Llovet JM. Medical therapies for hepatocellular carcinoma: a critical view of the evidence. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 34-42 [PMID: 23147664 DOI: 10.1038/nrgastro.2012.199]

11 **Sia D**, Tovar V, Moeini A, Llovet JM. Intrahepatic cholangiocarcinoma: pathogenesis and rationale for molecular therapies. *Oncogene* 2013; **32**: 4861-4870 [PMID: 23318457 DOI: 10.1038/onc.2012.617]

12 **Bridgewater J**, Galle PR, Khan SA, Llovet JM, Park JW, Patel T, Pawlik TM, Gores GJ. Guidelines for the diagnosis and management of intrahepatic cholangiocarcinoma. *J Hepatol* 2014; **60**: 1268-1289 [PMID: 24681130 DOI: 10.1016/j.jhep.2014.01.021]

13 **Kawahara N**, Ono M, Taguchi K, Okamoto M, Shimada M,

Takenaka K, Hayashi K, Mosher DF, Sugimachi K, Tsuneyoshi M, Kuwano M. Enhanced expression of thrombospondin-1 and hypovascularity in human cholangiocarcinoma. *Hepatology* 1998; **28**: 1512-1517 [PMID: 9828214]

14 **Shirabe K**, Shimada M, Tsujita E, Aishima S, Maehara S, Tanaka S, Takenaka K, Maehara Y. Prognostic factors in node-negative intrahepatic cholangiocarcinoma with special reference to angiogenesis. *Am J Surg* 2004; **187**: 538-542 [PMID: 15041507]

15 **Tang D**, Nagano H, Yamamoto H, Wada H, Nakamura M, Kondo M, Ota H, Yoshioka S, Kato H, Damdinsuren B, Marubashi S, Miyamoto A, Takeda Y, Umeshita K, Dono K, Wakasa K, Monden M. Angiogenesis in cholangiocellular carcinoma: expression of vascular endothelial growth factor, angiopoietin-1/2, thrombospondin-1 and clinicopathological significance. *Oncol Rep* 2006; **15**: 525-532 [PMID: 16465407]

16 **Park BK**, Paik YH, Park JY, Park KH, Bang S, Park SW, Chung JB, Park YN, Song SY. The clinicopathologic significance of the expression of vascular endothelial growth factor-C in intrahepatic cholangiocarcinoma. *Am J Clin Oncol* 2006; **29**: 138-142 [PMID: 16601431]

17 **Faivre S**, Delbaldo C, Vera K, Robert C, Lozahic S, Lassau N, Bello C, Deprimo S, Brega N, Massimini G, Armand JP, Scigalla P, Raymond E. Safety, pharmacokinetic, and antitumor activity of SU11248, a novel oral multitarget tyrosine kinase inhibitor, in patients with cancer. *J Clin Oncol* 2006; **24**: 25-35 [PMID: 16314617]

18 **Sweeney CJ**, Chiorean EG, Verschraegen CF, Lee FC, Jones S, Royce M, Tye L, Liau KF, Bello A, Chao R, Burris HA. A phase I study of sunitinib plus capecitabine in patients with advanced solid tumors. *J Clin Oncol* 2010; **28**: 4513-4520 [PMID: 20837944 DOI: 10.1200/JCO.2009.26.9696]

19 **El-Khoueiry AB**, Rankin CJ, Ben-Josef E, Lenz HJ, Gold PJ, Hamilton RD, Govindarajan R, Eng C, Blanke CD. SWOG 0514: a phase II study of sorafenib in patients with unresectable or metastatic gallbladder carcinoma and cholangiocarcinoma. *Invest New Drugs* 2012; **30**: 1646-1651 [PMID: 21748296 DOI: 10.1007/s10637-011-9719-0]

20 **Bengala C**, Bertolini F, Malavasi N, Boni C, Aitini E, Dealis C, Zironi S, Depenni R, Fontana A, Del Giovane C, Luppi G, Conte P. Sorafenib in patients with advanced biliary tract carcinoma: a phase II trial. *Br J Cancer* 2010; **102**: 68-72 [PMID: 19935794 DOI: 10.1038/sj.bjc.6605458]

21 **Faivre SJ**, Bouattour M, Dreyer C, Raymond E. Sunitinib in hepatocellular carcinoma: redefining appropriate dosing, schedule, and activity end points. *J Clin Oncol* 2009; **27**: e248-e250; author reply e251-e252 [PMID: 19901099 DOI: 10.1200/JCO.2009.25.0670]

22 **Ronot M**, Bouattour M, Wassermann J, Bruno O, Dreyer C, Larroque B, Castera L, Vilgrain V, Belghiti J, Raymond E, Faivre S. Alternative Response Criteria (Choi, European association for the study of the liver, and modified Response Evaluation Criteria in Solid Tumors [RECIST]) Versus RECIST 1.1 in patients with advanced hepatocellular carcinoma treated with sorafenib. *Oncologist* 2014; **19**: 394-402 [PMID: 24652387 DOI: 10.1634/theoncologist.2013-0114]

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