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REVIEW

Role of hepatitis B virus in development of hepatocellular carcinoma: Focus on covalently closed circular DNA

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Abstract

Chronic infection with hepatitis B virus (HBV) remains a major global health problem, especially in developing countries. It may lead to prolonged liver damage, fibrosis, cirrhosis, and hepatocellular carcinoma. Persistent chronic HBV infection is related to host immune response and the stability of the covalently closed circular DNA (cccDNA) in human hepatocytes. In addition to being essential for viral transcription and replication, cccDNA is also suspected to play a role in persistent HBV infections or hepatitis relapses since cccDNA is very stable in non-dividing human hepatocytes. Understanding the pathogenicity and oncogenicity of HBV components would be essential in the development of new diagnostic tools and treatment strategies. This review summarizes the role and molecular mechanisms of HBV cccDNA in hepatocyte transformation and hepatocarcinogenesis and current efforts to its detection and targeting.

Key Words: Hepatitis B virus; Covalently closed circular DNA; Hepatocellular carcinoma; Hepatocarcinogenesis

Core Tip: The covalently closed circular DNA (cccDNA) of hepatitis B virus (HBV) in human hepatocytes serves as the template for viral replication machinery. HBV cccDNA is also related to host immune response and persistent HBV infection leading to the development of hepatocellular carcinoma. This review summarizes current knowledge on cccDNA in hepatocarcinogenesis and comprehensive efforts to its detection and targeting.

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INTRODUCTION

Hepatitis B virus (HBV) is a DNA virus belonging to the *Hepadnaviridae* family. In humans, HBV may cause both acute and chronic infections in the liver that can lead to an increased risk of hepatocellular carcinoma (HCC) following persistent chronic infection[1].

HBV genome

The genome of HBV is a relaxed-circular DNA (rcDNA) that is 3.2 kbp in length. The small genome size of HBV causes the genome to be extremely compact, encoding four open reading frames (ORFs) that are overlapping: C, P, S, and X. These ORFs produced functional viral proteins: HBc and HBe antigens (HBcAg and HBeAg) and precore protein from C, polymerase (Pol) from P, surface antigens L-HBs, M-HBs, and S-HBs from S, and HBV X protein (HBx) from X[2].

HBV genome also contains four unidirectional promoters, core, SPI, SPII, and X, that are responsible for the initiation of transcription at different positions. Upon entry into cells, the rcDNA is converted into covalently closed circular DNA (cccDNA), which will be converted into different lengths of RNA (3.5 kb, 2.4 kb, 2.1 kb, and 0.7 kb) depending on transcription initiation from the different promoters.

The 3.5 kb RNA is the preC RNA that encodes for HBe and Pol. Another 3.5 kb RNA is the pregenomic RNA (pgRNA) that encodes for HBc protein and Pol. pgRNA serves as a template for both translation and reverse transcription[1]. Pol contains four different domains: A terminal protein (TP) domain that acts as primer to initiate minus strand DNA synthesis and for binding to pgRNA; a spacer domain; a reverse transcriptase (RT) domain that is necessary for reverse transcription and DNA-dependent DNA polymerization; and a ribonuclease H (RNase H) domain that is responsible for the digestion of pgRNA following reverse transcription[1,2]. The 2.4 kb preS1 RNA encodes for L-HBs protein, while the 2.1 kb RNA preS2 or S RNA encodes for the overlapping M-HBs and S-HBs. These HBs proteins form the HBV surface antigens that surround the viral nucleocapsid, and promote receptor binding during viral entry into target cells. Meanwhile, the 0.7 kb X RNA encodes for HBx protein. HBx protein promotes the production of new viral particles *via* the promotion of viral transcription and replication, and plays a role in the development of HBV-related HCC[1,2]. In addition, HBx protein has also been reported to have a role in cccDNA formation[3,4].

HBV replication

The HBV life cycle starts with its entry to hepatocytes (Figure 1A). HBV attaches to the host cell surface *via* binding to heparan sulfate proteoglycans (HSPGs) in a non-specific and low-affinity manner. This process is then followed by more specific and high-affinity interaction between the virus surface proteins and their respective receptors on the cell surface[5]. Multiple studies[2,6-10] have shown that sodium taurocholate co-transporting peptide (NTCP/SLC10A1) specifically expressed in the liver is the main receptor for HBV entry.

Recent studies[11,12] have reported that HBV internalization into hepatocytes, *via* endocytosis, is triggered by the direct interaction between epidermal growth factor receptor, a tyrosine kinase receptor, and NTCP. Upon endocytosis, the viral envelope and the host cell-derived vesicular membrane fuse to release the nucleocapsid to the cytoplasm. This process might be facilitated by the N-terminus of HBV preS1 domain which could contain the fusogenic sequence[13]. Meanwhile, another study had suggested an alternative process that HBV nucleocapsid delivery into the cytoplasm is based on membrane translocation instead of membrane fusion[14].

The nucleocapsid in the cytoplasm is translocated into the nucleus by intracellular trafficking, mainly *via* microtubules and importin[15-17]. Successful HBV infection is achieved when the HBV genome is delivered into the host cell nucleus. Once in the nucleus, the HBV rcDNA is converted into cccDNA with the help of Pol and other protein factors[18-21].

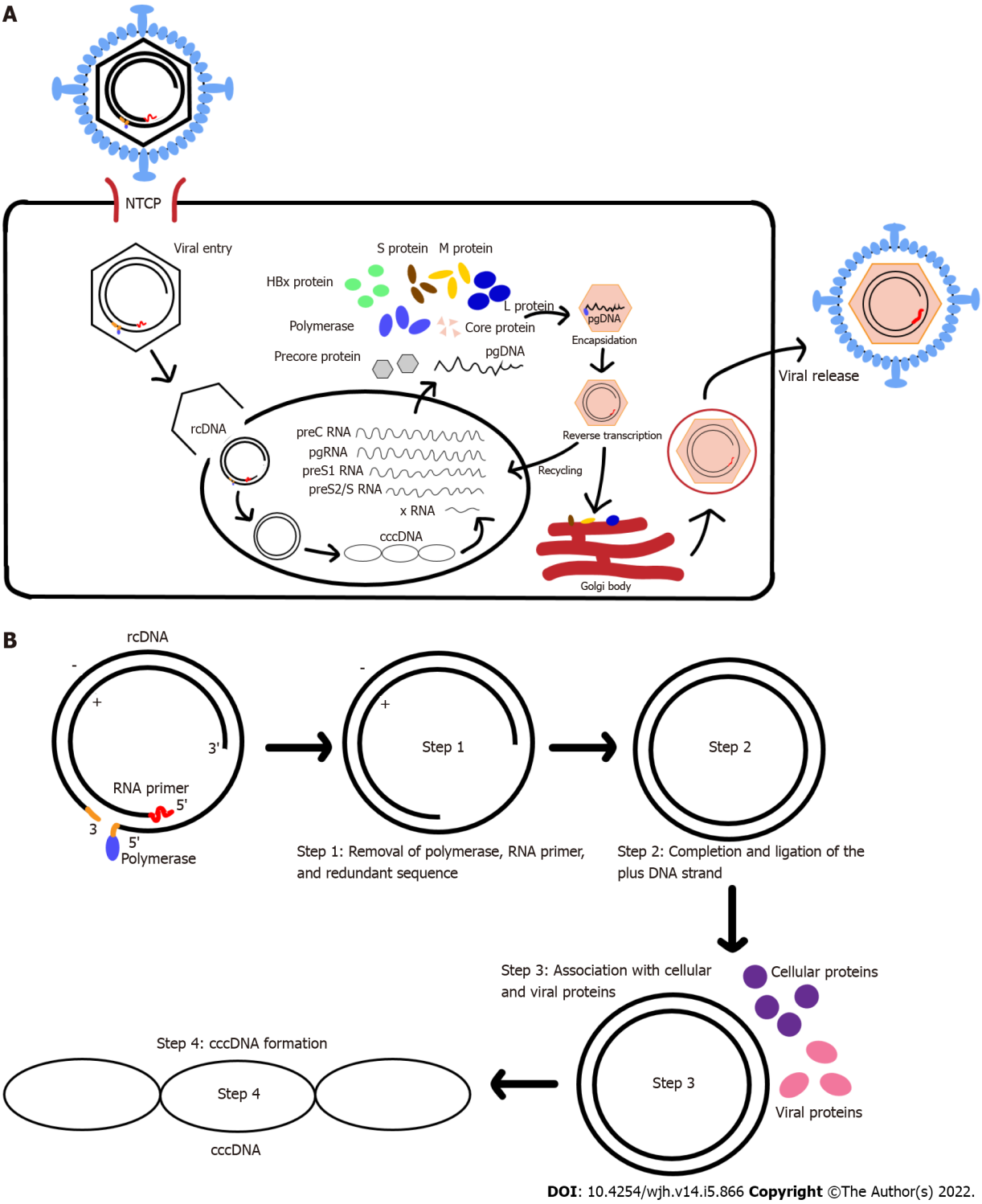

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Figure 1 Hepatitis B virus life cycle and covalently clos circular DNA. A: Hepatitis B virus entry and replication in host cell; B: Relaxed-circular DNA (rcDNA) conversion into covalently closed circular DNA (cccDNA). HBV: Hepatitis B virus; cccDNA: Covalently clos circular DN; rcDNA: Relaxed-circular DNA.

The cccDNA is generated (Figure 1B) by removing the Pol-linked terminal sequence at the 5′-end of the minus-DNA strand and the RNA oligonucleotide attached to the 5′-end of the plus-DNA strand. The gaps in both the minus- and plus-DNA strands are filled and ligated to produce the cccDNA[4]. The resulting cccDNA acts as the template for the transcription of the four viral mRNAs (Figure 1A), which are regulated by four different promoters.

HBV replication is performed through reverse transcription of pgRNA, an RNA intermediate generated from cccDNA. The pgRNA contains the ε signal and poly-A tail, which serves as template for the synthesis of minus-DNA strand by reverse transcription and translation of viral polymerase, core protein, and precursor of early antigen[1]. The encapsidation process, in which the pgRNA, Pol, and core protein are assembled to form a nucleocapsid, marks the start of HBV replication[22]. This process

is mediated by RNA-binding motif protein 24 (RBM24) *via* the interaction of RBM24 with Pol and ε signal[23]. Furthermore, several host factors, such as eukaryotic translation initiation factor 4E (eIF4E), DEAD-box RNA helicase DDX3, and APOBEC3G, have been reported to also be incorporated into the viral nucleocapsid[24-26].

The polymerase enzyme interacts with the ε signal of pgRNA and forms the nucleocapsid in association with the core protein. HBV nucleocapsid formation begins when pgRNA, Pol, and HBcAg dimers are formed. The reverse transcription begins with packing of pgRNA-polymerase complexes into the lumen of assembling capsids. Initiation of the minus-DNA strand synthesis is done through the binding of polymerase that is covalently linked to a short DNA oligonucleotide to ε signal. Another initiation factor is a protein-priming mechanism. RNase H domain will also simultaneously degrade the pgRNA template during the minus-DNA strand synthesis, eventually resulting in a short RNA fragment that contains a capped 5′ terminal region. This capped 5′ terminal region RNA fragment will be translocated to the 3′ terminus and extended to the 5′ end of the minus-DNA strand[2,3]. Plus-DNA strand synthesis leads to the formation of rcDNA. After the DNA genome is synthesized, the nucleocapsid will interact with envelope protein in the endoplasmic reticulum (ER) to form new mature virions that will be released[1,22]. Alternatively, the nucleocapsid can also re-deliver their rcDNA to repeat the viral replication, which may cause the build-up of cccDNA within the nucleus[27].

HBV and HCC

Chronic HBV infection is a growing public health issue with around 300 million people infected worldwide[28]. Depending on the age and route of infection, around 25% of individuals with chronic HBV infection could develop HBV-related HCC[29-31]. Indeed, chronic HBV infection is one of the leading risk factors for HCC development in most parts of the globe[28].

An important intermediate factor for HCC development from chronic HBV infection is the development of HBV-associated cirrhosis[32]. Studies have observed a strong relationship between cirrhosis from chronic HBV infection and the development of HCC, in which around 70%-90% of all HCCs developed from decompensated cirrhosis[29,33]. The repeated hepatitis flares or continuous recruitment of inflammatory cells and cytotoxic T cells (CTLs) to the liver may eventually lead to fibrosis and cirrhosis, and increase the risk of developing HCC[34-36]. It is a general understanding that HCC development from chronic HBV infection is the result of multifactorial mechanisms[28]. However, most studies had identified three major contributing factors for HBV-related hepatocarcinogenesis: (1) Chronic inflammation with continuous cycles of destruction and regeneration of hepatocytes; (2) cccDNA persistence and HBV DNA integration into the host genome; and (3) expression of oncogenic viral proteins and/or consequence of HBV-mediated alterations of various cellular pathways[30,31,37-40].

HBV CCCDNA

Function of cccDNA

cccDNA is generated from rcDNA as a plasmid-like episome that is retained in the nucleus of host cells. cccDNA forms a minichromosome with around 3 to 50 copies per infected cell, which decrease as the host cell divides[41]. However, cccDNA distribution among daughter cells is presumed to be unequal during cell division, which allows the cccDNA to form distinct pools that differ in their degradation susceptibility[42,43]. Therefore, the number of cccDNA copies during cell division can be maintained from the newly synthesized rcDNA-containing nucleocapsids that are imported into the nucleus. The intracellular amplification of cccDNA occurs during intracellular recycling and plays a major role in the early phases of HBV infection[41,44].

Fundamentally, cccDNA acts as a template for the transcription of all viral RNAs[44], including pgRNAs and other viral RNAs that are essential for viral proteins production. As such, cccDNA is very important for viral replication and progeny generation[45,46]. The pgRNA generated from cccDNA may also be reverse transcribed to form rcDNA for viral replication. The cccDNA function is heavily regulated by HBx protein, and inhibition of HBx protein will decrease HBV replication[47].

In addition, cccDNA is also speculated to play a role in persistent HBV infection or hepatitis relapse since cccDNA is very stable in non-dividing human hepatocytes. Furthermore, cccDNA can survive for the entire life span of the hepatocytes, thus acting as a persistent viral reservoir[46]. As cccDNA is established in the infected hepatocytes, viral replication can occur without stimulating the intrinsic antiviral defense mechanisms[46], hence making it possible for chronically infected individuals to develop hepatitis relapse after stopping the antiviral treatment. Moreover, cccDNA also mediated both HBV persistence and occult HBV infection (OBI), with OBIs resulting from epigenetic inactivation of cccDNA[48,49].

Epigenetic control of cccDNA

During HBV life cycle, the rcDNA conversion into cccDNA occurred through a repair process *via* an intermediate form called protein-free rcDNA[44,50]. The plus-strand DNA is synthesized *via* a gap-

filling mechanism, and viral polymerase and RNA primers that are attached to the 5'-termini of the minus-strand and plus-strand DNAs were removed to generate cccDNA[48]. Several host factors have been demonstrated to be involved in cccDNA formation. An *in vitro* study has shown that the host tyrosyl-DNA-phosphodiesterase 2 (TDP2) may be involved in the removal of viral polymerase that is covalently linked to the 5'-end of the minus-strand DNA[21]. However, *TDP2* gene knockout[18] resulted in uninhibited cccDNA formation in HBV infection of permissive hepatoma cells and intracellular amplification of duck HBV cccDNA. Meanwhile, *TDP2* gene knockdown resulted in increased cccDNA formation[18]. Another host protein that has been suggested to be involved in cccDNA formation is topoisomerase, although the detailed mechanism is still unclear[51].

The transcription of cccDNA is controlled in a similar way to the regulation of host chromatin. cccDNA transcription is regulated by two enhancer elements and four distinct promoters, which relies on the dynamic interplay of various transcription factors, co-activators, co-repressors, and chromatin-modifying enzymes[48]. cccDNA also contains many binding sites for ubiquitous and liver-specific transcription factors, which have been demonstrated to be involved in the transcription of viral RNAs [52].

cccDNA forms minichromosomes in the nucleus by associating with histone proteins H2a, H2b, H3, and H4 as well as linker H1, and non-histone proteins such as viral core and HBx protein[44,45,53]. Studies in hepatoma cell lines have indicated that cccDNA transcription is regulated by the acetylation status of cccDNA-bound H3 and H4 histones, as well as the acetylation status of non-histone proteins [54]. Furthermore, studies in HBV-infected patients also showed that histone hypoacetylation and histone deacetylase 1 (HDAC1) recruitment into the cccDNA were correlated with low HBV viremia [54]. Similar *in vitro* and *in vivo* studies have also demonstrated that HBV transcriptional activity and viral load were affected by the acetylation degree of cccDNA-bound histones H3/H4 and the association between cccDNA and histone-modifying enzymes[49].

HBV CCCDNA AND HEPATOCARCINOGENESIS

Role of HBx protein

cccDNA formation occurs early in the viral cycle, and due to its stability, it may persist inside the nucleus even without active viral replication as long as the infected cells survive[55,56]. cccDNA acts as the transcription template for the other viral RNAs. However, in the latter stage of infection, cccDNA activity is greatly regulated by HBx, which is required for efficient cccDNA transcription[57]. HBx protein as an oncoprotein plays crucial roles in the pathogenesis of HBV infection[58] and in the development of HCC[59]. HBx protein mainly affects the cell cycle regulation and DNA repair mechanisms to stimulate oncogenic transformation of the liver cells[60,61].

HBx protein, as the viral component of the cccDNA minichromosome, is required to initiate cccDNA-driven transcription of HBV RNA[58,62]. HBx protein regulates cccDNA function and activity by binding to cccDNA and modifying its epigenetic regulation[44]. The key roles of HBx include the degradation of structural maintenance of chromosome 5/6 (Smc5/6) restriction factors[58], the prevention of cccDNA transcriptional repressor recruitment[62,63], and the regulation of coding and non-coding RNA promoters[64]. HBx protein-mediated degradation of Smc5/6 may act as a host restriction factor that suppresses the transcription of cccDNA. HBx protein also binds to damaged DNA-binding protein 1 (DDB1), promoting the interaction of Smc5/6 with Cul4, a component of E3 ubiquitin ligase. This interaction triggers the ubiquitination and degradation of Smc5/6 complex, thus promoting cccDNA transcription[65].

In addition, HBx protein has also been demonstrated to recruit chromatin regulators such as p300 and other acetyltransferases to cccDNA, which further enhances viral transcription[62]. Consequently, mutations in HBx protein caused impaired viral replication and severely impaired acetyltransferases recruitment. Failure in recruiting acetyltransferases causes cccDNA to be deacetylated by histone deacetylases, thus reducing both viral transcription and replication[62].

On the other hand, the absence of HBx protein results in rapid silencing of cccDNA which is then maintained in a close state. HBx protein promotes the de-silencing of cccDNA, which converts the cccDNA into the open state and activates the cccDNA. This de-silencing process is usually done by stimulating the activating modifications, blocking the repressive modifications, or both[45]. As cccDNA activity is tightly regulated by HBx, the effect of cccDNA on HBV-related carcinogenesis may occur directly through its stability and persistence in infected cells and indirectly through HBx-related effect and interaction with numerous host factors that regulate cell cycle and cell death. The proposed effects of cccDNA in HBV-related hepatocarcinogenesis including the involvement of HBx and several host proteins are described below and are presented in Figure 2.

Modulation of HBx/STAT3/miR-539/APOBEC3B: Long non-coding RNAs (lncRNAs) are transcripts of more than 200 bp that are not translated into proteins. Recent transcriptome sequencing has revealed that some lncRNAs may contain pseudogenes, which are ancestral copies of protein-coding genes[66,67]. HULC (Highly Upregulated in Liver Cancer) is one of the first and most studied lncRNAs in HCC.

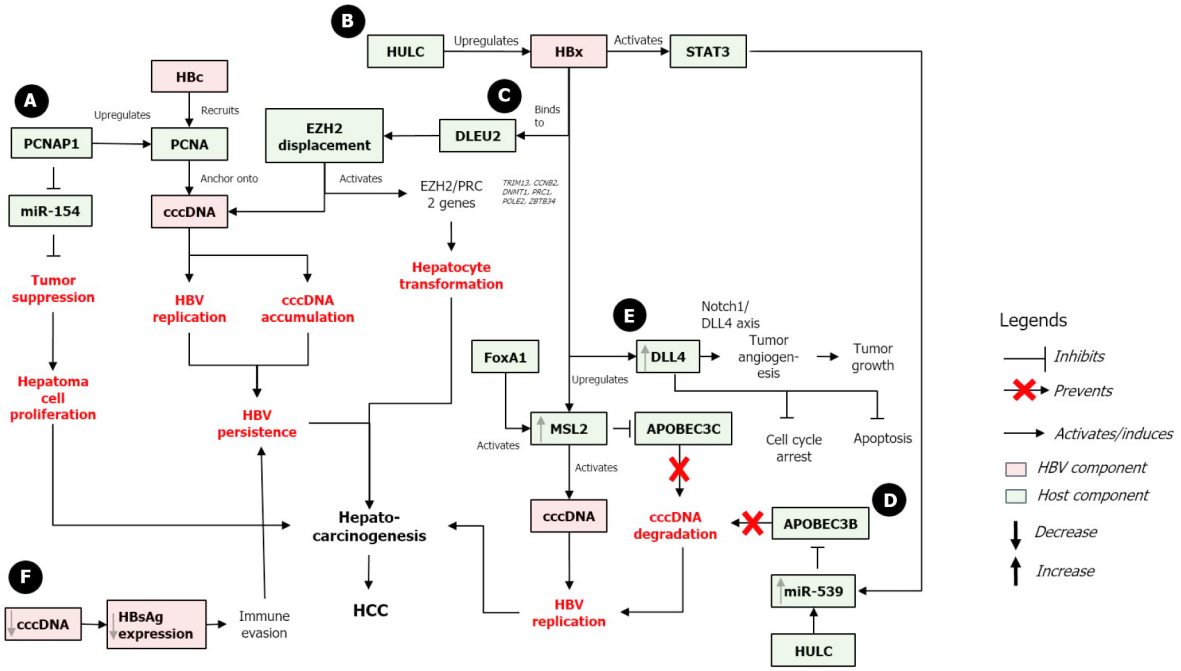

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Figure 2 Proposed mechanisms for the role of covalently closed circular DNA in hepatocarcinogenesis. A: Modulation of miR-154/PCNA/covalently closed circular DNA (cccDNA) signaling; B: Modulation of HBV X protein (HBx)/STAT3/miR-539/APOBEC3B; C: Positive feedback loop of HULC and HBx/MSL2/cccDNA; D: HBx/DLEU2 interaction to activate cccDNA; E: HBx/DLL4/Notch 1 signaling pathway; F: Reduction of cccDNA levels to avoid immune recognition.

It is found highly expressed in HCC tissues[68].

In HBV-infected HCC cells, HULC enhanced the stability of cccDNA by preventing its degradation by APOBEC3B, thus activating HBV and promoting the growth of cancer cells. Further, HULC also significantly increased the levels of HBeAg, HBsAg, HBx, and cccDNA to activate more HBV replication. At the same time, HULC upregulated miR-539, which targeted *APOBEC3B* mRNA for deactivation. APOBEC3B is also responsible for cccDNA elimination by inducing its deamination. Therefore, APOBEC3B inhibition allows for active cccDNA and promotes HBV replication. Simultaneously, HULC also upregulated HBx which co-activated STAT3. The activation of STAT3 stimulated activation of miR-539 promoter, which further downregulated APOBEC3B and enhanced hepatocarcinogenesis by promoting hepatoma cell growth[69].

Positive feedback loop of HBx/MSL2/cccDNA: HBV cccDNA is deaminated by APOBEC3A and APOBEC3B, and overexpression of these APOBECs resulted in decreased cccDNA levels[58]. HBx protein has also been documented to modulate degradation of Smc5/6 complex by hijacking DDB1-containing E3 ubiquitin ligase[65].

An E3 ubiquitin ligase, male-specific lethal 2 (MSL2), is upregulated in HCC compared to adjacent non-tumorous liver tissues, suggesting that MSL2 might be involved in hepatocarcinogenesis. MSL2-induced cells have elevated levels of cccDNA, while MSL2 knockdown resulted in the opposite. These suggest that MSL2 may activate cccDNA in hepatoma cells to accelerate HBV replication leading to hepatocarcinogenesis. Moreover, MSL2 induced the degradation of APOBEC3C, thus further suggesting that MSL2 can activate and maintain the levels of HBV cccDNA in hepatoma cells[70].

HBx protein also contributes to the upregulation of MSL2 in hepatoma cells. In clinical HCC samples, high level of HBx mRNA is accompanied with high level of MSL2. MSL2 upregulation was associated *via* YAP/FoxA1 signaling, where upregulation of FoxA1 further activated the *MSL2* promoter. Altogether, the positive feedback between HBx, MSL2, and cccDNA may contribute to HCC development by further enhancing the growth of hepatoma cells[70].

HBx/DLEU2 interaction and cccDNA activation: HBx protein can sustain the transcription of cccDNA and HCC-related genes by binding to DLEU2 (Deleted in lymphocytic leukemia 2), an lncRNA expressed in the liver. DLEU2 was upregulated in HBV-related HCC and in HBV/HBx-expressing cells [71]. HBx protein binding to DLEU2 activated DLEU2, resulted in increased cccDNA transcription and HBV replication. Furthermore, HBx-mediated DLEU2 upregulation and HBx recruitment to the target gene regulatory sequence increased chromatin accessibility and activated a subset of EZH2/PRC2 targets in both HBV-replicating cells and HBV-related HCCs. EZH2 (enhancer of zeste homolog 2) is the major cellular H3K27 trimethyl-transferase that catalyzes the addition of methyl groups at lysine 27 of

H3 histone[72]. EZH2 is found to be overexpressed in many cancers including HCC.

Furthermore, *in silico* modeling and biochemical evidence suggested that HBx and EZH2 compete for the same binding sites in DLEU2 intron 1, and co-recruitment of HBx and DLEU2 on cccDNA displaces EZH2 from the viral chromatin to boost both viral transcription and replication. DLEU2-HBx association with the target host promoters relieved EZH2 repression, which eventually led to the activation of a subset of EZH2/PRC2 targets in HBV-replicating cells and in HBV-related HCCs. Several regulatory genes (*TRIM13, CCNB2, DNMT1, PRC1, POLE2*, and *ZBTB34*) that play roles in DNA replication, cell cycle, and mitosis were also co-regulated by HBx, DLEU2, and EZH2[73]. These data suggested that co-recruitment of HBx and DLEU2 may modulate the infected hepatocytes cell cycle, which may induce hepatocyte transformation and hepatocarcinogenesis.

HBx/DLL4/Notch 1 signaling pathway: The role of HBx-mediated DLL4 (Delta like canonical notch ligand 4) upregulation and Notch signaling in hepatocarcinogenesis has been reported[74]. DLL4 is a Notch ligand that plays a role in angiogenesis including tumor angiogenesis. It can act as both an oncogene and a tumor suppressor gene[75]. Overexpression of HBx protein in HCC cell line upregulates the expression of all Notch ligands, suggesting the role of the Notch pathway in oncogenesis[76]. Silencing of DLL4 led to cell cycle arrest and increased apoptosis of HCC cells. Meanwhile, HBx overexpression resulted in DLL4 upregulation in HCC cells. The HBx-mediated DLL4 upregulation activates Notch signaling *via* Notch1/DLL4 axis to induce angiogenesis, thus promoting tumor growth[74,77].

Modulation of miR-154/PCNA/cccDNA signaling

PCNA (Proliferating Cell Nuclear Antigen) has been identified to play a role in hepatocarcinogenesis [67]. PCNA is a coordinator of DNA polymerase that plays a role in genomic integrity maintenance at both genetic and epigenetic levels, thus having multiple roles in DNA replication and repair by interacting with various proteins[78]. PCNA has been associated with the expression of miR-154[79], a tumor suppressor that inhibits tumor cells proliferation and metastasis[80]. Notably, miR-154 appears to be downregulated in multiple types of cancers, including HCC.

Similarly, the lncRNA PCNAP1 has been shown to promote HBV replication and cccDNA accumulation. PCNAP1 expression in HBV+ cells was 10- to 20-fold higher compared to HBV- hepatoma cells, and its level were significantly higher in HCC relative to the adjacent non-tumorous liver tissues. HBV DNA and cccDNA were upregulated *in vitro* in PCNAP1-transfected cells, while PCNAP1 knockdown resulted in the opposite effect. This *in vitro* result was supported by the clinical observation that PCNAP1 expression was significantly higher in cccDNA+ HCC tissues compared to cccDNA- HCC tissues[81]. Interestingly, PCNA (the ancestor of PCNAP1) was found anchored onto cccDNA *via* the interaction with HBc protein. HBc recruited and anchored PCNA onto cccDNA to induce HBV replication and cccDNA accumulation, thus further contributing to HBV persistence that may lead to hepatocarcinogenesis[81].

In relation with microRNA, PCNAP1 competed with miR-154 to enhance PCNA expression, resulting in the inhibition of miR-154. The inhibition of miR-154 led to unregulated cell proliferation and could induce hepatocarcinogenesis. PCNAP1 and PCNA significantly promoted the growth of hepatoma cells both *in vitro* and *in vivo*, suggesting the effect of PCNAP1/PCNA on the growth of HBV-related HCC [81].

Immune evasion by reduced cccDNA levels

A recent study has investigated the relationship between serum HBsAg and intrahepatic cccDNA in HBV-associated HCC. This study showed that the levels of serum HBsAg and intrahepatic cccDNA were significantly reduced in HBV-associated HCC tissues. The cccDNA reduction is speculated as the result of host tumor suppressor activity which controls the proliferation of cancerous cells by inducing the eradication of intrahepatic cccDNA. This cccDNA reduction also led to reduced expression of HBsAg, which could consequently contribute to immune evasion of the cancerous cells. Thus, this immune evasion strategy may further contribute to HBV persistence and eventually induce hepatocarcinogenesis[82]. However, the actual significance of these cccDNA and HBsAg reductions, and the exact mechanisms and the host factors involved in the cccDNA reduction in HBV-associated HCC tissues remain unclear and require further investigation.

DETECTION OF CCCDNA

The detection of cccDNA in a patient's serum and/or liver biopsies is important for the treatment of CHB. There have been numerous detection methods for cccDNA developed throughout the years. Southern blot is the gold standard for quantitative cccDNA detection; however, it is quite complicated and not suitable for high-throughput screening. Several more sensitive and simpler methods have been utilized, such as PCR-based methods, invader assays, *in situ* hybridization, and surrogates or substituted markers as described in Table 1[83].

Table 1 Methods to detect covalently closed circular DNA

Method	Advantages	Disadvantages	Ref.
Blotting			
Southern blot	Specific detection of DNA sequences; Able to distinguish cccDNA from other viral DNA species; Reliable and reproducible	Complicated, time-consuming, and costly	[101-102]
PCR-based methods			
Conventional qPCR	Simple, accurate, and sensitive; Suitable for high-throughput screening; Rapid and economical	Specificity is reduced if high concentration of rcDNA is present	[100,102]
Semi-nested and nested qPCR	Sensitive and specific; Allows for cccDNA quantification		[100,102,104, 106]
Competitive qPCR	Sensitive; Can readily distinguish cccDNA from rcDNA; Allows for cccDNA quantification		[100,102,107]
Droplet-digital PCR	Very sensitive and accurate; Can detect a single copy of cccDNA precisely; Allows for cccDNA quantification		[102,108-114]
Rolling circle amplification qPCR	Very sensitive; cccDNA is visible at single-cell resolution	Effective amplification may be hindered by cross-linked proteins or diffusion of DNA	[100,102,115-117]
Magnetic capture hybridization PCR	Sensitive and specific; Allows for selective isolation of cccDNA; Reproducible	Unable to capture all cccDNA completely; Complicated and costly	[100,102,118-119]
Invader Assay	Specific, simple, and reproducible		[100,102,121-124]
In situ hybridization	Specific; Can distinguish different types of nucleic acids; Visible at single-cell resolution	Complicated probe design	[100,102,125-126]
Indirect method			
Surrogate markers	Non-invasive; Convenient and cost-effective; Suitable for high-throughput screening	Indirect	[100,114,126-128,131-135]

cccDNA: Covalently closed circular DNA.

Southern blot

Southern blot is a molecular biology method for the detection of a specific DNA sequence in DNA samples. It is a straightforward and reliable method for cccDNA assay using cell culture samples[84]. Due to its specificity, cccDNA detection using Southern blot may also distinguish the cccDNA from other viral DNA species by the differential migration rate during electrophoresis. Southern blot is performed in sequential steps including probe preparation, electrophoresis, transmembrane hybridization, and detection[84]. It is a reliable and reproducible method, with a limit of cccDNA detection of around 2×10^6 copies. However, Southern blot procedures can be complicated, time-consuming, and costly[85].

PCR-based methods

PCR-based methods for cccDNA detection include conventional qPCR, competitive qPCR, real-time PCR, droplet-digital PCR, rolling circle amplification qPCR, and magnetic capture hybridization qPCR. Conventional qPCR is a simple method that has been used for cccDNA detection with a limit of detection of 2×10^3 copies/mL[85]. It is a rapid, accurate, economical, and sensitive method, which makes it suitable for high-throughput screening. However, the specificity of conventional qPCR is compromised if a high concentration of rcDNA is present in the sample. This is due to the shared partial homology of rcDNA and cccDNA, which reduces the specificity of conventional qPCR towards cccDNA [83]. Thus, chimeric sequences may be used to improve the specificity of conventional qPCR. These chimeric sequences consist of two different segments: Segment A which is complementary to HBV DNA plus-strand from nucleotide number 1615 to 1604, and segment B which is consensual to the HIV LTR region and dissimilar from HBV DNA[86].

A similar detection technique is the semi-nested and nested qPCR, which are sensitive and specific with a limit of cccDNA detection of 3.0×10^2 copies/mL[83,85]. Semi-nested qPCR includes two PCR reactions where the second PCR reaction uses the generated products from the first PCR as the templates. The first PCR reaction can only generate products when the template cccDNA is above a certain concentration as the primer pairs were only partially complementary[87]. Similarly, nested qPCR also includes two PCR reactions, in which the first PCR reaction uses outer primers, while the second PCR reaction uses inner primers with the first PCR products as the template. Nested PCR has been used to quantify cccDNA in peripheral blood mononuclear cells and bone marrow mononuclear cells[88]. The

PCR sensitivity and accuracy can be increased by using two hybridization fluorescence resonance energy transfer (FRET) probes in real-time PCR. This modification maintains a cccDNA:rcDNA specificity ratio greater than 1:10000[89].

Competitive qPCR is a more sensitive method for cccDNA detection compared to Southern blot, as it can readily distinguish between rcDNA and cccDNA with a limit of detection of 2×10^4 copies[85]. However, its specificity is compromised in the presence of a high concentration of rcDNA[83]. Competitive qPCR involves two templates: A competitor template with known quantity and a target template with an unknown quantity. These two templates will combine and compete for the same cccDNA-specific primers with comparable amplification efficiency during PCR. Thus, the length of the PCR product templates will be different and can be quantifiable[90].

Droplet-digital PCR (ddPCR) is a super sensitive and accurate method of detecting cccDNA with a limit of detection of only one copy[85]. The number of cccDNAs that persist in infected cells after antiviral therapy is usually very scarce, thus a more sensitive and reliable system is needed to detect and quantify cccDNA in these cells. This issue can be overcome by utilizing ddPCR[91-96], as it uses specific primers that can precisely detect one single copy of HBV cccDNA. In ddPCR, samples are partitioned in water-in-oil droplets into tens of thousands of droplets, and each droplet acts as an independent reactant for a conventional PCR. Thus, a droplet that contains a detectable fluorescent signal is scored as a positive event, while droplets with no detectable signal are scored as a negative event[83]. This approach allows ddPCR[91-96] to accurately detect low copy number of HBV cccDNA in the samples. The high specificity and accuracy of ddPCR for cccDNA detection had been confirmed using a cohort of OBI patients, where cccDNA was able to be detected and quantified in half of the examined OBI cases [97].

Rolling circle amplification qPCR is developed to increase the sensitivity and specificity of cccDNA detection in formalin-fixed paraffin-embedded (FFPE) liver biopsy tissues, since regular qPCR is unusable in this type of sample. It is also designed to minimize the interference of integrated HBV DNA in FFPE liver biopsy tissue, as cccDNA quantity in FFPE liver tissue is usually 100-fold less than that in cryo-preserved liver tissue[98,99]. This method is very sensitive and cccDNA is visible at single-cell resolution with a limit of detection of two copies per cell[85]. However, effective amplification may be hindered by diffusion of the amplified DNA into neighboring cells or by cross-linked proteins[83]. A modified rolling circle amplification-*in situ* qPCR technique has since been developed to accurately visualize the distribution and localization and also quantify the number of cccDNA copies in the liver tissue[100].

The magnetic capture hybridization qPCR allows for selective cccDNA isolation as well as enrichment for specific cccDNA quantification[101], with a limit of detection of 90 IU/mL[85]. This method is sensitive, specific, and reproducible. However, it is not able to completely capture all cccDNA, and can be complicated and costly[83]. The magnetic beads used to capture the cccDNA are synthesized using the reverse microemulsion method and further modified with streptavidin[102]. The captured cccDNA is released through denaturation and further processed using conventional qPCR [101].

Invader assay

Invader assay is a non-PCR signal amplification assay that is used for genotyping and gene expression monitoring, and able to detect only one strand of double-stranded DNA[103]. This assay was first used to quantify cccDNA in CHB patient serum[104]. It is specific, simple, and reproducible[83], with a limit of detection of 10^4 copies/mL[85]. Invader assay requires two oligonucleotides (a primary probe and an invader probe) and a FRET cassette. The two oligonucleotides will hybridize to the target DNA to form a partially overlapping structure. This overlapping structure is cleaved by a cleavase enzyme to generate a 5′-flap from the primary probe. Meanwhile, another primary probe will cycle to the target DNA and hybridize with the invader probe to form an overlapping structure. The released 5′-flaps will increase proportionally to the concentration of the target DNA. The FRET cassette will react with the released 5′-flaps to generate fluorescent signals that can be measured using real-time PCR. The differences between cccDNA and other forms of HBV DNA are used for the design of primary and invader probe sequences, resulting in positive signals for cccDNA and negative signals for non-cccDNA [105-107].

In situ hybridization

In situ hybridization was first performed[108] using digoxigenin-labeled single-stranded probe in HBV producing HepG2 cells, but was not done in liver tissues. *In situ* hybridization is specific, as it can distinguish and locate different DNAs, RNAs, and proteins without diffusion of the amplified products, and is visible at a single-cell resolution[83]. However, this method has a complicated probe design and its limit of detection is only one copy even under optimal conditions[85]. Recently, a highly sensitive and specific modification of *in situ* hybridization from ViewRNA assay was designed by using a probe set that spans the gap in rcDNA[109].

Surrogate markers

Indirect methods can also be used for cccDNA detection by using different surrogate markers that

correlate with the quantity of cccDNA in an infected cell. This approach allows for non-invasive method that is more convenient and cost-effective, and is also suitable for high-throughput screening[83]. Numerous markers have been correlated with cccDNA concentration, including hepatitis B core-related antigen (HBcrAg), HBsAg, HBeAg, and anti-HBc-IgG[83,97,109,110].

HBcrAg is detectable in HBsAg-negative CHB patients with undetectable HBV DNA. The decrease of HBcrAg levels is significantly associated with a hopeful HCC prognosis, as HBcrAg was shown as a reliable marker to predict HCC occurrence. Furthermore, HBcrAg is also correlated with both serum HBV DNA and intrahepatic cccDNA levels[110]. A study using chemiluminescent enzyme immunoassay to measure the HBcrAg levels in 130 CHB patients has found that HBcrAg level was correlated with serum HBV DNA, intrahepatic HBV DNA, pgRNA, and cccDNA levels[111]. Furthermore, patients who were negative for HBcrAg had less liver cccDNA and lower cccDNA activities than patients who were HBcrAg-positive. These finding suggest that HBcrAg may be used as a reliable surrogate marker for intrahepatic cccDNA and its transcriptional activities[111].

HBsAg has also been correlated with cccDNAs. The decline of cccDNA in liver biopsies was correlated with the decline in serum HBsAg during therapy, suggesting that quantification of serum HBsAg may represent a non-invasive surrogate marker for intrahepatic cccDNA pools[112,113]. A study has shown that serum HBsAg levels are significantly correlated with both intrahepatic HBsAg and cccDNA levels in matched non-cancerous tissues[109]. This finding is also supported by another study with similar results[114]. However, serum HBsAg levels were found not correlated with intrahepatic HBsAg and cccDNA levels in HCC tissues, while intrahepatic HBsAg levels were significantly correlated with intrahepatic cccDNA both in matched non-cancerous tissues and in HCC tissues. Further, the intrahepatic cccDNA levels in HCC tissues were significantly lower than those in matched non-cancerous tissue[109]. These findings were also supported by other similar studies[114,115]. In contrast, two different studies had showed opposite findings, and found no significant differences in intrahepatic cccDNA levels between tumor and non-tumor liver tissues in HBV-related HCC patients [116,117].

In addition to HBcrAg and HBsAg, HBeAg levels have also been shown to be correlated with cccDNA concentrations. Thus, HBeAg reporter assay may be a convenient and cost-effective tool for high-throughput screening for cccDNA targeting drugs[83,118]. Serum anti-HBc-IgG level has also been associated with intrahepatic cccDNA, as such titer of anti-HBc-IgG may be useful as a surrogate marker to predict the risk of OBI reactivation especially in immunosuppressed patients[97].

EFFORTS TO TARGET CCCDNA

The virological key to persistent HBV infection is cccDNA that persists in the nucleus of infected cells. However, current therapies for CHB infection, interferons and nucleoside analog inhibitors, are unable to effectively remove and/or eliminate cccDNA. cccDNA persistence also resulted in HBV reactivation when antiviral treatment is stopped and in immunosuppressed condition. It has been proposed that any mutations occurring in the cccDNA may be highly conserved during HBV life cycle and can quickly give rise to circulating mutant viruses that may result in antiviral resistance[119]. This may lead to both virological and clinical breakthrough in patients and faster progression to cancer development. Even so, cccDNA reduction or loss has been reported in small numbers of patients, through yet unclear mechanisms, but most likely achieved through a combined processes of reduced cccDNA formation due to rcDNA depletion, degradation of pre-exisiting cccDNAs, and loss/turnover of infected cells[56]. Thus, effective elimination of cccDNA is required to reduce HBV-related liver disease progression and to achieve complete cure.

Recently, more studies have been conducted to find effective strategies to eliminate HBV cccDNA, which include: Silencing of cccDNA expression by gene editing techniques or silencing of cccDNA transcription *via* epigenetic modifications[41,120]. These approaches are still in early stages of development and has to tackle many issues before any viable clinical application. Nevertheless, some have shown good potential as an effective approach for cccDNA elimination. The summary of gene editing and epigenetic modification techniques to target and eliminate cccDNA is listed in Table 2.

Gene editing techniques

Synthetic RNAi: RNAi is an endogenous gene regulatory pathway that can be reprogrammed by exogenous RNA molecules to create synthetic RNAi that targets specific sequences. As viral RNA transcripts contain overlapping sequences, a single RNAi trigger can, in theory, be utilized to degrade all viral transcripts, resulting in prevention of viral proteins production by eliminating cccDNA and other viral RNA transcripts. RNAi triggers may also be used to target pgRNAs, thus contributing to the reduction of cccDNA reservoir in infected cells *via* inhibition of viral replication[121]. A clinical trial of an RNAi-based drug, ARC-520, showed that ARC-520 was active in both HBeAg-negative and -positive patients. However, the reduction in HBsAg level seemed to be hindered by the concomitant expression of HBsAg from the integrated HBV DNA. This result indicated that specifically designed RNAi that can also target the viral transcripts from the integrated HBV DNA will be crucial for the total elimination of

Table 2 Gene editing and epigenetic modification techniques to target and eliminate covalently closed circular DNA

Technique	Study model	Study results	Ref.
Gene editing			
Synthetic RNAi	Clinical trial	ARC-520 was well tolerated, with only two serious adverse effects. ARC-520 was active in both HBeAg-neg and HBeAg-pos patients, but only moderate reduction in HBsAg was observed	[122]
Zinc finger nucleases (ZFNs)	*In vitro* (AAV-mediated delivery of ZFNs in HepAD38 cells)	Completely inhibited HBV DNA replication and decreased HBV pgRNA level	[124]
	In vitro	Decreased pgRNA level, thus having the potential to target cccDNA	[123]
Transcription activator-like effector nucleases (TALENs)	*In vitro* and *in vivo* [murine hydrodynamic injection (HDI)]	Efficient disruption of target sites and suppression of viral replication markers; targeted mutation in 35% of cccDNAs was observed *in vitro* under mildly hyphothermic conditions and further confirmed *in vivo*	[125]
CRISPR/Cas9 System	*In vitro* (A64 cells)	Inhibited both HBV antigen expression and replication, excised the entire full-length of integrated HBV genome, and disrupted cccDNA	[126-131]
Epigenetic modification			
Dicoumarol	*In vitro* (HBV-infected cells HepG2-NTCP cells) and *in vivo* (humanised liver mouse)	Reduced HBx protein expression, therefore having a potent antiviral activity against HBV RNAs, DNA, HBsAg, and HBc protein; cccDNA-ChIP decreased active histone marks and increased repressive histone marks	[132]
	In vitro (NTCP-expressing HepG2 and primary hepatocytes)	Inhibited HBV replication in HBV-infected primary human hepatocytes by inhibiting the activity of cccDNA	[133]
Interferon-alpha (IFN)	*In vitro* and *in vivo* (chimeric uPA/SCID mice)	Hypoacetylation of cccDNA-bound histone and active recruitment of transcriptional corepressors to the cccDNA; Inhibited HBV replication and cccDNA transcription	[134]
	In vitro	Induced a prolonged suppression of human and duck HBV cccDNA transcription	[135]
Zinc finger proteins (ZFPs)	*In vitro* (male longhorn hepatoma cells)	ZFPs binding to HBV enhancer region inhibited viral replication by inhibiting cccDNA transcriptional activity	[136]
Curcumin	*In vitro* (HepG2.2.15)	Reduced HBsAg and cccDNA levels up to 58% and 76%, respectively	[137]

cccDNA: Covalently closed circular DNA; HBV: Hepatitis B Virus; HBx: HBV X; ZFPs: Zinc finger proteins.

CHB infection[122].

Zinc finger nucleases: Zinc finger nucleases (ZFNs) are custom DNA endonucleases that are utilized to create DNA double-strand breaks in a specified target site and repair that double-strand break by creating sequence alterations at the cleavage sites[41]. ZFN treatment in HBV-infected cells has been shown to decrease the HBV pgRNA levels[123]. Further, HBV-targeted ZFNs were able to produce a sustained HBV level suppression for around 3 wk after the ZFN treatment[124]. This was achieved by using three specifically designed ZFNs to target HBV P, X, and C genes, which were delivered to HepAD38 cells *via* self-complementary adeno-associated viral (AAV) vectors[124].

Transcription activator-like effector nucleases: Transcription activator-like effector nucleases (TALENs) are also nucleases, similar to ZFNs. However, TALENs comprise a nonspecific nuclease that is fused to a sequence-specific DNA-binding domain, in which the DNA-binding domain is highly repeated and derived from transcription activator-like III effectors[41,120]. The efficiency of HBV cccDNA-targeting TALENs in reducing HBV replication in cell culture was first reported in 2013[125], using four TALENs that target specific sites within the S/pol, C/pol, and pol ORFs of the HBV genome. The S and C TALENs disrupted the intended target sites efficiently and suppressed other markers of viral replication. Subsequent experiment in HepG2.2.15 cells, which were triple transfected with the S TALEN under mildly hyphothermic conditions, resulted in targeted mutation in around 35% of cccDNAs. These results were further confirmed *in vivo*, where mice subjected to hydrodynamic injection of the S and C TALENs showed overall reduced markers of viral replication and accumulation of viral mutation in the targeted sites. Together, these results demonstrated the potential use of TALENs for targeted disruption of both HBV DNA and cccDNA[125].

CRISPR/Cas9 system: CRISPR/Cas9 system is the adaptive immune system of bacteria and archaea that acts against foreign DNA *via* the RNA-guided DNA cleavage[41,120]. The CRISPR-Cas9 system has been shown to successfully inhibit both HBV antigen expression and HBV replication in transfected A64 cells[126]. Furthermore, CRISPR/Cas9 system was able to excise the entire full-length of integrated HBV genome as well as disrupt cccDNAs. These findings were also supported by several other studies[127-

130]. Due to its effectiveness, CRISPR/Cas9 system is currently regarded as the best method for successfully inactivating HBV cccDNA and eliminating the entire length of integrated HBV genome in the liver cells[131].

Epigenetic modification

Dicoumarol: Dicoumarol is a competitive NADPH quinone oxidoreductase (NQO1) inhibitor that has been identified to inhibit the expression of HBx protein. Under normal circumstances, NQO1 binds to and stabilizes HBx protein by inhibiting the activity of 20S proteasome, thus preventing the proteasome-mediated degradation of HBx protein. Dicoumarol has been demonstrated to significantly reduce HBx protein expression, and has potent antiviral activity against HBV RNAs, DNA, HBsAg, and HBc protein in HBV-associated cells as well as in humanized liver mouse model[132]. Using cccDNA-ChIP (chromatin-immunoprecipitation) assay, dicoumarol treatment resulted in decreased active histone marks, but increased repressive histone marks in HBV-infected HepG2-NTCP cells. Therefore, dicoumarol exhibits a repressive effect on cccDNA transcription. This finding was also supported in an *in vivo* model that showed decreased levels of cccDNA-associated HBx protein in the dicoumarol-treated group[132]. A similar study[133] also showed that dicoumarol inhibited HBV replication in HBV-infected primary human hepatocytes by inhibiting the cccDNA activity.

Interferon-alpha: Interferon-alpha (IFN) has been shown to affect the epigenetic control of HBV cccDNA minichromosome by inducing persistent recruitment of co-repressors and components of the polycomb repressive complex 2 (PRC2) that target the acetylation and methylation of the histone tail. Therefore, IFN may provide an additional molecular mechanism for the repression of HBV transcription [134] to its immune modulating effect when used as antiviral treatment. IFN administration resulted in hypoacetylation of cccDNA-bound histone and active recruitment of transcriptional co-repressors to the cccDNA. These were achieved through the IFN effect on the reduced binding of STAT1 and STAT2 transcription factors to active cccDNA, thus mediating the epigenetic repression of cccDNA activity. IFN treatment also inhibited HBV replication and cccDNA transcription in both HCC cells and chimeric uPA/SCID mice[134].

A similar study[135] has also found that IFN treatment induced a prolonged suppression of both human and duck HBV cccDNA transcription, which was associated with a reduction of cccDNA-associated histone modifications that play a role in the activation of cccDNA transcription activity. On the other hand, downregulation of STAT1, structural maintenance of chromosome flexible hinge domain containing 1 (SMCHD1), or promyelocytic leukemia (PML) proteins resulted in increased basal level of cccDNA transcription activity and partially hindered the suppression activity of IFN towards cccDNA transcription. Meanwhile, ectopic expression of STAT1, SMCHD1, or PML can significantly reduce the activity of cccDNA. These findings indicate that IFN may modulate the epigenetic control of cccDNA function by affecting the recruitment of chromatin-modifying enzymes[134,135].

Zinc finger proteins: Zinc finger proteins (ZFPs) binding to the HBV enhancer region may inhibit viral replication by inhibition of cccDNA transcriptional activity. This was demonstrated by using six different ZFPs designed to bind to DNA sequences in the duck HBV enhancer regions[136]. The enhancer regions are the accessible parts of the cccDNA minichromosome which control the HBV core and surface promoters. Thus, ZFPs binding to these regions will interfere with viral transcription. The ZFPs were cloned into a eukaryotic expression vector and co-transfected into longhorn male hepatoma cells. The results demonstrated that ZFP treatment caused a significant reduction in viral RNA and HBV protein levels, indicating the effect of ZFPs on the transcription of viral proteins[136].

Curcumin: Curcumin is another compound that has been demonstrated to inhibit HBV infection by downregulation of cccDNA-bound histone acetylation in HepG2.2.15 cell line[137]. Additionally, treatment with 20 μmol/L curcumin for 2 d resulted in reduced HBsAg and cccDNA levels in HepG2.2.15 cells by up to 58% and 76%, respectively. Moreover, treatment with curcumin resulted in both time- and dose-dependent reductions of H3 acetylation levels, thus contributing to the reduction of H3- and H4-bound cccDNA. These findings indicate the potential use of curcumin as a cccDNA-targeting antiviral agent[41,137].

CONCLUSION

Chronic HBV infection remains a global health problem since it may lead to prolonged inflammation and subsequently more advanced liver diseases, including liver cancer. Furthermore, direct oncogenic properties of HBV viral components have been associated with their abilities to interact and alter the functions of various host genes, further contributing to HBV pathogenesis.

cccDNA is one of the most important HBV components. cccDNAs may persist in infected hepatocytes and serve as template for viral replication machinery. This highlights the need for an effective method for cccDNA detection and removal. Various methods have been developed for cccDNA detection and

targeted removal; however, their overall sensitivity and specificity are still far from satisfactory. The use of antiviral therapy and/or interferon has been shown to effectively reduce the viral load, improve the general health status, and prevent the development of HCC in chronic HBV patients. However, current antiviral therapy does not eliminate the cccDNA in the liver. Based on our review, we presume that the versatility of PCR-based technologies may be a potential approach for advancing effective methods for cccDNA detection and quantification. As for cccDNA targeting, the vast application of CRISPR/Cas9 system might be the most optimum resort to modify cccDNA function, and more importantly to inactivate the cccDNA activity.

Nevertheless, concentrated effort should be focused more on prevention of HBV infection and not on HBV treatment and elimination. As shown in our previous review[138], this preventative approach, which may be achieved through immunization, is crucial to prevent viral transmission to the new generations, particularly in endemic areas. At the same time, it is also necessary to improve the awareness of the general public for the consequences of the disease and to expand the national and regional surveillance program.

FOOTNOTES

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REVIEW

Emerging curative-intent minimally-invasive therapies for hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the most common cause of liver malignancy and the fourth leading cause of cancer deaths universally. Cure can be achieved for early stage HCC, which is defined as 3 or fewer lesions less than or equal to 3 cm in the setting of Child-Pugh A or B and an ECOG of 0. Patients outside of these criteria who can be down-staged with loco-regional therapies to resection or liver transplantation (LT) also achieve curative outcomes. Traditionally, surgical resection, LT, and ablation are considered curative therapies for early HCC. However, results from recently conducted LEGACY study and DOSISPHERE trial demonstrate that transarterial radio-embolization has curative outcomes for early HCC, leading to its recent incorporation into the Barcelona clinic liver criteria guidelines for early HCC. This review is based on current evidence for curative-intent loco-regional therapies including radioembolization for early-stage HCC.

Key Words: Hepatocellular carcinoma; Loco-regional therapy; Radiation segmentectomy; Transarterial radio-embolization; Ablation; Transarterial chemo-embolization; Curative intent

Core Tip: Accepted curative modalities for early hepatocellular carcinoma (HCC) include resection, liver transplant, and loco-regional therapies. In this manuscript, we review the curative-intent loco-regional therapies including recent evidence from the LEGACY study and DOSISPHERE trial demonstrating a curative role for transarterial radio-embolization in early HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common cause of liver malignancy and the fourth leading cause of cancer death across the globe[1]. Curative outcomes can be achieved for early stage HCC, which is defined using the Barcelona clinic liver criteria (BCLC) as 3 or fewer lesions less than or equal to 3 cm in diameter with preserved liver function and functional status. Patients with intermediate or advanced HCC who can be down-staged with loco-regional therapies (LRT) to resection or transplantation can also achieve curative outcomes. Traditionally, surgical resection, liver transplantation (LT), and ablation are considered curative therapies for early HCC.

In early HCC, resection and LT are often preferred over ablation when possible. However, many patients are not surgical candidates, whether due to medical comorbidities, inability to tolerate anesthesia, or tumor location. Additional drawbacks to surgical approaches include elevated post-operative morbidity and mortality rates[2,3], life-long immunosuppression in the case of LT[4], and high recurrence rates with resection[5]. Fortunately, loco-regional therapies such as radiofrequency ablation (RFA) make curative treatment possible for these patients. Studies demonstrating comparable overall survival and recurrence rates for ablation are now over a decade old, and ablation as a curative therapy has been present in the National Comprehensive Cancer Network (NCCN) guidelines since 2017[6]. However, recent work demonstrates that transarterial radio-embolization (TARE) is an effective, safe, and curative treatment option for early HCC.

This review discusses the evidence for curative outcomes in early HCC using LRT including ablation, TARE, transarterial chemoembolization (TACE), and combination therapy. We further review the role of these therapies in down-staging and bridging with curative intent for LT or resection.

CONCEPT OF CURE

While cure is the ultimate goal in the treatment of HCC, it is not always apparent what defines a curative-intent therapy. Cure for HCC using various modalities including LT, resection and ablation is reported in the form of overall survival and recurrence. When curative LT outcomes are considered for HCC, overall survival is > 70% at 5 years[7], with a recurrence rate of 6%-15%[8]. For resection, overall survival is 50%-70% at 5 years[3,9] and recurrent HCC is seen in > 60% of patients at 5 years[5,10]. Notably, these outcomes may be influenced by differing criteria between surgical and locoregional therapy candidacy; for example, surgical candidates are typically without significant portal hypertension[11]. For ablation, overall survival is around 60% at 5 years with a local recurrence rate of 3%-22% at 5 years in lesions up to 5 cm[12,13]. All three therapies are considered potentially curative in appropriate patients, and thus establish a standard for outcomes necessary to be considered curative (Table 1).

Of note, the modified Response Evaluation Criteria in Solid Tumors (mRECIST) assessment of the radiologic response to LRT validates the use of tumor response rate as a surrogate outcome for survival [14]. Generally speaking, tumor response rate utilizes established imaging criteria to group patients into non-responders, partial responders, and complete responders[15]. The commonly reported objective response rate (ORR) is the combination of partial and complete responders over all subjects. As might be expected, complete response is associated with the greatest improvement in outcomes[16].

Loco-regional therapies also play a neo-adjuvant role in the treatment of HCC. They can be used to bridge patients to definitive therapy with LT or can down-stage HCC to meet transplant criteria. Using LRT, down-staging is successful in approximately half of patients, regardless of whether TARE or TACE is used[17]. Importantly, bridging and down-staging do not worsen LT outcomes in terms of overall survival or recurrence[18-20].

Table 1 Outcomes for curative-intent therapies in hepatocellular carcinoma within Milan criteria

Modality	Overall survival at 5 yr (%)	Local tumor progression at 2 yr (%)	Local tumor progression at 5 yr (%)	Disease-free survival at 5 yr (%)
Transplant	≥ 70[21]	NDA	Cumulative recurrence < 15[22]	> 70[23]
Resection	60-80[24-26]	NDA	Resection margin recurrence 1-7 [27-29]	38-54[26,27,30]
Ablation ≤ 3 cm	44-69[13,25,31]	2-16[28,29,32]	9.7-22[13,33,34]	14-46[25,27]
TARE ≤ 3 cm	75[35]	2.4-6.1[36,37]	NDA	NDA
Ablation ≤ 5 cm	49-72[27,38,39]	6-9[40,41]	3-14[12,31,40]	50-59[27,40]
TARE ≤ 5 cm	57[35]	6.1-10[37,42]	28 for ≤ 5 cm[35]	NDA

While ablation is recommended for lesions < 3 cm, data for lesions up to 5cm is also included. Ablation studies included patients who were not surgical candidates. Data is derived from studies that included solitary and multiple lesions. Resection outcomes are limited to patients with Child-Pugh A liver function, while all other modalities include patients with Child-Pugh A and B. Data for transarterial radio-embolization should be considered preliminary. Included papers were published within the last ten years. NDA: No data available; TARE: Transarterial radio-embolization.

ABLATION

The most common ablation techniques for HCC are radiofrequency (RFA), microwave ablation (MWA), and cryoablation. In early stage HCC with preserved liver functions (Child-Pugh A/B), this is a potentially curative modality for patients who are not candidates for surgery or resection. In radiofrequency (RFA) and microwave ablation (MWA), probes are placed percutaneously into the tumor so that thermal energy may be used to directly induce tumor necrosis. In cryoablation, cold gas is delivered through hollow needles into the tumor tissue and frozen, inducing cell death[43]. For very early (BCLC 0) and early-stage (BCLC A) HCC patients with preserved liver function (Child-Pugh A/B), RFA and MWA offers survival outcomes comparable to resection despite lower baseline liver function[44-46]. These early-stage HCC patients are often disqualified from surgery by significant comorbidities, portal hypertension, poor hepatic function, intolerance to general anesthesia, or high-risk lesion location[47]. The use of ablation is limited by tumor location near central biliary structures, gallbladder, stomach, or sub-diaphragmatically given risk of unintended damage to these structures, as well as concern for the rare possibility of tumor tract seeding in case of sub-capsular tumors[35]. Additionally, ablation near large vascular structures decreases the ablative power as a heat sink effect from fluid flow draws thermal energy away from the target area[16]. A minority of patients experience a self-limiting post-ablation syndrome (PAS) characterized by fever, malaise, and chills in the first week[48]. Less than 4% of patients experience serious complications such as bleeding, abscess formation, liver failure, and damage to surrounding structures[49].

Ablation has been considered a curative treatment for early HCC ≤ 3 cm by the NCCN since 2017[39, 44]. A study including 120 patients with HCC ≤ 3 cm were randomized into either RFA or resection treatment groups. Results showed insignificant differences in the disease-free and overall survival rates at 1, 2, and 3 years. However, the RFA group exhibited meaningfully better hepatic function a week post-treatment, fewer incidences of postoperative complications, and shorter hospital stays[44]. Another study of RFA efficacy in 218 patients demonstrated complete response for lesions < 2 cm in more than 90% of the cases, with a local recurrence rate of < 1% and no mortality[16]. Studies have demonstrated that ablation of lesions up to 5 cm carries a 5-year overall survival rate of 60% with low rates of local recurrence[12].

RFA and MWA can also be considered in intermediate and advanced stage patients (BCLC B/C) for down-staging to transplantation, with demonstrable success when combined with TACE[50]. Additionally, no significant differences in overall survival were noted between patients who were down-staged *via* ablation to within Milan criteria before being transplanted, *vs* transplanted patients who defaulted within the Milan criteria[51]. Ablation can also be considered in patients within the Milan criteria for bridging to transplantation, with RFA leading to a lower waitlist dropout rate than bridging with TACE[52].

Future work should be focused on the role for new or less commonly used ablative modalities in early HCC, including high-intensity focused ultrasound[53], laser ablation[54], and irreversible electroporation[53] (Figure 1).

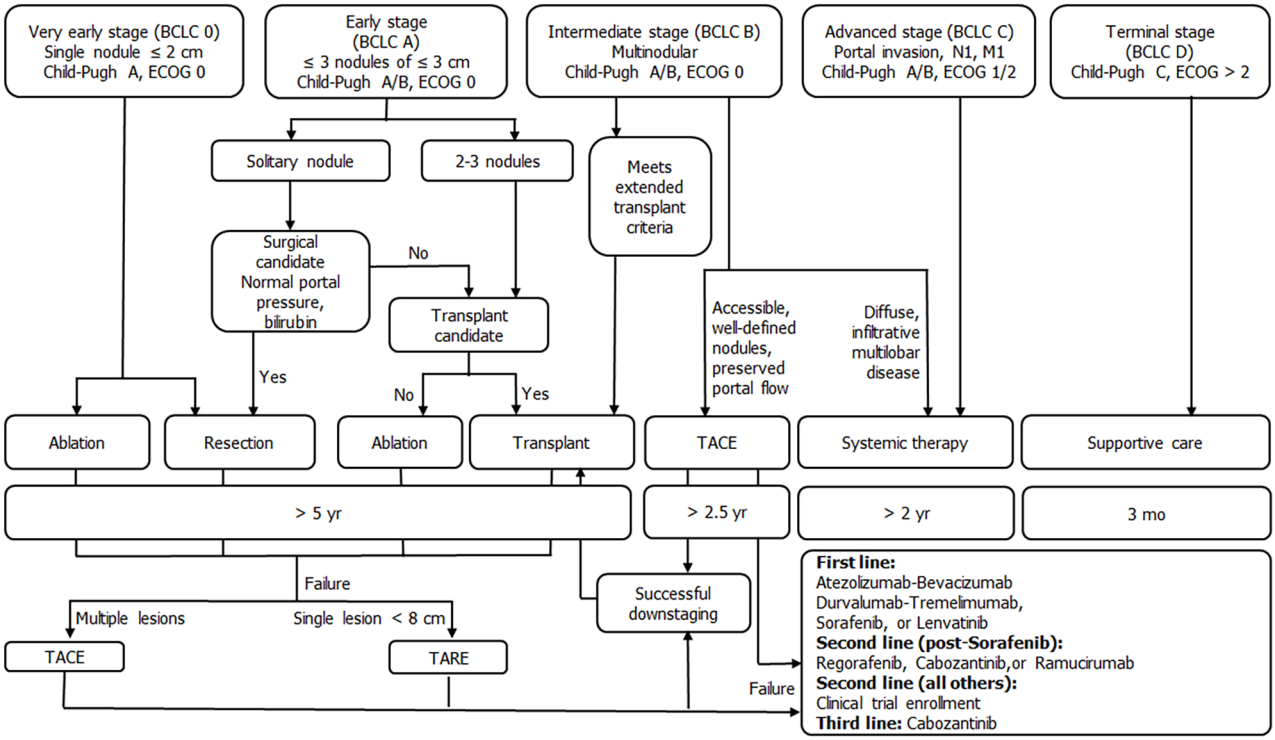

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Figure 1 Barcelona clinic liver criteria guidelines for hepatocellular carcinoma treatment.

TARE

TARE, also called selective internal radiotherapy (SIRT), is the administration of glass or resin microbeads coated in Yttrium-90 (^{90}Y) *via* a catheter into the hepatic artery supplying the tumor of interest[55]. This therapy targets tumors by taking advantage of the fact that they are preferentially supplied by hepatic arteries due to neo-angiogenesis, while liver parenchyma is supplied primarily by the portal vein[56]. Unlike for ablation therapy, tumor location in relation to other important structures is less of a concern for catheter-directed therapies; it is more important that the vascular supply to the tumor can be identified and accessed with an intravascular approach.

As a result of the studies reviewed below, TARE has recently been incorporated into the BCLC guidelines as a second-line therapy for early stage HCC. Contraindications include any shunting to the GI tract, excessive lung shunting, complete portal vein occlusion, severe liver dysfunction. Recent efforts to determine both optimal procedural approach and maximum tolerable radiation dose have led to data that suggest a curative role for TARE in early HCC[37,57]. Regarding the optimal procedural approach, there has been a trend towards increasingly selective TARE. In the past, where lobar or even whole liver radiation may have been administered, a segmental approach is now preferred[58]. Regarding radiation dose, recent work suggests targeted doses of 400 Gy or greater are well tolerated and demonstrate complete pathologic necrosis in all patients compared to prior thresholds of 190 Gy (complete response of 100% *vs* 65% respectively)[59].

Using these techniques, three studies demonstrated the potentially curative role for TARE in early HCC. In 2018, Lewandowski *et al*[35] published the results of a retrospective study on 70 patients with HCC who were treated with TARE alone. For patients with a single lesion ≤ 5 cm and preserved liver function (Child-Pugh A) who were not candidates for surgery or ablation, overall survival was (comparable to surgical resection) 98%, 66% and 57% at 1-, 3-, 5-year respectively. They also reported median overall survival (OS) of 6.7 years. This cohort with a single lesion ≤ 3 cm had 1-, 3-, 5-year overall survival rates of 100%, 82% and 75% respectively. In addition, this study reported 42.7% (100/234) patients with solitary HCC ≤ 5 cm were successfully down-staged to resection (n = 9) or LT (n = 91) after TARE.

In 2021, Salem *et al*[37] published the results of the retrospective LEGACY study which included 162 patients with solitary HCC up to 8cm (average size 2.7 cm), preserved liver function (Child-Pugh A), and preserved functional status (ECOG 0-1) who were treated with selective TARE. Patients with prior LRT, LT, resection, or systemic therapy were excluded, as were patients with vascular or extrahepatic disease or significant ascites or encephalopathy. In this study, overall survival at 3-years was 86.6% for patients treated with TARE alone (median dose 410 Gy), and 92.8% for patients who underwent TARE and successfully down-staged to LT (21%; 34/162) or resection (6.8%; 11/162). Local recurrence rate was

reported in only 5.6%. Despite the segmental delivery of radiation doses exceeding 400 Gy, there were no cases of REILD, and severe adverse events potentially related to treatment occurred in 5.6% of patients. Of note, analysis of patients who were bridged or down-staged to transplant after treatment with TARE shows similar outcomes to typical liver transplant recipients in terms of overall survival[60,61].

Garin *et al*[57] further published the DOSISPHERE trial in 2021 on role of TARE. This is a phase 2 multicenter trial comparing lobar TARE using a 120 Gy radiation dose ("standard dosimetry") to delivery of a radiation dose of > 205 Gy to the tumor itself ("personalized dosimetry"). Included patients had local, unresectable advanced disease with at least one lesion ≥ 7 cm. Patients with micro-aggregate albumin (MAA) studies demonstrating poor targeting of the tumor were excluded from the study. Personalized dosimetry was associated with significantly better response rates, defined as partial and complete responders at 3 mo using the European Association for the Study of the Liver (EASL) criteria. Partial and complete responders at 3 mo in the largest lesion were significantly higher in the personalized dosimetry group compared to the standard group: 71% (20/28) *vs* 36% (10/28). TARE achieved down-staging to resection in 36% (10/28) patients in the personalized dosimetry group and 3.5% (1/28) in the standard dosimetry group, including patients with portal vein tumor thrombus. Overall survival was improved in the personalized dosimetry group (26.6 mo *vs* 10.7 mo). This trial, while not attempting to demonstrate cure, is significant for its rigorous design, inclusion of patients with larger lesions and more advanced disease (including portal vein thrombus) and remarkable outcomes. Using more selective approaches and higher radiation doses, these studies demonstrates that the best possible outcomes for TARE are yet to come.

While the aforementioned studies used glass beads, future work will clarify appropriate dosing with resin beads. The ongoing DOORwaY90 trial will provide data on overall response and duration of response for resin beads, as well as data on safety, quality of life, bridging and down-staging (NCT04736121) (Table 2).

TACE

TACE refers to the delivery of chemotherapy *via* a catheter within the hepatic artery supplying the tumor. Chemotherapy can be delivered as a liquid solution followed by embolics or as drug-eluting beads. To date, TACE is not generally considered a curative-intent therapy. However, it may be offered to patients with early HCC as second-line therapy for those who are not candidates for surgical approaches or ablation in a process that has been termed stage migration. Typical contraindications include extrahepatic disease, main or lobar portal vein thrombus, and poor liver function (Child-Pugh C). Relative contraindications include elevated total bilirubin (≥ 2-3 mg/dL), as this increases the risk for radio-embolization-induced liver disease (REILD) in TARE and liver failure in TACE.

Novel technical approaches that have demonstrated improved survival are reviewed here. Selective TACE, defined as administration to a segmental artery, and super-selective TACE, defined as administration at the distal portion of a sub-segmental hepatic artery, both improve survival compared to lobar administration[62]. Another described technique is ultra-selective TACE, in which lipoidal is administered to the hepatic artery until opacification of the tumor's portal venous supply is seen[62,63]. In theory, this prevents post-procedural compensatory increase in portal venous supply to the tumor ensuring complete tumor ischemia and multiple studies have demonstrated improved local tumor response using this method[64,65]. While outcomes for selective and super-selective TACE include complete response rates around 40%-50%[62,66] and 5-year overall survival around 20%-35%[67], it is important to consider that TACE has primarily been studied in intermediate and advanced HCC, as opposed to early HCC. In fact, a small prospective study of selective TACE in early HCC (BCLC 0 or A) demonstrated a 3-year survival of 80%[68]. Patients most likely to benefit from TACE include those with fewer lesions and preserved liver function (BCLC A)[63,69]. Additional developments include the use of modified techniques including balloon-occlusion (B-TACE) and microvalve infusion catheters. These have demonstrated improved tumor targeting and greater rates of tumor necrosis but have not yet demonstrated improved clinical outcomes[70,71]. In the case of B-TACE, higher rates of complete response have been observed when compared to conventional TACE[70,72]. Furthermore, there is well-established evidence for the use of TACE in bridging and down-staging to transplantation[73,74].

Barriers to improved outcomes in TACE include lack of technical standards, specific chemotherapeutic agents, and the embolic effect of therapy, which prevents treatment of distal vessels. Thus, future work for curative TACE will require the development of technical standards and improved chemotherapeutics that are both tolerable and effective.

CHOOSING TACE *VS* TARE

In accordance with recent updates to the BCLC guidelines, TACE and TARE are now both acceptable second-line therapies for early stage HCC. TARE is indicated for single lesions less than 8 cm, whereas

Table 2 Comparing key features of the LEGACY study and DOSISPHERE trial

	LEGACY	DOSISPHERE
Study design	Multi-center single-arm retrospective study	Multicenter randomized control phase ii trial
Objective	To assess clinical outcomes of Y-90 glass microsphere treatment in patients with unresectable solitary HCC lesions	To compare clinical outcomes of lobar TARE using 120 Gy (SDA) versus > 205 Gy (PDA) in patients with intermediate/advanced HCC
Inclusion criteria	Unresectable solitary lesions (≤ 8 cm); BCLC A or C (ECOG 0-1); Child-Pugh score A	≥ 1 unresectable lesion ≥ 7 cm; BCLC A, B, or C
Exclusion criteria	Patients with vascular or extrahepatic disease, significant ascites, encephalopathy, or prior LRT, LT, resection, or systemic therapy	Patients with micro-aggregate albumin (MAA) studies demonstrating poor tumor targeting
Overall survival	At 3 yr, 86.6% for patients treated with TARE alone (median dose 410 Gy) and 92.8% for patients who down-staged *via* TARE	Overall survival was improved in the personalized dosimetry group (26.6 mo *vs* 10.7 mo)
Downstaging	21% successfully down-staged to LT; 6.8% to resection.	36% patients in the PDA group and 3.5% (1/28) in the SDA group down-staged to resection[1]

[1]Including patients with portal vein tumor thrombus.

HCC: Hepatocellular carcinoma; BCLC: Barcelona clinic liver criteria; LRT: Locoregional therapy; LT: Liver transplant; PDA: Personalized dosimetry group; SDA: Standardized dosimetry group; TARE: Transarterial radio-embolization.

TACE is recommended for multifocal disease. Overall survival for TACE *vs* TARE in HCC has not been directly compared in an RCT. However, a small randomized study demonstrated that TARE led to significantly increased time to progression (> 26 mo *vs* 6.8 mo) compared to TACE[75]. Other work has suggested an increased time to progression and higher quality of life for TARE compared to TACE[76]. Between TACE and TARE, the 2021 MERITS-LT trial demonstrated no differences in the rate of or time to successful downstaging to LT when either LRT was the initial downstaging treatment[77].

Considerations for choosing one over the other may be guided by patient characteristics. For example, patients with prior biliary instrumentation are higher risk for the development of hepatic abscess after TACE[78]. Despite its name, the ischemic effect of TARE is minimal compared to TACE. As a result, TARE is generally preferred for patients with significant portal vein tumor thrombus, given concerns for excessive ischemia using TACE[79-81].

COMBINATION THERAPY

In solitary HCC lesions up to 7 cm in size, combination therapy with ablation and TACE improves outcomes compared to ablation alone[82]. In 2021, Zhang *et al*[82] in a RCT of 189 patients, demonstrated superior overall survival for RFA plus TACE compared with RFA alone for early HCC < 7 cm (5-year and 7-year OS of 52% and 36% *vs* 43% and 19%, respectively). The benefit was particularly pronounced in tumors > 3 cm. This is consistent with prior studies demonstrating the benefit of combination ablation plus embolization in 3-5 cm tumors[83,84].

Given the high rates of recurrence after resection and ablation, the phase 3 STORM trial examined whether the addition of adjuvant sorafenib could reduce the recurrence rate after curative-intent treatment compared to active surveillance, but was unable to demonstrate benefit[85]. Ongoing trials explore the potential for other systemic therapies as adjuvants, including pembrolizumab monotherapy (NCT03867084), nivolumab monotherapy (NCT03383458), atezolizumab plus bevacizumab (NCT04102098), and durvalumab plus bevacizumab (NCT03847428).

The recent addition of TARE as an acceptable stage migration treatment modality for early HCC[86] suggests that future trials on combination treatments for early HCC may increasingly incorporate TARE as a treatment modality.

CONCLUSION

The treatment of early HCC is evolving, with improved outcomes for transplant, resection, and ablation. Most recently, evidence demonstrates curative outcomes for catheter-directed transarterial therapies for early HCC and suggest that a fourth modality may soon join the list of curative options. Longer follow up periods, technique standardization, and larger randomized controlled trials comparing loco-regional therapies to other curative modalities and defining patients who are most likely to benefit from TARE are needed to confirm these findings.

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MINIREVIEWS

Saving time and effort: Best practice for adapting existing patient-reported outcome measures in hepatology

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Abstract

It is increasingly recognised that collecting patient reported outcome measures (PROMs) data is an important part of healthcare and should be considered alongside traditional clinical assessments. As part of a more holistic view of healthcare provision, there has been an increased drive to implement PROM collection as part of routine clinical care in hepatology. This drive has resulted in an increase in the number of PROMs currently developed to be used in various liver conditions. However, the development and validation of a new PROM is time-consuming and costly. Therefore, before deciding to develop a new PROM, researchers should consider identifying existing PROMs to assess their appropriateness and, if necessary, make adaptations to existing PROMs to ensure their rigour when used with the target population. Little is written in the literature on how to identify and adapt the existing PROMs in hepatology. This article aims to provide a summary of the current literature and guidance regarding identifying and adapting existing PROMs in clinical practice.

Key Words: Patient reported outcome measures; Adaptation; Content validation; Hepatology; Patient reported outcomes

Core Tip: In the last few years, there has been a rapid increase in the number of patient-reported outcome measures (PROMs) in hepatology and, therefore, the choice between which of these PROMs to use can be difficult. This paper aims to illustrate ways of identifying existing PROMs and outlines key considerations and good practice with respect to their adaptation in clinical practice or research in hepatology.

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INTRODUCTION

What are patient reported outcome measures?

Patients are treated by healthcare providers with the primary goal of improving their health and well-being. Historically this improvement in health has been judged by improvement in biochemical, histological, radiological or clinical assessments. This approach does not always correlate with improvement from the patient perspective. From a patient perspective improving health is reflected in the documentation of their symptoms and experience of healthcare provision, which are more appropriately collected directly from the patient[1]. With a move towards shared-decision making and patient-centred care, there is growing recognition within the healthcare community of the importance of the patient perspective and the need to consider patient reported outcomes (PRO) as a key component of a holistic approach to patient care.

The U.S. Food and Drug Administration (FDA) defines a PRO as "any report of the status of a patient's health condition that comes directly from the patient, without interpretation of the patient's response by a clinician or anyone else"[2]. The European Medicines Agency state that "Any outcome evaluated directly by the patient himself and based on the patient's perception of a disease and its treatment(s) is called a patient-reported outcome (PRO)"[3]. Patient reported outcome measures (PROMs) can be broadly classified as generic or disease-specific instruments. Generic PROMs assess general aspects of health and can be applied across multiple conditions. Disease-specific PROMs, on the other hand, assess specific aspects that are related to a particular condition. PROMs are designed to measure aspects of health that can neither be directly observed or are not feasible to observe[4]. Broadly speaking, collection of PROMs from the patient can be classified into three main categories based on the outcomes measured: Health status and quality of life: patients' health and well-being as indicated by patient report; Patient satisfaction: patient-reported satisfaction with their medical treatment or care; Resource use: patients' reported use of health services and resources; Patient knowledge questionnaires: patients' understanding of medical conditions and the treatment.

What is currently driving the use of PROMs?

PROMs were initially developed for research use and many regulatory authorities such as the European Medicines Agency (EMA) and the FDA advocate their use[2,5,6]. Recent consensus guidance also recommends the inclusion of PROMs in clinical trial designs[7]. The collection of PROMs aligns well with the increased drive within healthcare organisations for value based healthcare, whereby organisations aim to achieve the best possible outcomes for patients with the available resources[8]. As more clinicians recognise the benefit of collecting PROMs in addition to measuring clinical outcomes, PROMs have seen an increased use in routine clinical practice[9].

As a consequence of the drive to collect PROMs, there has also been an increase in the number of PROMs developed, validated, and used. The King's Fund report reflects on this as ''a growing recognition throughout the world that the patient's perspective is highly relevant to efforts to improve the quality and effectiveness of health care'' and that PROMs are likely to become ''a key part of how all health care is funded, provided, and managed''[10]. This has been illustrated in the United Kingdom when, in 2009, the United Kingdom Government implemented the routine collection of PROMs in England for four routine elective surgical procedures — hip and knee replacement, groin hernia repair, and varicose vein surgery (https://digital.nhs.uk/data-and-information/data-tools-and-services/data-services/patient-reported-outcome-measures-proms), in order to compare performance between providers. It is likely that routine PROMs collection will be extended to more conditions in the future.

LITERATURE SEARCH TO IDENTIFY BEST PRACTICE FOR THE ADAPTATION OF EXISTING PROMS

In order to identify relevant literature regarding best practice and guidance for the adaptation of existing PROMs we undertook a scoping review of the literature. This scoping review aimed to explore the extent of the literature within the PROMs field regarding best practice/guidance for PROM adaptation without describing findings in detail[11,12].

We undertook a review of the literature to identify key papers/guidelines for the adaptation of existing PROMs. We searched PubMed and the Cochrane database (https://www.cochrane.org/). In order to limit the search, we searched for literature in the English language published within the last 10 years. Reference lists of relevant identified publications were also hand searched to identify further relevant literature. We also undertook a Google™ search to identify relevant publications.

Details of the search strategy are presented in Table 1. The searches were conducted on 17 February 2020. The inclusion and exclusion criteria are listed in Table 2.

We carried out an initial title screening, then abstract screening to identify relevant papers that fitted the inclusion criteria, which we then reviewed fully. We identified specific themes related to the adaptation of existing PROMs which we regarded as recommendations/good practice and have structured the paper according to these identified themes.

FINDINGS

Supplementary Table 1 illustrates the publications identified as part of the scoping review of the literature. The guidance identified within these publications is organised under the specific headings of: defining the requirements of a PROM, identifying and appraising existing tools, adapting existing PROMs, issues of content validity and getting the right people involved.

Defining the requirements of a PROM

In order to provide meaningful information, PROMs need to be appropriately developed and validated according to robust criteria. The psychometric validation of PROMs can be complex and time-consuming and requires evidence of numerous facets including validity, reliability and responsiveness [13,14]. Given the growth in the number of available PROMs, even within the same condition, the old adage "don't reinvent the wheel" should be the first principle applied before taking the decision to embark on the development of a new PROM. Consequently, to enable researchers to appraise the quality of existing measures with the aim of ascertaining whether a new measure is needed, researchers must first establish a clear definition of what is required of the PROM.

The requirements of the PROM need to be identified at the outset[15]. Consideration should include what the PROM aims to measure, whether the PROM should be generic or disease specific, the clinical condition of interest, the specific population for which the PROM will be applied and whether it will used as part of routine clinical care or research. These factors will help to determine whether existing PROMs are suitable or can be adapted. A useful overview and starting point for deliberations is provided by Luckett and King[16].

A generic PROM may allow comparison of patient outcomes across different conditions, however it will have less focus on specific symptoms relating to a condition. A disease-specific PROM will have a more defined focus on the condition itself and will be more sensitive to changes in the condition over time and its associated symptoms but may be longer and therefore the burden to the patient may be greater. If a disease-specific PROM is required, one needs to define the specific population. For example, a PROM developed to measure pruritus in primary biliary cholangitis may not be suitable for measuring pruritus in intrahepatic cholestasis of pregnancy as the patient experience of dyspnea may differ across these two clinical conditions[2,16]. It is also important to consider whether the PROM will be used within a routine clinical setting or a research setting[17]. In a clinic setting where time may be limited, the burden to the patient and the feasibility of completing the PROM need consideration. Within a research setting, time may not be as limited and longer, more detailed PROMs can be considered[17]. The proposed method of administration of the PROM is also important and authors planning on using a PROM should ensure that it has been appropriately validated for their proposed administration method[2]. Issues such as respondent and administrator burden — length, formatting, font size, instructions, privacy, literacy levels *etc.* also need to be considered[2]. Figure 1 provides an overview of the first steps required before choosing to develop a new PROM.

Luckett *et al*[16], have provided useful principles to consider when selecting a PROM: Selection of PROMs should be considered early during study design — selection should be driven by the research objectives, samples, treatment and available resources; For the primary outcome, choose as 'proximal' a PROM as will add to knowledge and inform practice — 'proximal' (symptoms) *vs* 'distal' (overall quality of life); Identify candidate PROMs primarily on the basis of scaling and content — which items/scales offer best coverage of the impacts of interest and which aspects of score distribution will be most meaningful to consider? Appraise the reliability, validity and 'track record' of candidate PROMs

Table 1 Keyword combinations used in the literature search

Keyword combinations		
Patient Reported Outcome Measures OR patient reported outcome measure[1] OR PROM[1] OR PRO[1] OR patient reported outcome[1] OR patient outcome[1] OR clinical outcomes assessment	AND	Guidance OR guidelineOR recommend[1] OR good practice OR best practice OR instrument development OR adapt[1] OR modif[1] OR develop[1] OR establish[1] OR efficien[1] OR standard[1] OR measurement properties

[1]It indicates where truncated versions of the wrord was used. Search was restricted to the last 10 yr and publications in the English lang.

Table 2 Inclusion and exclusion criteria used when screening identified studies

Inclusion criteria	Exclusion criteria
(1) Articles presenting guidance and recommendations for use or adaptation of existing PROMs; and (2) PROMs methodology papers	(1) Papers detailing development and validation of new PROMs; (2) Papers not presenting guidance or recommendation for adaptation of PROMs; (3) Study protocols; (4) Papers that focus on implementation of PROMs in research or clinical practice; (5) Conference abstracts; (6) Not published in English; or (7) Published longer than 10 yr ago

PROMs: Patient reported outcome measures.

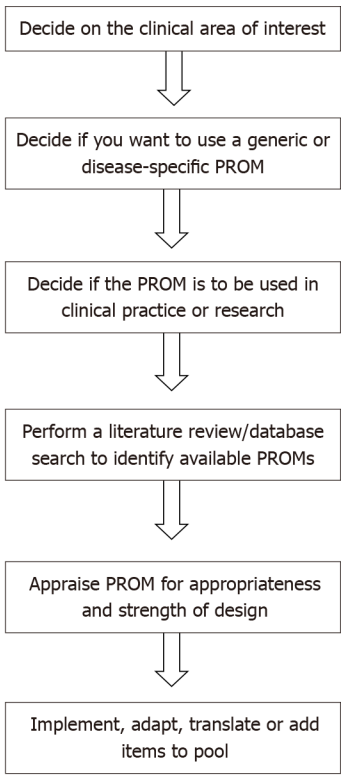

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Figure 1 Initial steps required prior to deciding whether to develop a new patient reported outcome measure. PROM: Patient reported outcome measure.

— look beyond articles that focus on evidence of validity and reliability; Look ahead to practical considerations — patient and staff burden, methods of administration, cost, availability of translated versions, guidelines for scoring and interpretation; Take a minimalist approach to ad hoc items — where content is similar, PROMs with proven psychometric properties are preferable to ad hoc measures developed by the researcher[17].

The EAPC[17] have provided guidance regarding the selection and measurement of PROMs in palliative care. This guidance reflects on the content of the PROM and the feasibility of its collection within the clinical environment. Some of the points made are particularly useful when considering adapting existing measures and can be usefully applied to other conditions: Use PROMs that have been validated with relevant populations and make sure these are sufficiently brief and straightforward; Use multi-dimensional measures; Use measures that have sound psychometric properties; Use measures

that are suited to the clinical task being delivered and are also suited to the aims of your clinical work and the population you work with; Use valid and reliable measures in research that are relevant to the research question and consider patient burden when using measures[17,18].

Identifying and appraising existing tools

Once one has defined the scope of the PROM, possible candidate measures can be identified. This process will determine whether there is a need for a new PROM. It will also allow for the identification of PROMs that could, be adapted, shortened, translated or expanded.

Given the large number of available PROMs, there are several ways in which possible candidate PROMs may be identified. Identifying systematic reviews of PROMs for a particular clinical area may prove to be particularly fruitful as good reviews will assess the methodological quality[13-16] of the PROMs identified and provide a summary of the PROMs that offer the most promise. In addition to undertaking literature reviews, there are also databases and online resources that can be searched to identify existing PROMs. Some of these resources are generic and cover many conditions, whilst others provide a resource for disease-specific PROMs. Table 3 provides some examples of resources that can be used to identify candidate PROMs for adaptation.

If these strategies do not identify any PROMs, conducting a new systematic review may uncover PROMs for consideration or items/questions in existing PROMs that could be included in the development of a new PROM[14]. Prinsen *et al*[13], have formulated a useful ten step process for conducting such systematic reviews of PROMs[13,14]. Such a systematic review should be conducted in accordance with the guidelines outlined by internationally recognised COnsensus-based Standards for the selection of health Measurement INstruments (COSMIN). COSMIN provide detailed information and tools to aid this process on their website (https://www.cosmin.nl/tools/guideline-conducting-systematic-review-outcome-measures/). This will ensure that the methodological rigor of the PROMs identified is appropriately appraised.

Once PROMs have been identified, the tools should be reviewed for their content and appropriateness for the desired application[13,14-22]. This process will also help to identify relevant questions/items that could be used to develop a new PROM or adapt an existing one.

Adapting existing PROMs

Researchers need to consider the existing PROM literature to determine whether an adequate instrument exists to assess and measure the concepts of interest. If no PROM exists, a new PROM can be developed or in some situations, a PROM can be adapted by modifying an existing instrument[2].

Examples of instrument modifications include: (1) Making minor cultural/Language adaptations within the same source language; (2) Undertaking a cross-cultural adaptation that includes translation into a different language; (3) Including additional items/questions; and (4) Shortening the original instrument. Such PROM modification may be necessary to enable the PROM to be used with a different population or with a different population age group (for example, modification of an adult PROM for a paediatric population), to facilitate its use in a different language, for use in a different disease stage or treatment (for example cancer stage, or for a newly diagnosed condition rather than a pre-existing condition), or to reduce patient burden.

The FDA state that when a PROM is modified, evidence of adequacy for its new intended use should be provided and that "additional qualitative work may be adequate" to test such modifications[2]. Such changes include: Changing an instrument from paper to electronic format; Changing the application to a different setting, population or condition; Changing the order of items, item wording, response options, or recall period or deleting portions of a questionnaire; Changing the instructions or the placement of instructions within the PROM.

Snyder *et al*[21], outline some requirements to revalidate a PROM when changes such as these are made to an existing PROM.

The search for PROMs may identify existing instruments that have proven validity for the population being studied and can be applied without requiring any adaptation. Alternatively, a PROM may be identified that appears appropriate but requires modification. Before engaging in any adaptation, it is important first to contact the PROM developer/copyright holder to ask for permission to make changes to the original PROM. Wild *et al*[22], have recently published guidance from the International Society for Quality of Life Research (ISOQOL) Translation and Cultural Adaptation Special Interest Group (TCA-SIG) regarding copyright of PROMs. Failure to gain appropriate permissions for use and adaptation may result in legal challenges due to breaches in copyright. The authors present recommendations to prevent future conflict that includes: Protecting the copyright of the original PROM; Writing a contract; Taking care when publishing; Establishing rules; Making the copyright notice visible; Maintaining copyright of the PROM and any derivatives with the original author; Centralising distribution; Getting legal counsel; Clarifying the copyright situation with respect to legacy PROMs.

It is therefore prudent for researchers considering the adaptation (including the translation of existing PROMs) to identify and obtain agreement from the copyright holder prior to any adaptation[22].

Table 3 Some examples of online resources that can be used to identify candidate patient reported outcome measures for adaptation

Resource name and web address	Resource Information
ePROVIDE™ (https://eprovide.mapi-trust.org/)	This is an online service provided by Mapi Research Trust and is the official licensor and distributor of more than 450 clinical outcome assessments (or PROMs). This resource allows you to search for PROMs within a specific clinical area and presents: a summary of each tool; the authors of the tool; different version of the questionnaire; the copyright owner; the specific condition/disease in which the PROM has been used; the original language the PROM was developed in; references to the original PROM development publications; and a list of any validated translations of the original questionnaire. If a PROM is deemed appropriate but no valid translation exists, there is also an opportunity to submit a request to undertake a linguistic validation of the questionnaire
COnsensus-based Standards for the selection of health Measurement INstruments (COSMIN) (https://database.cosmin.nl/)	The COSMIN initiative (https://www.cosmin.nl/) aims to "develop methodology and practical tools for selecting the most suitable outcome measurement instrument). Their mission statement is: "to improve the selection of outcome measurement instruments of health outcomes by developing and encouraging the use of transparent methodology and practical tools for selecting the most suitable outcome measurement instrument in research and clinical practice". The COSMIN website provides a link to the COSMIN Database for Systematic Reviews which can be searched to identify literature reviews that have been undertaken within specific clinical areas. The database provides a summary of the review and the PROMs that formed part of the review and links to the original publications. Examination of these reviews is useful in assessing whether an existing PROM may be appropriate to use. Many of these reviews will also present a synthesis of each PROM with an assessment of its methodological quality and validity according criteria outline in more or more of the guidance documents available[2,44,61-65]
International Consortium for Health Outcome Measurement (ICHOM) (https://www.ichom.org/)	As part of a wider initiative ICHOM publish Standard Sets. ICHOM Standard Sets are defined as 'standardized outcomes, measurement tools and time points and risk adjustment factors for a given condition. Developed by a consortium of experts and patient representatives in the field, our Standard Sets focus on what matters most to the patient'
Measures for Person Centred Coordinated Care (http://p3c.org.uk/about)	Set-up as a result of an NHS England funded project. This online resource describes itself as providing information "about measures for Person Centred Coordinated Care ("P3C") for people with long-term conditions (LTCs), multiple-LTCs, and those at the end of their life (EoL)". It provides a compendium of measures — defined as PROMs and patient reported experience measures (PREMs) — that can be utilised within programs that aim to deliver or evaluate P3C in the target populations"
European Organisation for Research and Treatment of Cancer (EORTC) (https://www.eortc.org/tools/)	Amongst other resources, the EORTC website provides a list quality of life questionnaires that have been developed and validated for cancer patients that are available for academic use
Oxford University Innovation/University of Oxford Clinical Outcomes Assessments (https://innovation.ox.ac.uk/clinical-outcomes/patient-reported-outcomes-measures/)	The PRO portfolio is made up of condition-specific questionnaires aimed at assessing the outcome for patients being treated for a range of medical conditions

PROMs: Patient reported outcome measures.

CROSS CULTURAL ADAPTATION

If a suitable PROM is identified and has appropriate content validity for the population of interest, but was developed and validated in a different language, cross cultural adaptation represents an efficient way of adapting an existing PROM. Cross-cultural adaptation manages language translation and cultural adaptation issues with the aim of ensuring a PROM is sensitive to the linguistic and cultural needs of the target population[22]. A PROM that has undergone rigorous cross-cultural adaptation is suitable for use in multinational and multicultural studies.

It is important that any cross-cultural adaptations of PROMs are undertaken rigorously. Guidance regarding the process of cross-cultural adaptation has been described in a wide range of publications [22-25]. The lack of 'gold standard' guidance for cross-cultural validation prompted the Patient Reported Outcome (PRO) Consortium, to update and develop further guidelines for best practice in the translation process[26]. These guidelines are based on the ISPOR Task Force guidelines, updated with greater detail through a further consensus process[22].

The aim of cross-cultural adaptation is to provide equivalence between the source PROM and the adapted version. Equivalence has many definitions; however, most current guidelines follow the universalist approach proposed by Harachi *et al*[27], which gives consideration to the influence of culture on how people respond to any given item on a questionnaire. Questions therefore not only require linguistic translation, but they must also be adapted to fit culturally to the target country[26]. For example, a question about difficulty using a fork in eating may not be applicable in a country where a fork is not used in eating[28]. Equivalence can be divided into five categories plus a summary category [22,28] (see Table 4), and this has formed the basis of many guidelines for the cross-cultural adaptation of outcome measures.

Ultimately, all of the available guidelines are broadly based on a core set of principles that need to be considered when cross-culturally adapting an existing PROM: (1) Preparation. The initial stage of the process is to identify the team that will be responsible for the work, identify suitable translators and

Table 4 Categories of equivalence

Categories	
Conceptual equivalence	The domains of the questionnaire have the same relevance, meaning and importance in both cultures
Item equivalence	Individual items have the same relevance in both cultures
Semantic equivalence	The meaning of the items is the same in both cultures
Operational equivalence	The questionnaire can be used in the same way by the target population in both cultures
Measurement equivalence	The two versions have similar psychometric properties
Functional equivalence	This is meant as a summary category of the preceding five categories. It is an overall statement that identifies if both versions "do what they are supposed to do equally as well"

gain permission from the original instrument design team to carry out a translation process; (2) Forward translation. Translation of the original language version into the new, target language. It is considered best practice for this to be performed at least twice by different translators from the target country; (3) Reconciliation. Comparison of multiple forward translations and merging them into one translated version; (4) Back translation. The newly translated version is translated back into the original source language; (5) Back translation review. The back-translated version is compared to the original version to assess for equivalence in text and meaning; (6) Harmonisation. All translated versions are reviewed for consistency in language and conceptual meaning; (7) Proofreading. All copies of the questionnaire are proofread to remove mistakes; (8) Cognitive interviewing. The newly translated questionnaire is piloted on a minimum of five people in the target population. Cognitive interviews are performed to identify problems with the questionnaire, difficulties in understanding and meaning of items and any other concerns; (9) Cognitive interview review. Results of the cognitive interviews are reviewed and changes made to the questionnaire if required; (10) Final review and publication. The final version of the translated questionnaire is agreed upon and published for use in the target population; and (11) Cross-cultural validation. Following the production of a culturally and linguistically valid and similar version of the original questionnaire, the adapted questionnaire must then be psychometrically validated against internationally recognised criteria[28,29].

The degree of cross-cultural adaptation required varies depending on the proposed use of the adapted PROM. The intended use of the PROM may influence the number of steps of the above that require completion[29]. Table 5 illustrates five different scenarios where differing adaptation needs are required[25,29]. These range from a situation in which no adaptation is required (*i.e.*, the questionnaire used in the same population, in the same culture and language as originally designed), to full translation and cross-cultural adaptation (*i.e.*, where the questionnaire is to be used in a different country and language).

ADDING TO EXISTING PROMS

If an existing PROM is identified as largely meeting the requirement for the population of interest but following patient and expert consultation and/or exploration of the literature it is perceived to be missing in one or more key areas, there is the potential to adapt the PROM by adding new questions/items. There are various ways in which items can be sourced[17,28]: By asking patients. Patients can be asked to identify additional items and domains that do not exist in the current version of the PROM. Patients are essential to item generation, ensuring item content is both relevant and provides full coverage of the target construct. Qualitative methods such as patient focus groups, interviews and surveys are useful for generating potential new items[30-32]; By evaluating the PROMs identified as a result of reviewing the literature or online resources. This can be an efficient way to generate new items. There are benefits to sourcing items in this way, most notably that there are likely to be a limited number of ways to ask questions about a specific problem such as abdominal pain, vomiting, *etc.* Moreover, items in existing PROMs have been repeatedly used and validated in many studies and trials; By identifying possible items from clinical observations. These items can be derived by clinicians based on their experience; By asking experts. This is a commonly used approach to generate new items. Similar methods (for example interviews, focus groups and surveys) to those used with patients can be used for gathering information about possible items for inclusion. Although useful for generating items, expert involvement should be used in tandem with other methods and should not be used in place of patient input; By utilising item banks. Item banks are a source of validated items that can be added to existing PROMs. One such item bank, the Patient-Reported Outcomes Measurement Information System (PROMIS™) initiative was established in 2004, with the main goal of developing and evaluating,

Table 5 Scenarios in which different degrees of cross-cultural adaptation are required[25]

	Results in a change in			Adaptation required	
	Culture	Language	Country of use	Translation	Cultural adaptation
Use in same population. No change in culture, language or country	-	-	-	-	-
Use in established immigrants in source country	Yes	--	-	-	Yes
Use in another country, but same language	Yes	-	-	-	Yes
Use in new immigrants, not source language speaking but in the source country	Yes	Yes	-	Yes	Yes
Use in another country and another language	Yes	Yes	Yes	Yes	Yes

for the clinical research community, a set of publicly available, efficient and flexible measurements of PROs[33]. PROMIS™ (http://www.healthmeasures.net/explore-measurement-systems/promis/intro-to-promis/List-of-adult-measures) provides item banks that offer the potential for PRO measurement that is efficient (minimizes item number without compromising reliability), flexible (enables optional use of interchangeable items), and precise (has minimal error in estimate) measurement of commonly-studied PROs[33]. The PROMIS group has developed and tested several hundred items measuring 11 health domains[33]. These core PROMIS domains reflect common, generic symptoms and experiences that are likely to apply to people in a variety of contexts or with a variety of diseases[33]. With additional validation, these banks may provide a common metric of represented constructs across a range of patient groups, thereby reducing the large number of different measures currently used in research and allowing researchers to compare these constructs across patient groups in different studies [33].

SHORTENING OF EXISTING PROMS

Although many single-item and short-form symptom measures exist, one reason for adapting an existing PROM is to shorten it and reduce the number of items included in it. This can result in reduced patient burden and facilitate the use of a PROM as part of routine clinical care. As with other aspects of adaptation, it is essential to ensure that a shortened PROM is comprehensible to patients, includes all the relevant items and is fit for purpose. Like cross-cultural adaptation and adding existing items to a PROM, shortening will require further psychometric testing according to recognised criteria[30].

Issues of content validity

Where any adaptation is planned, the PROM will still need to show evidence that it is 'fit for purpose' with the intended population[33]. In 2007, the International Society for Pharmacoeconomics and Outcomes Research (ISPOR) Health Science Policy Council recommended that an ISPOR Patient-Reported Outcomes taskforce on the use of existing instruments and their modifications be established. This resulted in the publication in 2009 of their report detailing good research practices[16]. A major aspect of this report related to the content validity of PROMs, and stated "evidence of content validity should be obtained from an analysis of the relationship between the instrument's content and the construct it intends to measure"[16]. This report highlights the key issues relating to content validity issues that should be considered when selecting and modifying existing PROMs[16]: Name and define the concept; Target population and end point; Identify candidate PROMs; Identify or formulate a conceptual framework for the PROM; Assemble and evaluate information on development methods — elicitation focus groups and interviews; cognitive interviews; transcripts; Conduct any needed qualitative work; Assess adequacy of content validity for purpose; Determine the need for modifications or new PROM development.

This guidance has since been updated to include further recommendations from the ISPOR good practice task force[34]. Additional guidance regarding content validity and its consideration with respect to PROM development and adaptation have also been published and includes best practices for undertaking qualitative research to explore content validity, including differences between establishing content validity for new measures compared with existing measures[14,35].

Assessment of PROM content is an important process when adapting an existing PROM and this should involve engagement with, most importantly, patients and also clinicians.

Getting the right people involved

Having identified a candidate PROM for adaptation it is important to ensure that it is appropriate for the patient population being studied. This is particularly important to undertake if the PROM is being

adapted for use with a new clinical population. Pre-testing the PROM with patients, clinicians, and subject-matter experts will provide evidence of the PROM's content validity and help to ensure that any problems are rectified prior to applying the PROM in a large-scale study or implementing the instrument in routine clinical practice.

GETTING PATIENTS INVOLVED

In 2009 FDA guidance suggested that an important first step in establishing that a measure is fit for purpose is to develop a conceptual framework for the PROM and generate relevant items on the basis of direct input from patients with the clinical disease[2,34,35].

Recent guidance[36,37] highlights the various roles that patients and patient advocates can play in PROM studies. These include: PROM design and selection — bringing knowledge of the disease, symptoms and attributes of care with the greatest impact on patients' lives; PROM implementation and administration — the patient can bring insights based on their experience to guide practical decisions around PROM administration and implementation; Linguistic and cultural input — patients can contribute to the language used in the PROM to ensure it is straightforward and understandable to patients.

Guidance regarding how the patients can be recruited to PROM studies, how to engage with patients, defining the role, provision of training and remuneration[38] has also been provided.

In addition, a framework for fully incorporating public involvement (PI) into PROMs has recently been published[38] which illustrates the extent to which patients can be involved in the adaptation process (see Table 6).

Existing measures can be reviewed to ensure they match the domains of interest and if further modification may be required[16]. Recent research that explored the level of involvement of patients in the development of PROMs has concluded that what patients consider important can differ from what health-professionals regard as important[30,31]. Content validity is often cited as a PROM's most important measurement property as unless the PROM can be shown to be measuring the construct of interest from the patient perspective, all other measurement properties may be considered inconsequential[13]. This highlights the importance of engaging with patients as part of the PROM adaptation process.

A variety of qualitative methods can be used by researchers to engage with patients with the aim of maximising a candidate PROM's content validity (relevance and comprehensiveness) and to pre-test an adapted PROM for comprehensibility and acceptability of instructions to respondents, its items and response format(s). In a recent study examining the developers' perspective of including patients, the methods used were interviews and/or focus groups, cognitive interviews and feedback questionnaires [30,31]. Maitland and Presser advocate a diverse range of methods, both qualitative and quantitative, for appraising the quality of PROM items and the ability of the items to generate reliable and valid responses[39].

Interview and focus groups are often used to gain insight into the experiences of the target population in relation to the construct of interest and, therefore can be used to generate content for new or additional questions. Cognitive interviews, on the other hand, are normally used to refine item candidates and their response scales. Cognitive interviews capture problems with the cognitive processes associated with item response[40], thereby enabling the developers to evaluate the relevancy, comprehensiveness, comprehensibility and acceptability of the instrument's items and response scales.

Feedback questionnaires can also provide patients insight regarding their experience of using a health status questionnaire. The QQ10 is one such validated, self-completed questionnaire. It is made up of 10-items scored using a 5-point Likert scale (0 = strongly disagree to 4 = strongly agree) covering two factors, "value" and "burden". It contains specific items developed to assess a PROM's content validity (*i.e.*, relevance, comprehensiveness) from the patient perspective[41,42].

GETTING EXPERTS INVOLVED

The assessment of a candidate PROM from the expert clinical, researcher and academic perspective is also important. This can be achieved *via* focus groups and interviews, by questionnaire survey methods or by employing expert review panels[34]. Ideally, these panels should include clinicians with experience of treating the defined population, PROMs methodologists and researchers. The COSMIN standards recommend a minimum sample size of seven professionals for studies evaluating a PROM's content validity[14].

Experts can also be utilised to calculate content validity indices (CVI) based on ratings of item relevance. A minimum of three experts is recommended for the purposes of calculating a CVI[43]. A CVI is a consensus indicator of the content validity of an item or scale[44]. It represents the proportion of reviewers who agree that an item is content valid, adjusted for chance agreement. If all reviewers are in agreement, the CVI value for an item (I-CVI) will be 1.00.

Table 6 Stages within an patient reported outcome measure adaption process where patients may be involved

Stage	How can PI be involved
Establishing a need for a new or refined PROM	Review existing PROMs; Critique existing PROMs; Determine whether a new PROM is needed
Development of a conceptual model	Review of conceptual model to ensure validity
Identifying item content	Input on study design; Input on culturally appropriate issues; Input on participant facing documents; Input on ethics and governance issues
Item development	Analysis and interpretation of qualitative interviews; Advice and input on wording of potential items
Item reduction	Identify potentially redundant items; Identify items that could benefit from rewording; Input and advice on ordering of items
Pre-testing of items (cognitive interviews/debriefing)	Input on study design, methodology, recruitment, design and content of public facing documents and conducting the interviews; Analyse and/or interpret results
Selection of items for the PROM	Advice on final selection of items; Consideration of number of items to be included; Advice and input into how PROM may be used in clinical settings
Design of the PROM	Advice and input on format and layout of PROM; Advice on instructions of how to complete the PROM, framing of questions, wording of response options, and order of items

PI: Public involvement; PROMs: Patient reported outcome measures.

Employing different psychometric methods to PROMs development and adaptation

Traditionally the development and psychometric evaluation of PROMs has been based on classical test theory (CTT). CTT is probably still the most commonly applied method in validation studies[20,45]. CTT assumes that the expected value of all the random error will equal zero[46]. There are, however, some disadvantages with CTT, such as sample dependency. This is where the item and scale statistics can only in theory, apply to the specific group of patients who took the test and as such further validation is required for a different population[29]. There is also the assumption of item equivalence, where it is assumed that all items contribute equally to the final test score and no item weightings are applied[46,47].

As a result of the disadvantages of CTT, modern psychometric methods of item response theory (IRT) and Rasch measurement theory (RMT) have been developed[48]. Rather than considering the questionnaire as a whole, as in the case of CTT, these methods allow analysis at the individual item level [49]. They also provide sample-free measurements (*i.e.*, the results are applicable to all similar groups once the validation process has occurred). In IRT, additional model parameters are used to model the relationship between the individual's trait, the item property and the probability of endorsing an item. The assumption in IRT is that the "probability of answering any item in the positive direction is unrelated to the probability of answering any other item positively for people with the same amount of the trait" [28,29]. RMT differs somewhat in that the data are assessed to see if they fit the Rasch model. RMT allows for the creation of linear, interval-level measurement from categorical data. In the case of non-fitting data (items or persons), data can be further examined to understand why they do not fit or removed from the data set. Rasch analysis can be used to examine the properties of previously constructed scales as well as in the construction of new scales, and is important in making interval scales [50].

Although there has been a general shift towards using IRT in more recent years for developing and validating a PROM, there are some drawbacks to its use over CTT. One issue relates to the sample size required. It is recommended that sample sizes based on CTT should be large enough for the descriptive and exploratory pursuit of meaningful estimates from the data, starting with a sample of 30 to 50 subjects may be reasonable in some circumstances[51]. At later stages of psychometric testing, various recommendations have been given for exploratory factor analyses with recommendations of at least five cases per item and a minimum of 300 cases or to enlist a sample size of at least 10 times the number of items being analysed, so a 20-item questionnaire would require at least 200 subjects[51]. For IRT, sample sizes of a minimum of 150-250 patients has been proposed, with around 500 patients recommended for the latter stages of validation[51,52].

In addition to the inflated sample size recommendations for IRT, additional expertise in the study team is often required, and this may consequently result in greater development costs. Furthermore, strict assumptions in the model can mean that items may be rejected even when they have good content validity if they do not fit the IRT model. CTT should therefore not be disregarded and indeed, most authorities will agree that aspects of both CTT and IRT have a role to play in the validation process of a modern PROM.

CLINICAL USE OF PROMS IN HEPATOLOGY

Most health providers treat patients with the aim of improving radiological, biochemical, histological and clinical assessments[1]. Historically, health outcomes have focused on death or clinical indicators such as infection rates, readmissions, re-operations and adverse events[10]. Many of the symptoms that are experienced by patients in hepatology may be undetected by clinical tests or underestimated by clinicians[53]. The need to assess the impact of health treatment on patients and to demonstrate the value of the care provided to the patient by the provider is now recognised[9]. There is constant pressure on healthcare providers to improve the quality of healthcare provided and make it more patient-centered[54]. Given how much money is spend on treatment, it is important to assess if the treatment given offers value for the money. Clinical applications of PROMs can be divided into: Clinical research and trials: Health regulatory bodies such as the FDA and the National Institute for Health and Care Excellence (NICE) require PROMs to be incorporated into the assessment of new treatments, health technologies or medical devices; Quality improvement projects: PROMs can be very helpful in assessing the impact of a new service or project from the patient perspective. However, PROMs must be integrated into clinical practice with strong incentives to encourage their routine use in such quality improvement projects; Clinical practice: Measuring PROMs in clinical practice contributes to patient-centeredness and measures clinical effectiveness from a patient perspective.

There has been widespread adoption of PROMs use within the research field, especially since FDA and EMA recommended that PROMs should form part of the outcome assessment for new drug trials[2,5]. Reporting guidance for PROMs has also now been incorporated as an extension to CONSORT reporting for trials[7]. The value of collecting PROMs data routinely is now recognised as an important part of driving the delivery and organisation of healthcare and can thereby help to improve healthcare quality[9].

Although individual hospitals and clinicians have started to implement routine PROM collection, widespread adoption is largely restricted to England, Sweden and parts of the United States[9,55]. PROMs have now been implemented in England for the routine collection following some elective surgery (https://digital.nhs.uk/data-and-information/data-tools-and-services/data-services/patient-reported-outcome-measures-proms). Their potential to be used in other clinical areas, such as oncology [56,57], multiple sclerosis is now also recognised. The routine collection of PROMs is not without its challenges however[1,9,55-60].

Some of the practical challenges to routine integration include: the selection of the most appropriate tool; difficulties with patient completion (for example, lack of comprehension, elderly and frail or sick populations); clinical reluctance; achieving high rates of patient participation; operational difficulties; lack of clarity about the PROM; times pressure for patients and clinicians; lack of human resources; recognition of the three dimensions of quality (safety, effectiveness and experience); attributing outcomes to the quality of care; providing meaningful outputs from PROMs data for differing audiences; and avoiding misuse of PROMs[1,9,55,59-65].

McDowell and Jenkinson[66], have developed a series of key strategic priorities that should be considered when implementing PROMs in real-world situations: Ensure international collaboration across multiple stakeholders to agree on a standardised approach to PROM assessment; Develop a comprehensive standard set of recommendations, methods and tools that are applicable to the generation of real-world evidence; Formulate a clear governance process including an ethical framework for how patients should be consented, who selects patients, who has access to data and how data will be used; Establish standard sets of PROMs, electronic tools and administration schedules; Develop and use electronic PROMs where possible; Minimise workload and technical complexity for patients and clinicians; Consider the objectives of the PROM assessment, timings, length of follow-up, strategies for managing missing data and inclusion of diverse patient populations; Ensure data collection adheres to the FAIR (findable, accessible, interoperable and reusable) guidance; Provide guidance on interpretation and use of the data; Ensure both patients and clinicians gain value from PROM collection to tailor their needs.

FUTURE OF PROMS

With new treatment and technologies, mortality is reducing and more patients are living with their illness for longer. As such, there is a growing need to develop and implement PROMs to facilitate the translation of clinical research into practice and, in keeping with the principles of shared clinical decision-making as part of routine clinical practice.

The increased use of digital media presents an exciting opportunity for PROM capture and adaptation. By utilising new technologies to aid PROMs capture and support interpretation, more clinicians may be encouraged to use PROMs as part of their routine clinical care. For example, innovative delivery methods using app or web-based based technology [for example, through data platforms such as REDCap- Research Electronic Data Capture (https://www.project-redcap.org/)] are helping to streamline data capture from patients by facilitating PROM completion on tablets, mobile

phones and the internet.

Employing digital media also allows novel methods such as ecological momentary assessment (EMA) [64-68] to be used. EMA refers to a collection of methods often used in behavioural medicine research where a patient repeatedly reports on, for example, their symptoms or quality of life close in time to when they experience them and in their own environment[64]. EMA data can be collected in various ways, including written diaries, electronic diaries and telephone. EMA using mobile phones, for example, could facilitate the collection of PROM data in real-time and overcomes some of the inherent problems of PROMs, such as patient recall accuracy.

The burden of data collection associated with the routine collection of PROMs data in practice can be reduced by simplifying data collection using techniques such as computerised adaptive testing (CAT). CAT involves using a computer to administer a PROM, one question at a time. The CAT then uses an algorithm to choose the subsequent question based on the previous answer given. For example if a PROM is assessing hand function and in response to the first question a patient answers that their hand function is 'normal', then there is little to be gained from asking increasingly granular questions about hand problems, which may be more appropriate to someone who has 'non-normal' hand function. By pre-selecting questions, the PROM score can be determined without having to ask all of the questions [61,62,69,70].

Another future technology is that CAT and electronic PROMs could be administered by virtual assistants (such as Siri or Alexa, or similar) using voice recognition software to avoid the need for manual form filling to further reduce the manpower required for data collection[59-62].

CONCLUSION

The process of developing a new PROM is often a complex and resource-intensive process. If possible, researchers should first consider whether any existing PROMs could be suitable candidates for use, or if they could be adapted. This review provides a general introduction to PROMs and some background regarding the recent drive to collect PROM data. It then reports on findings from a scoping review that identified good practice and issues that should be considered prior to adapting existing PROMs. These issues are organised under the specific headings of: defining the requirements of a PROM, identifying and appraising existing tools, adapting existing PROMs, issues of content validity and getting the right people involved. The review ends with some insights into different psychometric methods, clinical use of PROMs and future PROMs developments.

FOOTNOTES

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MINIREVIEWS

Loco-regional treatment of hepatocellular carcinoma: Role of contrast-enhanced ultrasonography

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Abstract

Hepatocellular carcinoma (HCC) is one of the few cancers for which locoregional treatments (LRTs) are included in international guidelines and are considered as a valid alternative to conventional surgery. According to Barcelona Clinic Liver Cancer classification, percutaneous treatments such as percutaneous ethanol injection, radiofrequency ablation and microwave ablation are the therapy of choice among curative treatments in patients categorized as very early and early stage, while transcatheter arterial chemoembolization is considered the better option for intermediate stage HCC. A precise assessment of treatment efficacy and surveillance is essential to optimize survival rate, whereas residual tumor requires additional treatment. Imaging modalities play a key role in this task. Currently, contrast-enhanced computed tomography/magnetic resonance imaging are considered the standard imaging modalities for this purpose. Contrast enhanced ultrasound (CEUS), using second generation contrast agents, plays an increasingly important role in detecting residual disease after LRTs. CEUS is a straightforward to perform, repeatable and cost-effective imaging modality for patients with renal failure or iodine allergies. Due to the ability to focus on single regions, CEUS can also provide high temporal resolution. Moreover, several studies have reported the same or better diagnostic accuracy as contrast-enhanced computed tomography for assessing tumor vascularity 1 mo after LRTs, and recently three-dimensional (3D)-CEUS has been reported as a promising technique to improve the evaluation of tumor response to therapy. Furthermore, CEUS could be used early after procedures in monitoring HCC treatments, but nowadays this indication is still debated, and data from literature are conflicting, especially after transcatheter arterial chemoembolization procedure.

Key Words: Hepatocellular carcinoma; Contrast-enhanced ultrasonography; Radiofrequency ablation; Transcatheter arterial chemoembolization; Ultrasound; Liver

Core Tip: Contrast enhanced ultrasound (CEUS) is playing an increasingly important role to evaluate locoregional treatments efficacy in hepatocellular carcinoma. In this paper, on the basis of personal experience and the relevant literature, we will review and discuss the CEUS technique. We will also highlight the importance of CEUS in evaluating the efficacy and post-procedural surveillance and their efficacy compared to the gold standard contrast-enhanced computed tomography and contrast-enhanced magnetic resonance imaging.

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INTRODUCTION

Hepatocellular carcinoma (HCC) represents the sixth most common neoplasm and the third cause of cancer death in the world[1,2]. HCC occurs more often in males than females (2.4:1), but the worldwide incidence is heterogeneous because of the variable prevalence of the risk factors, with a higher incidence in Eastern and Southern Asia and Middle and Western Africa[3].

Chronic liver disease and cirrhosis remain the most important risk factors for the development of HCC regardless of etiology. Hepatitis B and C, alcohol and nonalcoholic fatty liver disease and autoimmune or genetic conditions are independent risk factors for the development of cirrhosis[4].

Development of HCC is a complex multistep process that involves inflammatory liver damage, hepatocyte necrosis and regeneration and fibrotic deposition, leading to increasing liver function impairment. The accumulation of genomic alterations in addition to epigenetic changes runs in parallel with the progression to low-grade dysplastic nodules, high-grade dysplastic nodules, early HCC and advanced HCC[5].

HCC is one of the few cancers for which locoregional treatments (LRTs) are included in international guidelines and have emerged as a valid alternative to conventional surgery[6]. The choice of treatment in patients with HCC is therefore guided not only by tumor staging (defined by number and sizes of nodules, presence of vascular invasion, extrahepatic spread), as in the vast majority of cancers, but also by careful assessment of liver function and general health status[7].

Although several staging systems have been proposed to provide a clinical classification of HCC, the current guidelines of the European Association for the Study of the Liver, European Organization for Research and Treatment of Cancer and American Association for the Study of Liver Disease endorse the Barcelona Clinic Liver Cancer (BCLC) classification[8]. This classification defines the standard of care for each tumor stage. Percutaneous treatments and in particular percutaneous ethanol injection and thermal ablation are included among curative treatments in patients categorized as very early (BCLC stage 0) and early stage (BCLC stage A) when surgical resection or liver transplantation is not feasible or not available. On the other hand, transcatheter arterial chemoembolization (TACE) is considered the best option for intermediate stage (BCLC stage B)[9].

Finally, much hope has been placed in the recognition of novel targets and prognosis predictors through molecular profiling. The combinations of immunotherapy with LRTs are under investigation, representing a promising treatment strategy in advanced HCC[10]. In several preclinical studies this combination has demonstrated increased antitumor immune response due to LRTs effect to increment tumor immunogenicity by inducing inflammation and by releasing tumor-associated antigens[11]. Furthermore, observational and small non-randomized studies have demonstrated the safety of TACE in combination with sorafenib, with manageable toxicities, in patients with intermediate stage HCC and good liver function. However, the combination did not provide meaningful clinical benefit compared with doxorubicin-eluting beads (DEB-TACE) alone[12].

Unfortunately, HCC is known to be a multicentric tumor, often with a poor prognosis. Despite great efforts in terms of primary prevention, surveillance and multidisciplinary approach, incidence and mortality continue to rise. An accurate assessment of therapeutic response is of crucial importance, considering that complete treatment response significantly increases the likelihood of patient survival, whereas residual tumor requires additional treatment[13].

CONTRAST ENHANCED ULTRASOUND

Today, contrast enhanced ultrasound (CEUS) plays an increasingly important role in the management of HCC due to its numerous advantages in comparison with contrast-enhanced computed tomography (CECT) and contrast-enhanced magnetic resonance imaging (CEMRI)[14]

CEUS is a repeatable, cost-effective imaging modality that enables real-time dynamic assessment of liver lesions throughout the vascular phase[15]. Furthermore, CEUS has a much higher temporal resolution compared to other imaging modalities, without the associated radiation burden of CECT or the cost and the long examination time of CEMRI[16]. Compared with the latter, CEUS allows the reading, recording and tracking of every second of the study, without loss of information[17]. CEUS can perfectly depict the contrast-enhancement pattern of HCC[18]. In the arterial-phase, HCC is typically hypervascular and shows a mild and late washout, usually never before 60 s after the completion of contrast injection (Figure 1).

CEUS can be performed during the same interventional procedure session seamlessly. The excellent tolerance and high safety profiles of ultrasound contrast agents (USCAs) make them suitable for patients with renal failure, renal obstruction or allergic to iodine and can be administered more than once during the same examination. The high safety of USCAs was demonstrated by a meta-analysis of 23188 patients examined with CEUS for liver lesions, where the rate of serious adverse events was 0.0086%[19].

For these reasons, many international guidelines currently recommend CEUS as a fundamental imaging modality for the management of HCC, including surveillance, diagnosis, CEUS-guided treatment and treatment response evaluation[20,21].

USCAs for liver study

CEUS exam is performed by intravenously injecting microbubble-based contrast agents. The most widely used of these agents consists of particles with a radius ranging from 1 to 10 μm in diameter, and it is composed of a fluorinated low-solubility gas core encapsulated by a flexible phospholipid shell. Of note, microbubbles, in contrast to the most commonly used contrast agents for computed tomography (CT) and magnetic resonance imaging (MRI), are purely intravascular blood-pool agents. Due to their relatively large size, when injected intravenously they pass through the pulmonary filter and remain in the intravascular space, without an interstitial extravascular phase[17]. The USCAs currently used in diagnostic ultrasound (US) of the liver are: (1) SonoVue (sulfur hexafluoride), Bracco SpA, Milan, Italy, introduced in 2001. Licensed in Europe, China, Hong Kong, Singapore, India, Korea, New Zealand and Brazil; (2) Sonazoid (perfluorobutane), Daiichi-Sankyo, GE Tokyo, Japan, introduced in 2007. Licensed in Japan and South Korea; and (3) Definity/Luminity (octafluoropropane), Lantheus Medical, Billerica, MA, United States, introduced in 2001. Licensed in Canada and Australia[22].

These newer second generation USCAs strongly increase the backscatter of US, and the low solubility of the gas improves their stability and provides good resonance behavior at low-mechanical index (< 0.2). This allows a continuous real time scanning over several minutes to visualize all vascular phases. These features have led to a better sensitivity and the accuracy in the detection of micro- and macro-vasculature of liver tumors[23].

Of note, the only USCA presenting a post-vascular, liver-specific phase is Sonazoid, which is deemed to be taken up by the Kupffer cells of the liver[24].

Technical note

A baseline US examination is always performed before starting CEUS, including a color/power Doppler and pulsed Doppler analysis to choose the best acoustic window and to identify the target lesion. It is also important to select the best scanning plane either in the axial or long-axis plane and carefully evaluate the cooperation of the patient (*i.e.* positioning, breath holding), as the lesion has to be visualized during all phases of the CEUS examination. Once set, the US scan parameters, such as time gain compensation and focal zone, they should not be changed during the study. In order to minimize microbubbles disruption, the US scanner is switched to a low mechanical index contrast specific imaging module.

A standard CEUS protocol consists of the injection of 2.4 mL bolus of second generation USCAs followed by a flush of 5/10 mL of normal saline by using an intravenous access 20 G or greater than 20 G cannula. Some investigators use 1.2 mL, but the exact dose may depend on multiple factors, such as the software of the ultrasound equipment used, the size and depth of the lesion and others.

Activation of the timer coincides with the injection of the saline flush. Digital cineloops are acquired both during baseline and post contrast US scanning in 30-60 s intervals for up to 3-5 min to assess for all arterial, portal-venous phase (PVP) and late phase (LP). A post vascular or Kupffer phase for Sonazoid is registered 10 min after injection[22,23,25].

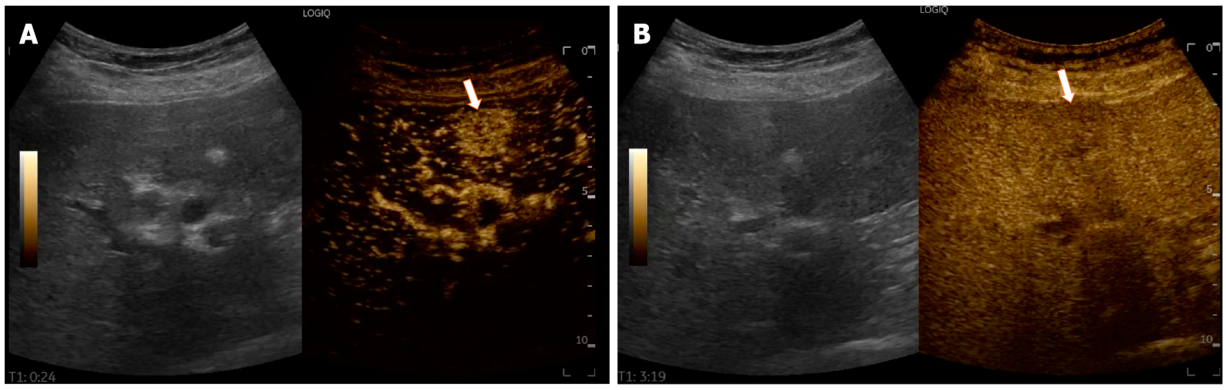

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Figure 1 Typical contrast-enhancement pattern of a hepatocellular carcinoma in a 72-year-old woman with hepatitis C virus related cirrhosis. A: In the arterial phase (24 s after the injection of a microbubble-based contrast agent) contrast enhanced ultrasound depicts a hypervascular tumor sized 2 cm in the IV liver segment (white arrow); B: In the late phase, 199 s after the injection, the lesion shows a mild wash-out (white arrow).

CEUS FINDINGS AFTER LRTs

A precise assessment of treatment efficacy and risk of local recurrence is essential to achieve a satisfactory survival rate, whereas residual tumor requires additional treatment[18,20,21,25].

According to modified Response Evaluation Criteria in Solid Tumors, a CECT or CEMRI procedure is deemed successful when there is no enhancing portion within the treated lesion or an irregular peripheral enhancement along the ablative margin during the arterial phase. Moreover, to achieve a complete tumor necrosis, the region ablated beyond the borders of the tumour, ideally, should measure 5 mm in its smallest width[26].

Although today CECT and CEMRI are considered the reference standard in imaging modalities for evaluating treatment after LRTs, CEUS is considered an effective alternative in patients who have kidney failure or iodine allergy[20].

In the last decade many studies supported the importance and validity of CEUS in this peculiar clinical setting[27-29]. Hai *et al*[30] examined 43 publications to evaluate the overall sensitivity and specificity of CEUS in detection of residual tumor after locoregional therapy, estimated, respectively, to 85% and 94% with a diagnostic accuracy of 93.5%[30].

CEUS findings after percutaneous ablative procedures

Percutaneous ablative procedures have been increasingly used for HCC treatment as minimally invasive procedures securing a good local control of tumor[31]. Radiofrequency ablation (RFA) is currently accepted as the best therapeutic choice for patients with very early and early-stage HCC when liver transplant or surgical resection is not a suitable option, providing excellent local control and acceptable morbidity and mortality rates[8,32-34]. Other forms of ablation are cryoablation, microwave ablation (MWA) and irreversible electroporation[35,36]. The latter is the most recent trend in ablation treatment of HCC, especially in patients with contraindications to other commonly used ablative techniques[37,38].

RFA induces thermal injury to tumoral tissue by creating resistive ionic heating (50–100 °C) through an electrode needle delivering high frequency alternating-current. The electrode needle is introduced into the lesion under US guidance[39]. MWA is a relatively new promising ablation procedure for the treatment of HCC. MWA systems uses an alternating electromagnetic field at 915 MHz or 2.45 GHz that has the ability to propagate through a tissue. Heat is generated when the alternating field interacts with tissue water and ions, generating greater ablation zones than RFA in perfused organs[29].

To date there are many studies in scientific literature that show the excellent accuracy of CEUS in detecting residual viable tumor. CECT and CEMRI are the most commonly used modalities for assessing the therapeutic response, however these imaging techniques are normally acquired 1 mo after and not immediately close to percutaneous ablative procedures[27].

CEUS assessment of LRTs can distinguish a complete response, a partial response and an equivocal treatment response. The lack of any nodular arterially enhancement portion within or at the edge of the ablated HCC is considered a complete response. In contrast, the detection of a residual viable tumor is considered a partial response[26,40]. Partial response may further divided in (1) an ingrowth-pattern when arterial phase hyperenhancement is detected within the edge of a treated nodule (Figure 2); and (2) outgrow-pattern when arterial phase hyperenhancement is detected immediately adjacent to the margin of the treated nodule (Figure 3).

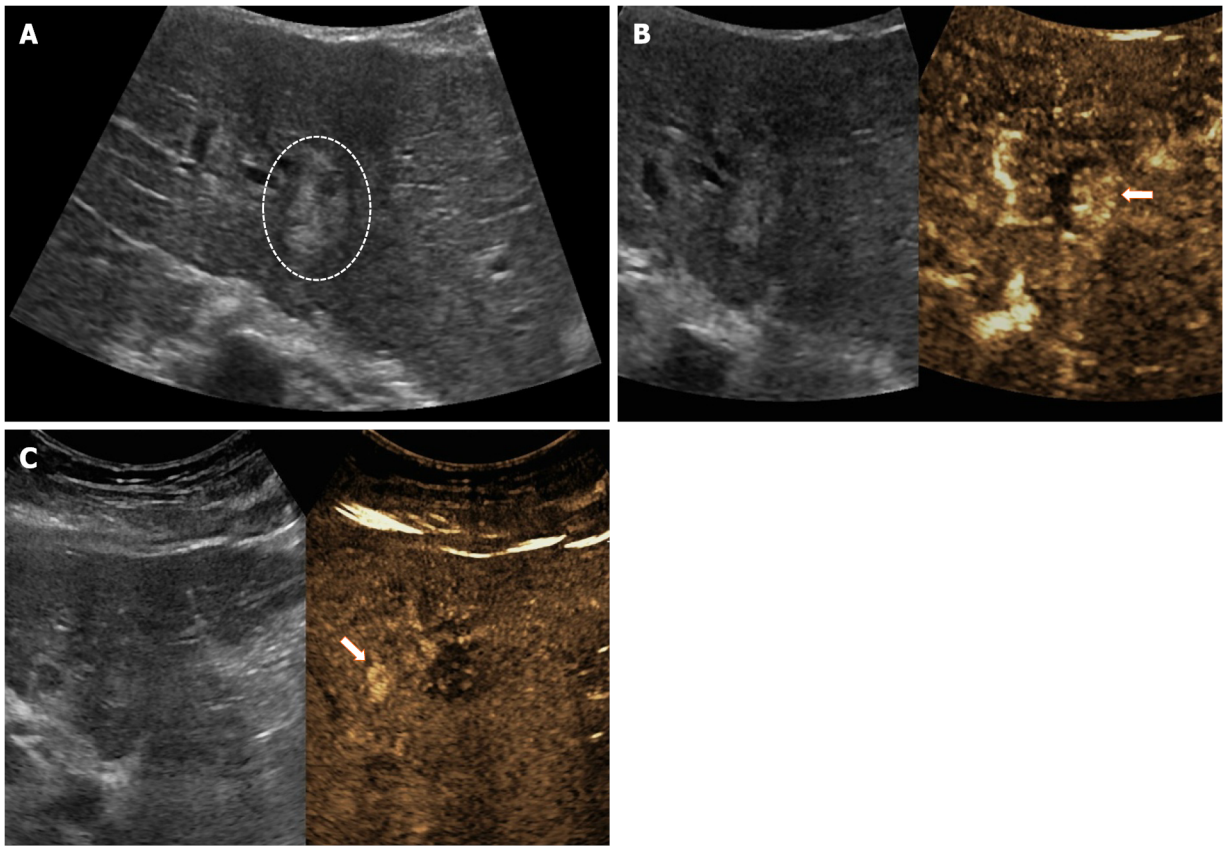

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Figure 2 Intrazonal recurrence after radiofrequency ablation in a 79-year-old woman with hepatitis C virus-related cirrhosis. A: Gray-scale ultrasound image achieved 1 yr after ablation shows a heterogeneous hyperechoic nodule (circle); B: Contrast enhanced ultrasound (CEUS) imaging achieved 15 s after injection shows a nodular token of arterial phase hyperenhancement (white arrow); C: CEUS image obtained at 2 min shows a wash-out. The patient underwent a new radiofrequency ablation in the same session. Simultaneously CEUS shows small hemangioma in the same segment (white arrow).

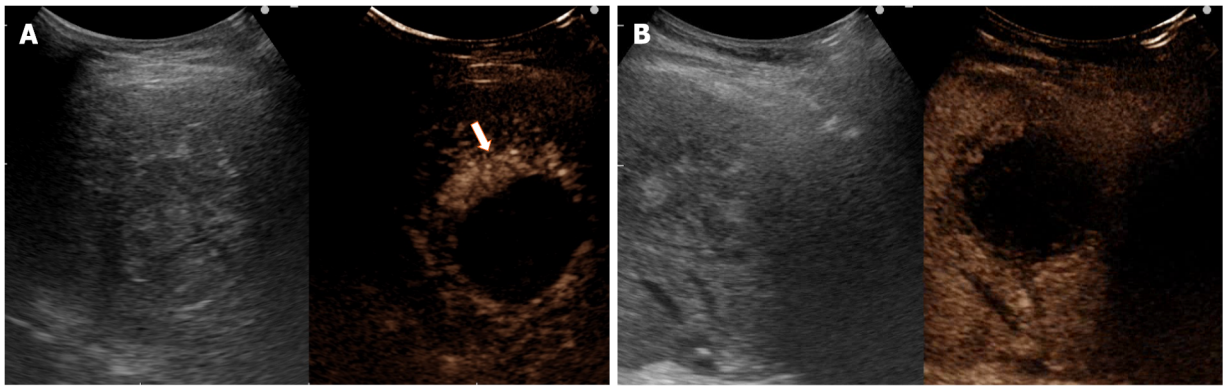

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Figure 3 Extrazonal recurrence. Follow-up of hepatocellular carcinoma treated with microwave ablation in a 79-year-old man with hepatitis C virus and ethanol alcohol-related cirrhosis. A: Contrast enhanced ultrasound image achieved 12 mo after treatment shows a peripheral arterial enhancement 20 s after injection (white arrow); B: Subsequent wash-out is evident at 3 min after injection.

Of note, there are some factors to be aware of when performing immediate postprocedural CEUS. First, as with CECT or CEMRI after RFA, the presence of a peripheral rim of enhancement can reflect a halo of inflammatory hyperemia surrounding the treated zone due to thermal damage. This finding disappears over time and can be distinguished from residual tumor for its characteristic to be a thin (4 mm to 8 mm) regular and peritumoral contrast enhancement and for the absence of wash-out in the PVP and LP (Figure 4). Furthermore, misinterpretation of this perilesional hyperemic halo as residual viable tumor can be avoided by comparing post-ablation images with pre-ablation scans. Hence, it is highly recommended to perform a pre-treatment CEUS study[22,26,41]. Second, during CEUS, the

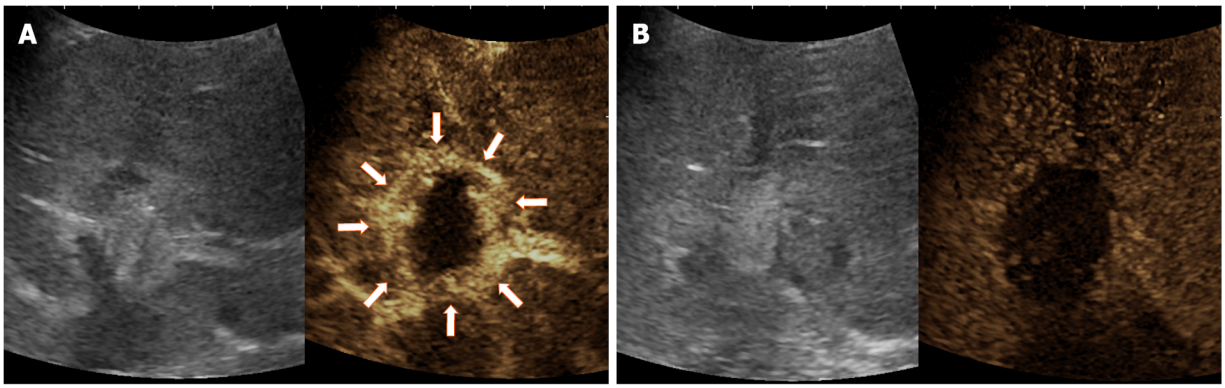

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Figure 4 Post procedural contrast enhanced ultrasound (20 min after microwave ablation) in a 72-year-old man with chronic hepatitis C virus-related hepatocellular carcinoma. Reactive hyperemia achieved immediately after the procedure (22 s). A: Thick and regular rim of arterial phase hyperenhancement surrounds the treatment area (arrows); B: Absence of wash-out in the late phase (4 min) confirms the reactive significance of hyperemia.

hyperechoic foci in the arterial phase detecting as viable tumor may not have clear washout in portal venous and LP as well as HCC. This underlines the importance of careful evaluation of the arterial phase[26]. Third, after thermal ablative therapies, an ill-defined gas cloud and a hyperechoic artifact are usually seen in the treatment bed. This occurs for the outgassing of water vapor produced by the deposition of high intensity energy. In most cases, it is enough to wait 10-15 min for this artifact to resolve[31].

CEUS and fusion imaging

To date, the latest advancement in fusion technology has permitted to match CT and MRI imaging, with real time US or real time CEUS used for ablation guidance[42,43]. According to recent studies, the fusion technology is better compared to conventional tumor ablation only under US or CEUS guidance, showing respectively an effectiveness rate up to 98.8%–100% compared to 67.7%–93.5%[42,44-46]. This approach exceeds the limit of conventional ablation, taking advantage of the high sensitivity of CT and MRI imaging, in which small HCCs are more detectable, with the coregistered real time US or CEUS for ablation guidance[42,47].

When CEUS is available, it is preferable to US. Indeed, CEUS has the advantage of showing the absence of any nodular arterially enhancement portion within or at the margin of the treated lesion in successfully ablated tumor or remaining enhancing residual tumor in uncompleted ablation[23,48]. Hence, the additional use of CEUS may improve lesion conspicuity, allowing a better rate of satisfying treatment[49]. The result is a more confidence during procedure guidance, especially for those tumors poorly visible with US or CEUS: Lesions located in depth or in the liver dome, small size lesions and hypovascular lesions in a severe cirrhotic liver[43,50].

In conclusion, CEUS/fusion imaging guidance has raised the clinical efficacy of ablation technique, particularly in poor conspicuous HCC, aiding in the visualization and ablation of initial and residual enhancing tissue.

CEUS findings after TACE

TACE is considerate the best option for intermediate stage liver cancer according to BCLC classification [8,11,12,51].

In conventional TACE protocols, chemotherapeutic drug emulsion containing iodized oil (Lipiodol, Guerbet, France) is delivered through the tumor feeding artery, followed by embolization[7]. CECT is one of the most commonly used modalities, also recommended by modified Response Evaluation Criteria in Solid Tumors criteria, to evaluate a successfully treated site, as indicated by the lack of arterial phase hyperenhancement[52]. However, many studies have shown that CECT may be inconsistent in detecting residual viable tumoral tissue, especially in the early assessment[53-56].

The estimation of tumor necrosis can be troublesome because the residual viable enhancing tumor may be obscured by the artifacts created by the dense accumulation of the ethiodized oil in TACE, not allowing an accurate interpretation of response. Additionally, radiation hazard and renal iodine contrast toxicity often limit repeated applications of CECT in patients with HCC[57]. In a study encompassing 178 patients, Bargellini *et al*[54] concluded that CECT may overestimate tumor response to TACE with a relatively low specificity (62%) in detecting complete necrosis[54]. Salvaggio *et al*[55] found that CECT is less sensitive to assess residual contrast enhancement than CEUS[55]. Liu *et al*[56] concluded that CEUS shows a better diagnostic performance than CECT and, as consequence, CEUS may be more sensitive and accurate to detect residual tumor after TACE, especially when the tumor is completely filled with lipiodol[56].

CEMRI is considered the most accurate imaging modality for the diagnosis and post-procedural assessment of HCC[58,59]. In comparison with CECT, the better contrast resolution of CEMRI allows a high sensitivity in the assessment of enhanced tissue of viable tumor[60-62]. In addition, the multiphasic contrast enhanced sequences are less affected by the intratumoral retention of iodized oil[61,62]. However, high cost, limited availability and breath holding related artifacts may constitute constraints to a widespread application of MRI. In 2015, in a pilot study by Cho *et al*[58], CECT and CEUS showed sensitivities of 75% and 100%, respectively, when compared with MRI in identifying the presence of residual tumor at 4 wk[58]. In the same study, CEUS also showed a great advantage in the early detection of tumor recurrence or in incomplete treatments[58].

CEUS has been advocated as having equivalent or superior efficacy for assessing HCC response to TACE than CECT, especially for the tumor completely filled with lipiodol[63].

CEUS TIMING STRATEGY

Immediate assessment

The utility of CEUS in this modality has been debated in literature[23,64]. Some studies found a low sensitivity of CEUS performed immediately after procedure owing to false negative results[65,66]. Moschouris *et al*[67] suggested that very early assessment may not be suitable in the case of TACE with doxorubicin capable beads because the level of necrosis could be underestimated[67]. These latter were in part associated to technical difficulties secondary to procedure-related artifacts such as gas or uncooperative patients still under conscious sedation or general anesthesia or in pain. Gas-related artifacts may be avoided by waiting at least 5–10 min to 20–40 min after procedure. However, these drawbacks are offset by low cost, ready availability, absence of radiation exposure and the unique CEUS ability of detecting still viable tumor during the RFA session allowing re-treatment in the same procedural setting.

The best advantage of CEUS in monitoring HCC treatments is the possibility to detect tumor vascularization immediately after ablation, permitting retreatment in the same session if needed. A study has shown that CEUS performed within 1 h after RFA had a good agreement with CECT or CEMRI performed 1 mo later[68]. In the same study, CEUS demonstrated a specificity of 94% but a sensitivity of only 40% in the detection of residual disease[68]. Furthermore, Lekht *et al*[69] showed that performing immediate postprocedural CEUS significantly decreased the incidence of residual tumor seen at follow-up imaging[69]. Moreover, in a study by Mauri *et al*[70] with 93 patients, residual disease was seen at CT or MRI 24 h after the procedure in 5.4% of patients who underwent immediate postprocedural CEUS, whereas residual disease was seen in 36.5% of the patients who did not undergo postprocedural CEUS[70]. These findings demonstrate the significant role of immediate postprocedural CEUS in optimizing patient management[27,41].

24–48 h follow-up

Alternatively, it is possible to perform CEUS at 24-48 h after LRTs[71]. This latter strategy has the disadvantage of not allowing retreatment during the same treatment. However, immediate postprocedural CEUS could be not accessible in all clinical settings, and the aforementioned technical problems could overcome[23].

Nevertheless, the usefulness of CEUS at 24-48 h is still a debated topic, considering that some studies confirm low sensitivity owing to peripheral hyperemia[65,66]. However, a recent study involving 94 patients showed sensitivity and specificity of CEUS performed at 48 h after LRTs (percutaneous ethanol injection, RFA, TACE, combined treatments), respectively, of 79.1% and 96.7% compared to CECT at 1 mo as reference standard[56]. The same authors thinks that tumor position may have affected the results in all false negative cases, confirming that CEUS may not be always practicable due to the inherent inconveniences associated with ultrasound in general. Moreover, peripheral reactive hyperemia may still be a confounding factor even 48 h after the procedure[56].

In our opinion, both CEUS for the immediate postprocedural assessment and CEUS performed at 24-48 h after LRTs could be considered the first choice exam to evaluate quickly the technical success of the ablation without having to wait 4 wk to evaluate response using CECT.

Secondary surveillance

CEUS performed 1 mo after therapy was demonstrated to be valid to identify residual disease after both percutaneous and intra-arterial treatments. Vilana *et al*[66] reported a sensitivity of 91% and a specificity of 97% after LRTs[66]. Another study demonstrated sensitivity and specificity of CEUS of 87% and 98.4%, respectively, in the evaluation of efficacy 1-mo after ablation treatment of hepatocarcinoma, showing an optimal diagnostic agreement with CT (94.6%)[72]. Hence, several reports have concluded that the sensitivity, specificity and diagnostic accuracy of CEUS in the detection of residual tumor performing at 1-mo is at least equal to that of CECT and CEMRI examinations[66,72-74]. Bansal *et al*[41] proposed their own protocol with CEMRI performed 1 mo after the treatment, and subsequent imaging performed at 3-mo intervals and the patient alternating between CEUS and MRI for the first 2 years[41].

In our experience, 1-mo CEUS with second generation contrast agents can be considered a good alternative imaging modality to CECT for evaluating the efficacy of HCC therapy, with sensitivity of 92.9% and specificity of 98.8%, when compared to MDCT. However, it must be considered that deep lesions in the liver and irregular reactive peripheral hyperemia could still represent a diagnostic problem even at 1-mo CEUS[23,75].

TWO DIMENSIONAL (2D)-CEUS *VS* 3D-CEUS

Three dimensional-CEUS consents the division of structures into tomographic slices in three orthogonal planes, giving additional information unachievable with 2D-CEUS[76]. The bidimensional nature of CEUS makes it impossible to visualize the entire ablated volume but only single planes. Moreover, the vasculature of HCCs may be heterogeneous over the 2D imaging planes, and this represents a limitation of the evaluation of treatment.

The development of real time 3D and four-dimensional US techniques may increase the diagnostic accuracy in detection of hypervascular contrast enhancement, presenting several slices continuously and allowing a superior representation of the whole tumor throughout the vascular phases after the injection of contrast agent[23,77]. During conventional 2D-CEUS there may not be enough time to evaluate in the whole lesion the rapid duration of hyperenhancement during the arterial phase. Covertly 3D-CEUS, permitting acquisition of the volume data of region of interest in a few seconds, could make it easier to evaluate the enhancement of the whole target area.

Furthermore, the volume data are able to be retrieved and thoroughly reviewed from various directions or slice by slice with a sub millimeter thickness. Therefore, 3D-CEUS may pick up some residual tumor tissue that is missed by 2D-CEUS[78,79]. Xu *et al*[78] used static 3D-CEUS to evaluate the treatment response of liver cancer (n = 107) after local therapies and found that 3D-CEUS improved diagnostic confidence relative to 2D-CEUS[78]. Luo *et al*[80] compared 3D-CEUS performed 1 d after RFA for the assessment of residual disease using 1-mo 3D-CEUS as reference standard. They have shown a good agreement and a sensitivity, specificity and accuracy of 1-d 3D-CEUS for detecting adequate ablation of 97%, 100% and 97%, respectively[80]. In our preliminary study, 2D- and 3D-CEUS have provided a comparable diagnostic performance in the assessment of therapeutic response of HCC treated with LRTs[77].

We think 3D-CEUS is an increasingly used technique. Current studies showed that 3D-CEUS is a promising technique to improve the evaluation of tumor response to therapy, providing supplementary information unachievable with 2D-CEUS.

CEUS LIMITATIONS

CEUS has the identical limitations as conventional B-mode ultrasound imaging. Suboptimal ultrasound technique can lead to ineffective surveillance on CEUS. Hence, general limitations in the use of CEUS in the liver are in patients who have a large body habitus, bad acoustic window, intervening bowel gas and poor physical condition. Moreover, tiny lesions deeply located in the liver parenchyma, especially at depth more than 12 cm, are difficult to interpret because of limited ultrasound wave penetration of a fatty liver[19]. Furthermore, CEUS is not comparable to CECT in case of intra-arterial treatments that include more than one lesion, since each tumor has to be evaluated separately to detect changes in arterial enhancement, even if reinjection of USCA is carried out[13,56].

CONCLUSION

In summary, CEUS enables in real-time assessment of the therapeutic effect of LRTs in HCC and provides an easy, repeatable and cost-effective way for detecting residual disease. Although CEUS is subject to the same limitation as B-Mode US and is inferior to CECT/ CEMRI in some aspects, CEUS can be considered the first-line exam for the early assessment of treatment efficacy during the interventional procedure to determine the necessity of immediate additional treatment.

CEUS could also be considered the first-line exam in monitoring the efficacy of TACE. Moreover, CEUS can be considered a reliable alternative imaging modality to CECT/CEMRI at 1 mo follow-up and could be used in conjunction with CECT/CEMRI in follow-up.

FOOTNOTES

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MINIREVIEWS

Benign focal liver lesions: The role of magnetic resonance imaging

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Abstract

Liver lesions are common findings in radiologists' daily routine. They are a complex category of pathology that range from solitary benign lesions to primary liver cancer and liver metastases. Benign focal liver lesions can arise from different liver cell types: Epithelial (hepatocytes and biliary cells) and none-pithelial (mesenchymal cells). Liver magnetic resonance imaging (MRI) is a fundamental radiological method in these patients as it allows with its multiparametric approach optimal non-invasive tissue characterization. Furthermore, advanced liver MRI techniques such as diffusion-weighted imaging and hepato-biliary contrast agents have improved the detection of focal liver lesions and can be highly effective in differentiating pseudotumor from tumors, as well as benign from malignant lesions, and can also be used for differential diagnosis. Although histological examination can be useful in making a definitive diagnosis, MRI is an important modality in the diagnosis of liver lesions with a significant impact on patient care. This aim of this review is to provide a comprehensive overview of benign liver lesions on MRI.

Key Words: Magnetic resonance imaging; Liver neoplasms; Biliary tract; Hepatocytes

Core Tip: Liver magnetic resonance imaging (MRI) is a fundamental radiological technique in patients with focal liver lesions as it allows, with its multiparametric approach, optimal non-invasive tissue characterization. Liver MRI can be highly effective in distinguishing pseudotumor from tumors, as well as benign from malignant lesions and can also be used for differential diagnosis. Although histopathological assessment sometimes has an important role in definitive diagnosis, MRI is a key imaging modality in the diagnosis of liver lesions with a great impact on patient management.

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INTRODUCTION

Liver lesions are common findings in radiologists' daily routine. They are a complex category of pathology that ranges from solitary benign lesions to primary liver cancer and liver metastases[1-4]. Benign focal liver lesions can arise from different liver cell types: Epithelial (hepatocytes and biliary cells) and nonepithelial (mesenchymal cells)[5]. The diagnosis is often straightforward, although sometimes distinguishing between malignant primary and secondary lesions may represent a diagnostic challenge due to atypical tumor appearance and features.

To avoid misdiagnosis, radiologists must cope with the key characteristics of the lesion and decide which imaging procedure [*i.e.*, ultrasound (US), computed tomography (CT) and/or magnetic resonance imaging (MRI)] will most likely provide the diagnosis[3,4,6-8].

The use of advanced liver MRI techniques such as diffusion-weighted imaging (DWI), multiarterial phase technique, hepatobiliary contrast agents and artificial intelligence have improved the detection and differentiation of different focal liver lesions[2-4,8-11]. Furthermore, liver MRI is often the last imaging technique used in the diagnostic algorithm before liver biopsy.

Therefore, it is important to know the characteristics of different focal liver lesions on MRI and how to differentiate between benign and malignant lesions in order to make the correct diagnosis, recommend the best follow-up when necessary, cut the costs of unnecessary diagnostic tests and, last but not least, reduce patient anxiety.

This aim of this article is to provide a comprehensive overview of benign liver lesions on MRI. A schematic representation showing MRI features of benign liver lesions is presented in Figure 1. Table 1 shows the histological classification that was used in this review. Table 2 provides an overview of the MRI contrast agents currently used in clinical practice.

EPITHELIAL TUMORS (HEPATOCELLULAR AND BILIARY)

Hepatocellular adenoma

Hepatocellular adenoma (HCA) is a rare benign monoclonal neoplasm of the liver, composed of hepatocytes arranged in sheets or in a cord-like fashion with a lack of portal venules and biliary ductules[12-14]. Hepatocyte sheets are separated by dilated sinusoids. They are perfused solely by high-pressure peripheral arterial feeding vessels, resulting in a remarkable hypervascular nature. Hepatocytes might contain a variable amount of intracellular fat or glycogen.

HCA has an annual incidence of about 1-1.3 million cases per year in North America and Europe; 85% of cases occur in women of childbearing age (15-45 years old)[15]. An increased incidence of HCAs has been reported since the 1960s following the introduction of oral contraceptive pills (OCPs)[14,16]: Long-term users of OCPs have a 25-fold increased risk of developing HCA compared to the general population[14]. Some studies suggested that OCP discontinuation might lead to tumor regression, but this remains controversial[17]. Similarly, long-term use of anabolic androgen steroids (AAS) has been associated with a high risk of developing HCA. Glycogen storage disorders (GSDs), in particular type 1 and type 3, have been linked to an increased incidence of HCAs[14].

Hepatic adenomatosis, first described in 1985, is a condition in which 10 or more adenomas are present in an otherwise normal liver[18]. These cases are at higher risk of complications: 63% risk of hemorrhage and 10% risk of malignant transformation[19]. Other risk factors for HCA are diabetes (both type 1 and 2), metabolic syndrome, obesity, Fanconi's anemia, familial adenomatosis polyposis,

Table 1 Histological classification of benign liver lesions

Epithelial tumors (hepatocellular and biliary)	Mesenchymal tumors	Pseudotumor
Hepatocellular adenoma	Hemangioma	Focal fatty infiltration
Focal nodular hyperplasia	Lymphangioma	Infection (liver abscess, *Echinococcus granulosus*)
Biliary cystadenoma	Solitary fibrous tumor	Inflammatory disorder of the liver (pseudotumor, sarcoidosis)
Biliary hamartoma (von Meyenburg Complex)	Mesenchymal hamartoma	

Table 2 Magnetic resonance contrast agent

Category	Molecule	Structure	Ioniity	Relaxivity	Recommended dose (mmol/kg)	Excretion
ECAs	Gadoterate meglumine (Dotarem)	Macrocyclic	Ionic	Standard	0.1	Renal
ECAs	Gadobutrol (Gadavist)	Macrocyclic	Non-ionic	Standard	0.1	Renal
ECAs	Gadoteridol (Prohance)	Macrocyclic	Non-ionic	Standard	0.1	Renal
ECAs	Gadopentetate dimeglumine (Magnevist)	Linear	Ionic	Standard	0.1	Renal
ECAs	Gadoversetamide (OptiMark)	Linear	Non-ionic	Standard	0.1	Renal
ECAs	Gadodiamide (Omniscan)	Linear	Non-ionic	Standard	0.1	Renal
HBA	Gd-EOB-DTPA (Eovist/Primovist)	Linear	Ionic	High	0.025	50% renal, 50% biliary
HBA	Gd-BOPTA (MultiHance)	Linear	Ionic	High	0.1	5% biliary, 95% renal
BPA	Gadofosveset trisodium (Ablavar)	Linear	Ionic	High	0.03	Renal

ECAs: Extracellular agents; HBA: Hepatobiliary agents; BPA: Blood pool agents.

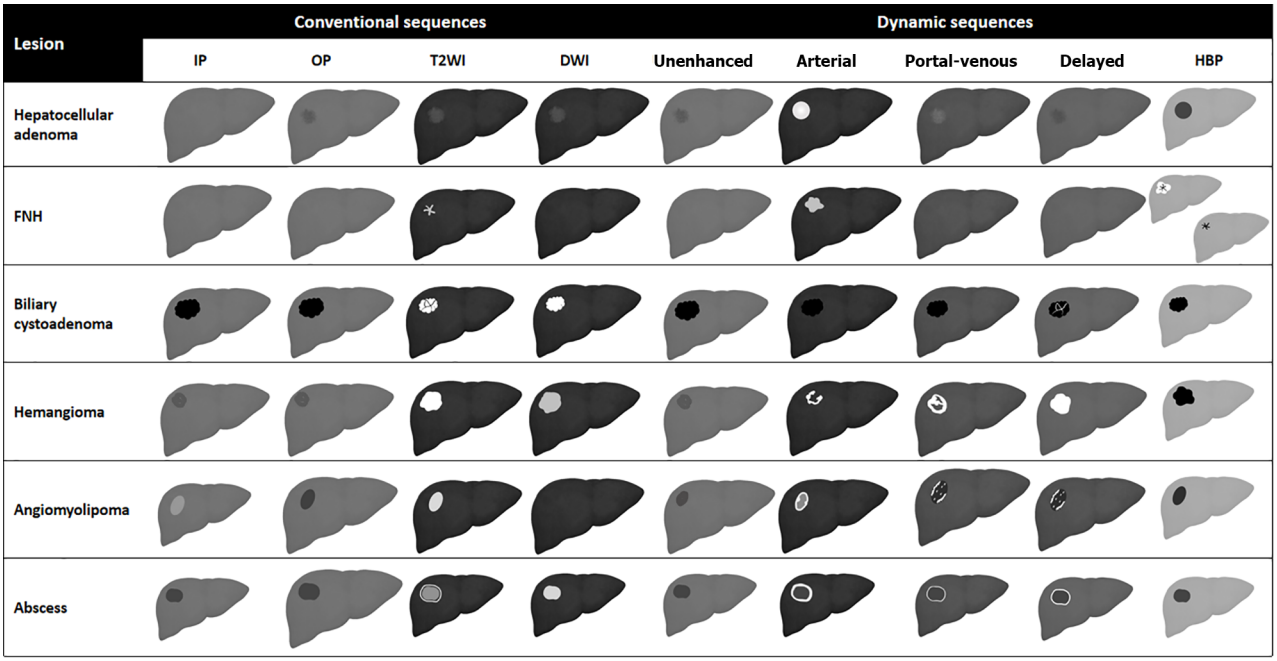

Figure 1 Schematic representation showing liver magnetic resonance imaging features of benign liver lesions. IP: T1-weighted in-phase imaging; OP: T1-weighted out-of-phase imaging; DWI: Diffusion weighted imaging; HBP: Hepatobiliary phase; FNH: Focal nodular hyperplasia.

beta thalassemia and tyrosinemia[14].

HCAs may be complicated by the presence of intralesional fat, hemorrhage, or malignant transformation with a subsequent wide range of imaging appearances resulting in a challenging diagnostic process. In 2006, four subtypes of HCA were identified based on genotype-phenotype analyses according to the genetic and histopathological features of the lesion: (1) Type 1: Hepatocyte nuclear factor (HNF)-1α HCA; (2) Type 2: Inflammatory HCA (I-HCA); (3) Type 3: β-catenin activated

HCA; and (4) Type 4: Unclassified HCAs[12].

Several hypervascular benign and malignant hepatic lesions may mimic HCA: Focal nodular hyperplasia (FNH), hepatocellular carcinoma (HCC), hemangioma (HA), angiomyolipoma and metastases. The use of hepatocyte-specific contrast agent allows differential diagnosis between HCA and FNH with very high sensitivity and specificity (respectively 91% to 100% and 87% to 100%)[20]. On the hepatobiliary phase (HBP), most HCAs present a low signal compared to surrounding parenchyma, while FNHs have an iso- or hyperintense signal. Some HCAs, however, generally I-HCAs, may have an iso- or hyperintense signal on the HBP.

Type 1: HNF-1α HCA

HNF-1α HCA is the second most common subtype of HCA, representing 30% to 35% of lesions; it is almost exclusively found in women, the majority of whom (> 90%) with a history of OCP use in anamnesis[20]. This type of HCA may be associated with maturity-onset diabetes of the young and familial adenomatosis. HNF-1α HCA generally has an indolent biological behavior and among all types of HCA it has the lowest risk of malignant degeneration. HNF-1α HCA may be associated with mutations of the transcription factor 1 gene on chromosome 12q24.43, an anti-oncogene involved in hepatocyte differentiation, whose inactivation can promote proliferation of such cell lines[14].

Due to its intracellular fat content, HNF-1α HCA typically exhibits diffuse intratumoral signal loss on chemical-shift T1-weighted imaging (*i.e.*, signal loss on opposed-phased images compared to in-phase images); this finding alone demonstrates 86.7% sensitivity and 100% specificity for this subtype. HNF-1α HCA is often hyper- or isointense on T1-weighted images and iso- or hyperintense on T2-weighted images, with no apparent signal restriction on diffusion imaging. HNF-1α HCAs often show hyper-enhancement in the arterial phase after contrast injection; however, this does not persist in the portal venous phase[14,21] (Figure 2).

Type 2: I-HCA

This is the most frequent subtype, accounting for 40% to 50% of all HCAs; it is linked to mutations in the interleukin 6 signal transducer gene, which is located on chromosome 5q11 and codes for glycoprotein 130. This mutation causes the Janus kinase (JAK) signal transducer to be activated indefinitely, resulting in aberrant hepatocyte proliferation. Type 2 HCA is most common in young women on OCP medication, as well as in those with metabolic syndrome or obesity.

I-HCA is the HCA type with the highest risk of bleeding, occurring in up to 30% of cases. Malignant transformation of HCAs into HCC occurs in 5% to 10% of cases. On MRI, I-HCA generally shows a heterogeneous high signal on T2-weighted sequences, more intense in the peripheral part of the lesion ("atoll" sign) and arterial enhancement that persists in the portal venous and delayed phases. Such features are due to the presence of dilated sinusoids. Intralesional steatosis is rare, more frequently focal or with a patchy and heterogeneous pattern. Intratumoral hemorrhage, reported in up to 30% of cases, results in increased lesion heterogeneity (Figure 3). Acute hemorrhage presents a high signal on T1-weighted sequences due to the presence of methemoglobin; chronic hemorrhage has a low signal on T1 and T2-weighted sequences due to hemosiderin content[14,21].

Type 3: β-catenin-activated HCA

β-catenin activated HCAs account for 10% to 15% of all HCAs. Mutations in the β-catenin gene (CTNNB1) on chromosome 3p21 cause continuous activation of the β-catenin protein, resulting in uncontrolled hepatocyte growth. It is more common in men and is linked to GSDs, AAS, and familial adenomatosis polyposis. β-catenin mutation is strongly associated with malignant degeneration[14].

β-catenin-activated HCAs do not have typical MRI features. Such lesions generally do not contain intratumoral fat. Malignant transformation may be suspected in the case of growth, local invasion, or contrast medium washout on portal-venous or delayed phases. β-catenin-activated HCAs present arterial enhancement and washout on the portal-venous and delayed phase, making differential diagnosis with HCC challenging[14,21,22].

Type 4: Unclassified HCAs

This category includes approximately 10% of all HCAs with an adenoma-like appearance but no distinguishing genetic and/or clinical characteristics. There is a scarcity of information about these lesions. Such tumors have no pathognomonic characteristics on MRI[14,23].

FNH

After HA, FNH is the second most common benign hepatic tumor (representing around 8%-9% of all primary liver tumors) and is frequently an incidental finding on imaging as most individuals are asymptomatic. FNHs are more common in females than in males (8:1), in the third to fifth decades, and, unlike HCAs, a relationship with OCPs is uncommon. Severe FNH syndrome is defined as the presence of multiple FNH lesions (up to 20%) and HAs. To ensure adequate treatment, it is crucial to distinguish FNH from other hypervascular liver lesions such as HCA, HCC, and hypervascular metastases[24].

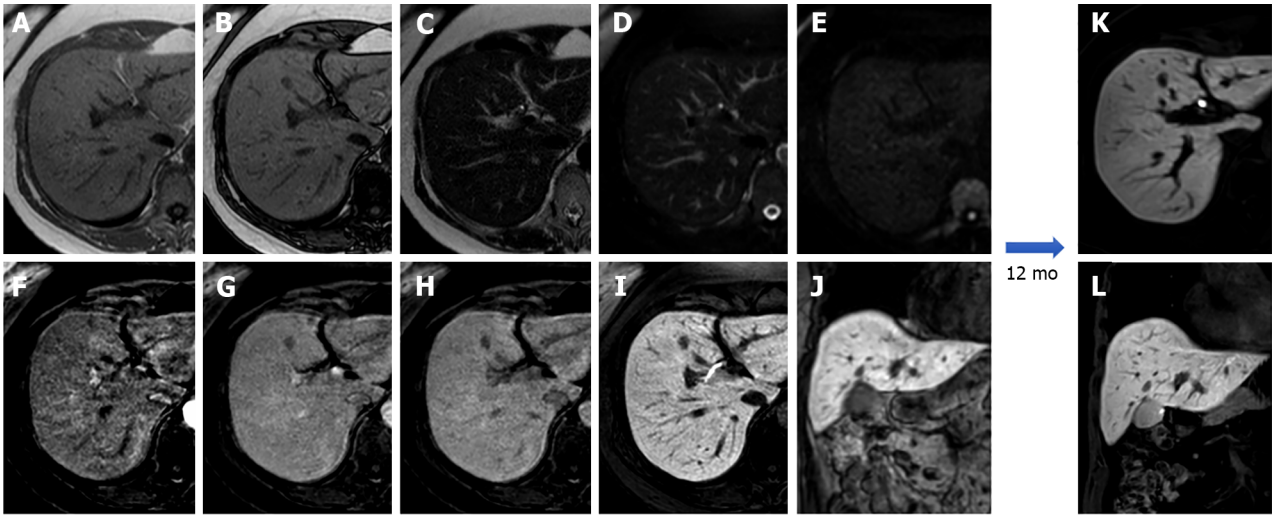

Figure 2 A 53-year-old patient presented with right hypochondrium pain and underwent abdominal ultrasound examination demonstrating a hyperechoic nodule in S4. Liver magnetic resonance imaging confirmed an isointense nodule on T1 in-phase sequence with loss of signal on opposed-phase T1-weighted images, isointense on T2 sequences, without increased signal intensity on diffusion weighted images, with minimum wash-in on the arterial phase and wash-out on the portal-venous and delayed phase, hypointense in the hepatobiliary phase, findings consistent with hepatocyte nuclear factor 1α - mutated hepatocellular adenoma. A: In-phase T1-weighted image; B: Out-of-phase T1-weighted image; C: T2-weighted image; D: T2-Spectral Attenuated Inversion Recovery; E: High *b*-value diffusion weighted imaging; F: Arterial phase magnetic resonance imaging (MRI); G: Portal venous phase MRI; H: Delayed phase MRI; I-L: Hepatobiliary phase MRI.

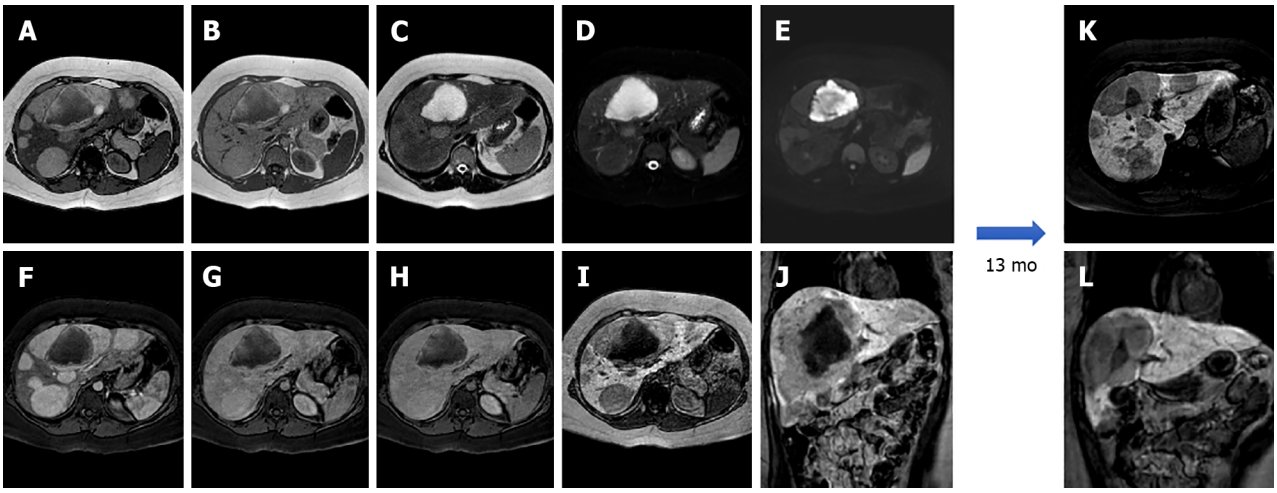

Figure 3 A 25-year-old female with 10-year history of oral contraceptive pill presented to the emergency department with diffuse abdominal pain. Liver magnetic resonance imaging demonstrated multiple hepatic lesions, the larger 15 cm in diameter in S4-S5-S8 shows iso-hyperintense signal on T1-weighted sequences, with dishomogenous hypointense central component with peripheral hyperintensity. The lesion is isointense on T2-weighted sequences with marked hyperintensity of the central component; on diffusion weighted images the mass presents a slight increase in signal while the central portion has a marked signal increase. At the dynamic study, the mass has wash-in that persists on the portal venous and delayed phase while the central portion illustrates minimum enhancement. The mass is hypointense on the hepatobiliary phase with a marked hypointense central portion. Liver biopsy demonstrated a hepatic adenoma with a bleeding component. At 13 mo follow-up, the lesion presented a slightly reduced diameter while the central bleeding component was almost completely reabsorbed. A: Out-of-phase T1-weighted image; B: In-phase T1-weighted image; C: T2-weighted image; D: T2-Spectral Attenuated Inversion Recovery; E: High *b*-value diffusion weighted imaging; F: Arterial phase magnetic resonance imaging (MRI); G: Portal venous phase MRI; H: Delayed phase MRI; I-L: Hepatobiliary phase MRI.

FNH should be regarded as a regenerative lesion rather than a neoplasm: It may be caused by the presence of a vascular abnormality (either of the arterial or portal vascular supply) that, when combined with hormonal stimulation, results in mass growth. Histologically, these lesions are known as hamartomatous malformations[25]. From an anatomopathological point of view FNH is a nodule with polycyclic contours, composed of organized hepatocytes, completely or incompletely surrounded by circular or short fibrous septa originating from a central scar that contains a hypertrophic arterial vessel, originating from the hepatic artery.

The surrounding septa connective tissue also contains numerous capillaries and ductules. The rich network of capillaries, that arise from the central artery, provide arterial blood to the hepatocytes and sinusoids, in accordance with the highly hypervascular nature of most FNH lesions on imaging[26]. The sinusoids, the malformed arteries and the vein of FNH drain into the hepatic vein. FNH does not have a portal venous supply. Normal liver tissue surrounds the FNH and there is no fibrous capsule at the interface of the lesion and the liver[27].

Due to its vascular physiopathology, FNH is commonly associated (20%-30%) with other hepatic lesions or conditions such as hepatic HA, arteriovenous malformations, anomalous venous drainage, HCA (possible but not proven), congenital absence of portal vein/portal vein atresia and portal shunts [28]. Because it contains hepatocytes, FNH shows an isointense signal on both T1- and T2-weighted sequences with occasional slight hypointensity and hyperintensity on T1 and T2, respectively.

MRI imaging has higher sensitivity (70%) and specificity (98%) for FNH than US and CT. Both on CT and MRI, after contrast medium administration, FNH shows homogenous and strong contrast enhancement in the arterial phase with the exception of the central scar[26]. During portal-venous and delayed phases, it becomes isointense to the liver parenchyma while the central scar remains relatively hypointense. On HBP FNH appears isointense to hyperintense compared to adjacent liver parenchyma without or with the presence of a central scar[21], which is hypointense (Figure 4).

On diffusion weighted imaging the average numerical value of the apparent diffusion coefficient (ADC) in benign hepatic lesions (FNH, HA, HCA) is about 1.88 (1.326-2.48) × 10^3 mm^2/s, while the ADC of malignant liver lesions [HCC, cholangiocarcinoma (CCC), colorectal cancer liver metastasis (CRCLM)] are significantly lower, around 1.15 (1.024-1.343) × 10^{-3} mm^2/s[29]. FNH and HCA have ADC values lower than normal liver but FNH has an ADC higher than HCA[30].

The central scar is more often detected with MRI than with CT (78% and 60%, respectively)[31]. The central scar appears slightly hyperintense on T2-weighted images and this is an important difference compared with the central scar of HCC that is generally hypointense in all sequences and is rarely hyperintense, mimicking that of FNH[32].

On MRI with non-hepatobiliary specific agents (and consequently also on CT imaging) the central scar shows enhancement in the delayed phase due to the presence of abundant myxomatous stroma. With different hepatobiliary specific agents the central scar manifests variously[32]: (1) With gadobenate dimeglumine (Gd-BOPTA - MultiHance®; Bracco, Milan, Italy) the central scar will enhance in the late phase (hepato-biliary excretion after 1-3 h); and (2) With gadoxetic acid (Gd-EOB-DTPA - Primovist®; Bayer-Schering, Berlin, Germany) the central scar will never enhance because the late venous phase overlaps the HBP (that comes on average after 10-100 min) making everything that is not of hepatocellular origin hypointense, including the connective central scar.

Atypical FNH

Currently, FNH is divided into two types: Classic and non-classic. Classic FNH is characterized microscopically by the presence of: (1) Abnormal nodular architecture; (2) Malformed vessels; and (3) Cholangiolar proliferation and on imaging appears with all the characteristics mentioned above. Non-classic FNH lesions lack one of the classic features but always show bile ductular proliferation[24]. Non-classic FNH may demonstrate "atypical appearance" at imaging that may lead to the following 5 subtypes: (1) No central scar FNH; (2) Large central scar; (3) Intralesional fat; (4) Presence of a pseudocapsule; and (5) Sinusoidal distension (Figure 5).

Biliary cystadenoma

Biliary cystadenoma (BCA) is an uncommon (less than 5% of cystic liver lesions) benign hepatic tumor that arises from intrahepatic biliary channels, most commonly in the right lobe (55%), and rarely from extrahepatic biliary ducts or the gallbladder[33]. Due to its high prevalence in middle-aged women (> 85%)[33] it is considered a congenital disorder with potential hormonal influence. Due to its high risk of recurrence (if non-completely removed) and its potential risk for malignant degeneration into biliary cystadenocarcinoma (BCAC), the differential diagnosis with other cystic lesions of the liver is mandatory[21].

US imaging reveals a massive, lobulated, well-defined hypoechoic or anechoic mass with hyperechoic interior septa or calcifications and solid papillary projections; contrast-enhanced ultrasonography (CEUS) may indicate enhancement of the walls and septa. These features may aid in identifying BCA from other liver cystic lesions[33].

CT confirms US appearance and CEUS behavior: A single multiloculated cystic lesion with a well-defined thick fibrotic capsule, mural nodules, and rarely calcifications of the capsule[33]. On MRI, BCA appears as a fluid-containing multilocular lesion that is significantly hypointense on T1-weighted sequences and hyperintense on T2-weighted images with septal hypointensity. On both T1- and T2-weighted images, the signal intensity may fluctuate due to the presence of proteinaceous material or blood products[21] (Figure 6). If thinner septa and a regular wall are seen in cystadenoma, polypoid excrescences, hemorrhage and evident vascularized septa are more suspicious for BCAC, but the differential diagnosis between such lesions may be challenging[21].

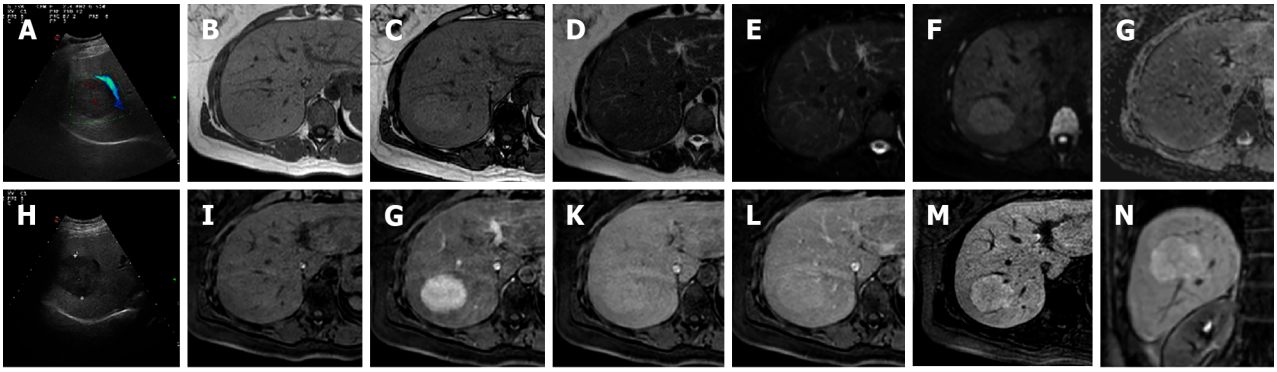

Figure 4 A 41-year-old female presented to the emergency department with diffuse abdominal pain and underwent ultrasound examination revealing a 5.3 cm solid hypoechoic lesion in S7. Liver magnetic resonance imaging confirmed an isointense lesion on T1-weighted sequence, isointense in T2 sequences, with minimum increased signal intensity on diffusion weighted images and isointense on the ADC map. After gadoxetic acid administration the lesion shows intense arterial enhancement; slightly hyperintense on the portal venous and delayed phases. The mass shows hyperintense signal on the hepatobiliary phase. These findings are consistent with typical focal nodular hyperplasia. A: Liver ultrasound image; B: In-phase T1-weighted image; C: Out-of-phase T1-weighted image; D: T2-weighted image; E: T2-Spectral Attenuated Inversion Recovery; F: High *b*-value diffusion weighted imaging; G: ADC map; H: Liver ultrasound image; I: Pre-contrast phase magnetic resonance imaging (MRI); J: Arterial phase MRI; K: Portal venous phase MRI; L: Delayed phase MRI; M and N: Hepatobiliary phase MRI.

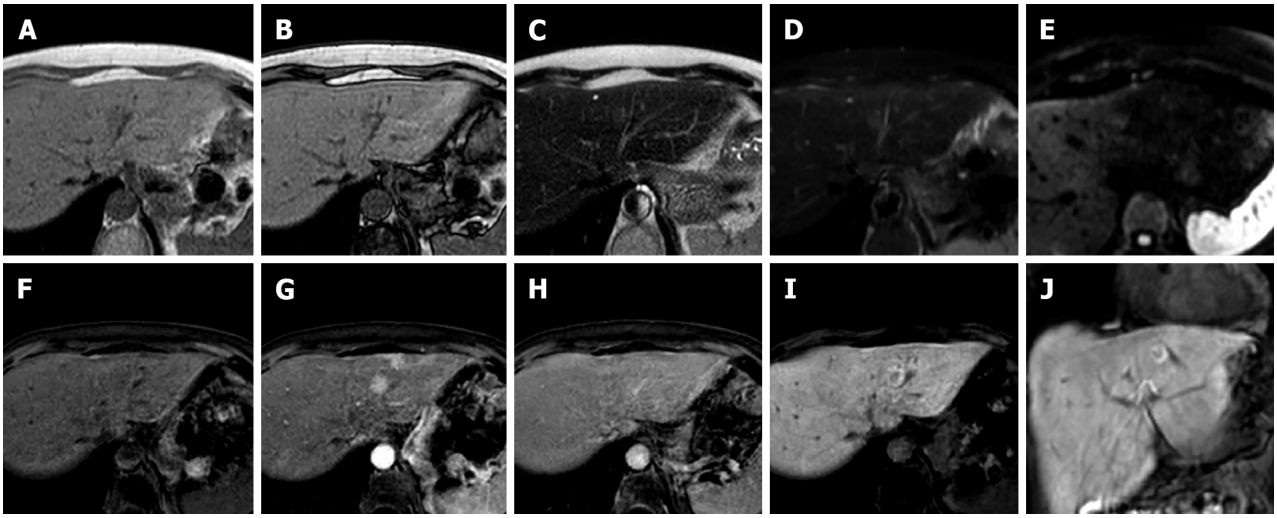

Figure 5 A 56-year-old female with ultrasound evidence of a hyperechoic nodular lesion in S8. Liver magnetic resonance imaging confirmed a nodule, slightly hypointense on T1-weighted, slightly hyperintense on T2-weighted sequences, without increased signal intensity on diffusion weighted images. After gadoxetic acid administration, the lesion shows homogeneous arterial enhancement, becomes isointense during the portal venous phase, and presents peripheral hyperintensity with a hypointense core on the hepatobiliary phase. Such findings are consistent with atypical focal nodular hyperplasia. A: In-phase T1-weighted image; B: Out-of-phase T1-weighted image; C: T2-weighted image; D: T2-Spectral Attenuated Inversion Recovery; E: High *b*-value diffusion weighted imaging; F: Pre-contrast phase magnetic resonance imaging (MRI); G: Arterial phase MRI; H: Portal venous phase MRI; I and J: Hepatobiliary phase MRI.

Biliary hamartoma (von Meyenburg Complex)

Multiple biliary hamartomas, also known as von Meyenburg Complex, are rare bile duct malformations, with a very low incidence ranging between 0.4% and 5.5%. There is a higher prevalence in females (F/M = 7/4) with a mean age of 48 years (range 33-68 years). They belong to the spectrum of "fibropolycystic liver disease" and are defined as small duct dilatations within thick bile. The cause is unknown, however it can be present in both non-cirrhotic and cirrhotic livers, as well as in children and adults. However, its prevalence rises dramatically with chronic liver illness, implying an acquired etiology[34].

Histologically, bile duct hamartomas are generally small, less than 1 cm, often subcapsular, and composed of small dilated biliary ducts surrounded by biliary epithelium[35]. On T1-weighted images they typically appear as small hypointense lesions, with a strongly increased signal on T2WI due to its intrinsic cystic component especially on heavily weighted T2 sequences. These lesions can show the presence of small mural nodules (1-2 mm) with an isointense signal on T1-weighted and an intermediate signal on T2-weighted images. On dynamic sequences, the mural nodules can show enhancement during the portal-venous phase in about 90% of cases with no core enhancement. On cholangiography sequences, both intra- and extra-hepatic bile ducts are normally represented[36,37],

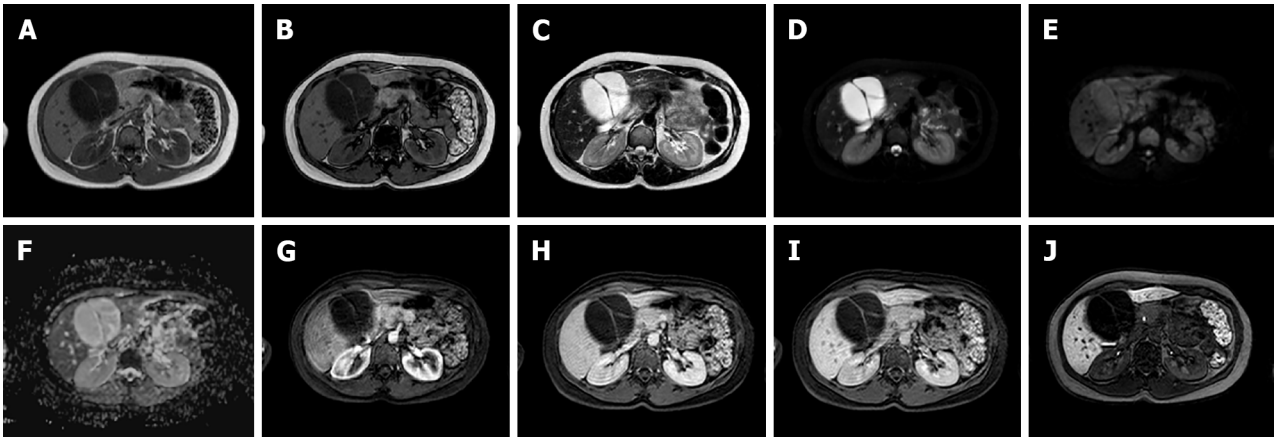

Figure 6 A 34-year-old female patient. Liver magnetic resonance imaging demonstrated a multiloculated cystic mass in S4b, hypointense on T1 sequences with isointense septa, hyperintense on T2 sequences, slightly hyperintense on diffusion weighted images and without significant restriction on the ADC map. On dynamic study after gadoxetic acid administration, the lesion presents no enhancement even in the septal components. On the hepatobiliary excretion phase the mass is hypointense. After surgical resection, histological examination demonstrated a biliary cystadenoma. A: In-phase T1-weighted image; B: Out-of-phase T1-weighted image; C: T2-weighted image; D: T2-Spectral Attenuated Inversion Recovery; E: High *b*-value diffusion weighted imaging; F: ADC map; G: Arterial phase magnetic resonance imaging (MRI); H: Portal venous phase MRI; I: Delayed phase MRI; J: Hepatobiliary phase MRI.

while multiple hyperintense cystic lesions on T2-weighted images are uniformly distributed in the liver with no communication with the bile ducts, appearing as a ''starry sky'' configuration[38] (Figure 7). Biliary hamartomas should be carefully recognized in order to distinguish it from metastases, microabscess, simple liver cysts, and Caroli disease (Figure 8).

MESENCHYMAL TUMORS

HA

Hepatic HAs are the most prevalent benign mesenchymal liver lesions, with an estimated frequency of around 20% in the general population, and are up to five times more common in females than males [39]. HAs are frequently an incidental finding in asymptomatic patients during routine radiological examinations; however, because of their high prevalence, the differential diagnosis can become complex in patients affected by primary liver cancer or liver metastases. HAs are categorized by size: Small HAs are 1-2 cm, typical HAs are 2-10 cm and giant HAs have a diameter of more than 10 cm. Recent retrospective cohort studies demonstrated that up to 40% of HAs may grow during follow-up, with a slow growth rate (2 mm/year in diameter and 17.4%/year in volume)[39].

Microscopically, HAs appear as cavernous vascular spaces lined by endothelium and containing a fibrous stroma. Larger HAs may contain a fibrous nodule or a collagen scar. HAs are usually fed by vessels from the hepatic artery circulation, with a slow blood flow within the vascular spaces. HAs have been histologically categorized in 3 different subtypes, with some overlap among them: Cavernous, capillary and sclerosed.

Cavernous HA

This is the most common subtype, generally smaller than 3 cm with few internal connective components and with a prevalence of large vascular spaces[21]. The high water content of HAs causes homogeneous hyperintensity on T2-weighted sequences and poor signal intensity on T1-weighted images during MRI [40]. Giant HAs may present a central T2-weighted hypointense component in the case of hyalinized or thrombosed areas. If calcifications are present, they show very low signal intensity on all sequences.

On diffusion weighted imaging, HAs show hyperintensity on b0 s/mm^2 images, with a progressive decrease in signal intensity at higher *b* values; the signal on the ADC map is greater than that of the nearby liver parenchyma. Some HAs have residual hyperintensity on high *b* value images (500-750 s/mm^2) because of the "T2 shine through effect" making them more difficult to characterize; however, quantitative assessment with the ADC map make differential diagnosis easy as their signal is always higher than that of surrounding liver[41]. Following contrast injection, typical cavernous HAs exhibit peripheral nodular enhancement on the arterial phase, with centripetal progression and progressive filling on the portal venous and delayed phases; due to a lack of hepatocytes, they display a hypointense signal on the HBP[21] (Figure 9).

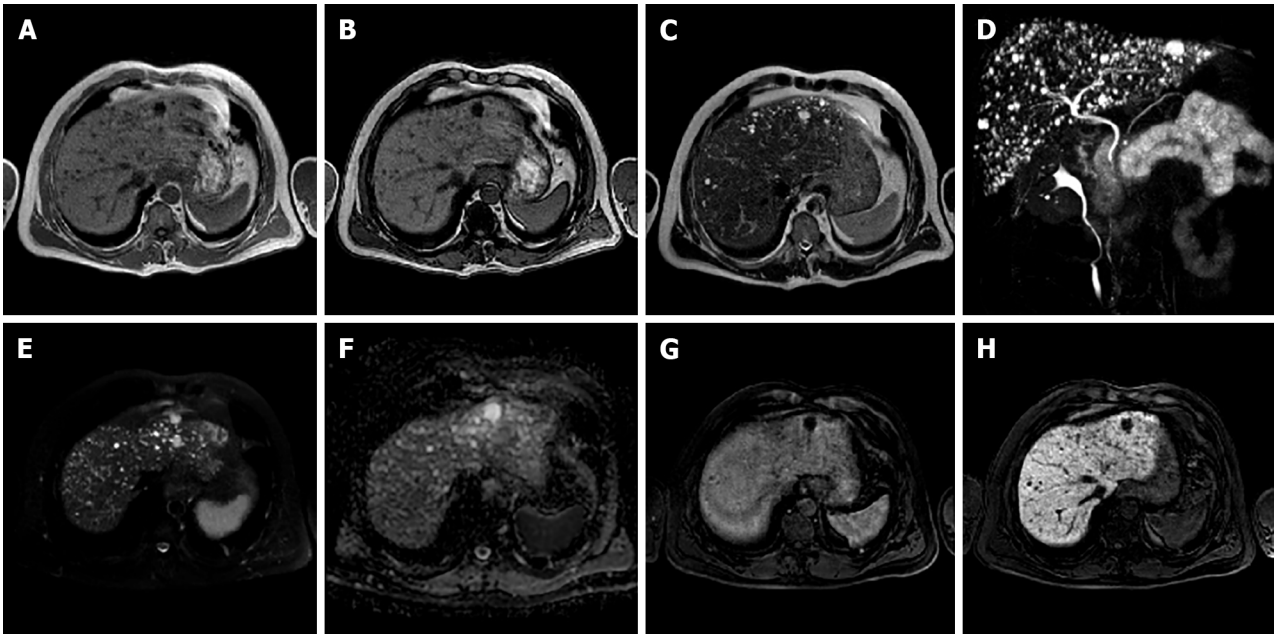

Figure 7 A 50-year-old male. Liver magnetic resonance imaging demonstrates the presence of multiple bilobar small nodular lesions, hypointense on T1 imaging, hyperintense on T2 sequences, without diffusion restriction on the ADC map and presenting no enhancement on the portal venous phase after contrast injection. These nodules are hypointense on the hepatobiliary phase. Such features and the lack of communications with the biliary tree demonstrated on maximum intensity projection images from magnetic resonance cholangiography are consistent with multiple biliary hamartomas (von Meyenburg complex). A: In-phase T1-weighted image; B: Out-of-phase T1-weighted image; C: T2-weighted image; D: 3D maximum intensity projection reconstruction magnetic resonance cholangiopancreatography; E: High *b*-value diffusion weighted imaging; F: ADC map; G: Portal venous phase magnetic resonance imaging (MRI); H: Hepatobiliary phase MRI.

Capillary HA

This accounts for almost 16% of all HAs, being smaller than 1 cm in about 40% of cases[42,43]. Microscopically it consists of small vascular spaces with abundant connective components. It presents high signal intensity on T2-weighted images and low signal intensity on T1-weighted sequences. In the case of hepatic steatosis, it shows a hyperintense signal on T1-weighted "chemical shift" images due to peritumoral sparing of fatty infiltration.

After contrast injection it is characterized by a "flash-filling" kinetic of contrast enhancement with early homogeneous enhancement, similar to that of the aorta in all phases[21]. Due to the presence of arterioportal shunts, fugacious perilesional parenchymal enhancement may be observed in extremely tiny lesions (less than 1 cm). Relative hyperintensity on delayed phases allows differential diagnosis with hypervascular metastases that are hypointense in the portal venous and delayed phases.

Sclerosed/hyalinized HA

Sometimes HAs deteriorate, resulting in the development of severe fibrosis, which usually begins at the core of the lesion, obliterating vascular gaps and causing changes in MRI signal intensity. Sclerosed HAs may only present slight hyperintensity on T2-weighted images, generally localized at the periphery of the lesion. Owing to histologic lesion heterogeneity, the contrast enhancement pattern may be unusual. In most cases no early enhancement is present and there is a slow and inhomogeneous contrast progression. Late centripetal filling of the central scar might be present. Due to its rarity and heterogeneous radiological presentation, that can mimic hepatic malignancies such as CCC or metastases, the final diagnosis of sclerosed HA is often histological[8,21,42,44] (Figure 10).

Angiomyolipoma

Hepatic angiomyolipomas (HAMLs) are mesenchymal benign tumors composed of arteries, fat, and smooth muscle cells that are categorized into four subtypes based on their composition: Mixed (the most frequent form), lipomatous (containing 70% fat), myomatous (containing 10% fat), and angiomatous.

The fat components of a lesion might range from 10% to > 90% of the total lesion volume. Patients with tuberous sclerosis complex are more likely to have these tumors (5%-10% of the time)[45]. HAML is classified as a "Perivascular Epithelioid Cell Tumor" and is immunohistochemically positive for beta-hydroxy-beta-methylbutyrate-45, a monoclonal antibody for melanoma: This positivity is a clear diagnostic criterion, while other hepatic tumors are negative for this marker.

The radiological appearance of HAML is completely dependent on the proportions of these three components. Lipomatous HAML appear hyperechogenic on US, hypodense on non-contrast enhanced CT, hyperintense on in-phase T1-weighted sequences, and hypointense on out-of-phase T1-weighted

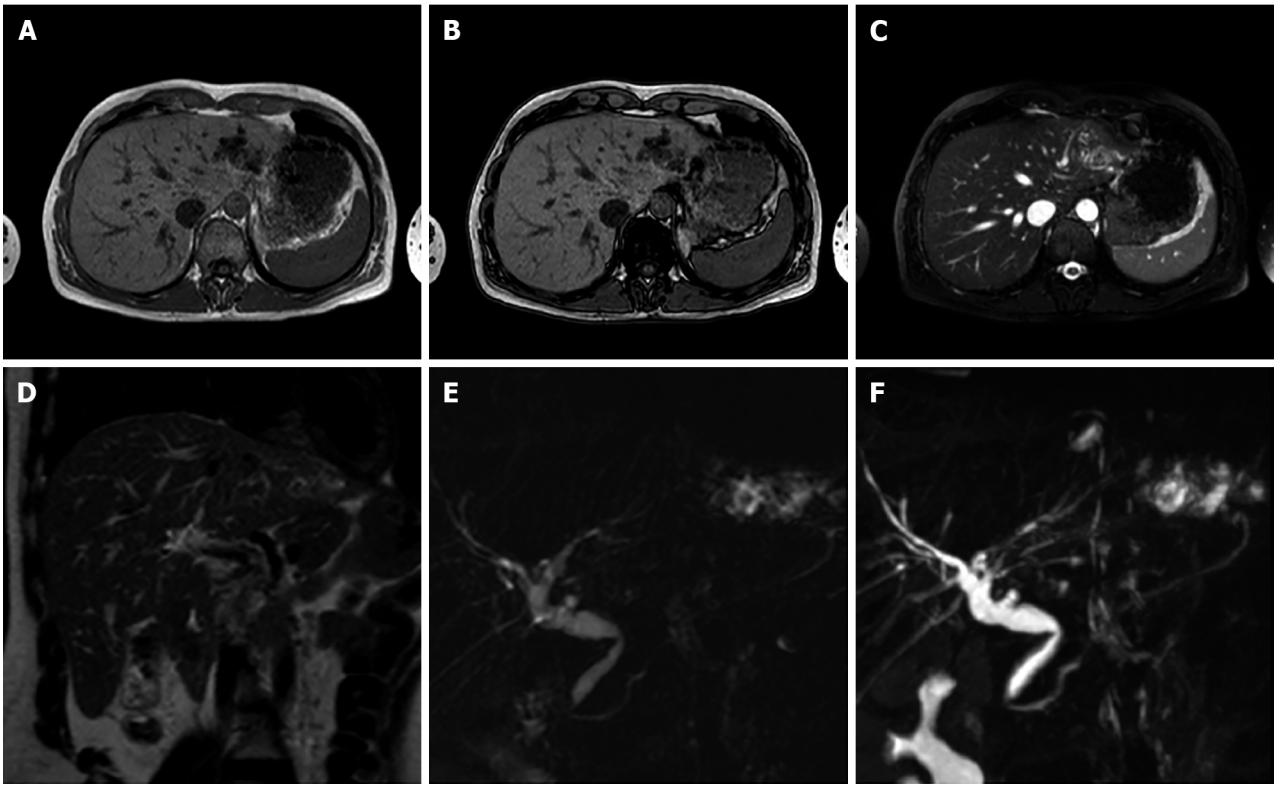

Figure 8 A 40-year-old female. Liver magnetic resonance imaging demonstrates dilatation of the left biliary hemisystem with multiple voids of signal on long TR sequences compatible with lithiasis. Pathology examination after left hepatectomy confirmed Caroli disease. A: In-phase T1-weighted image; B: Out-of-phase T1-weighted image; C: T2-Spectral Attenuated Inversion Recovery; D: T2-weighted image; E: Single-shot magnetic resonance (MR) cholangiopancreatography; F: 3D maximum intensity projection reconstruction MR cholangiopancreatography.

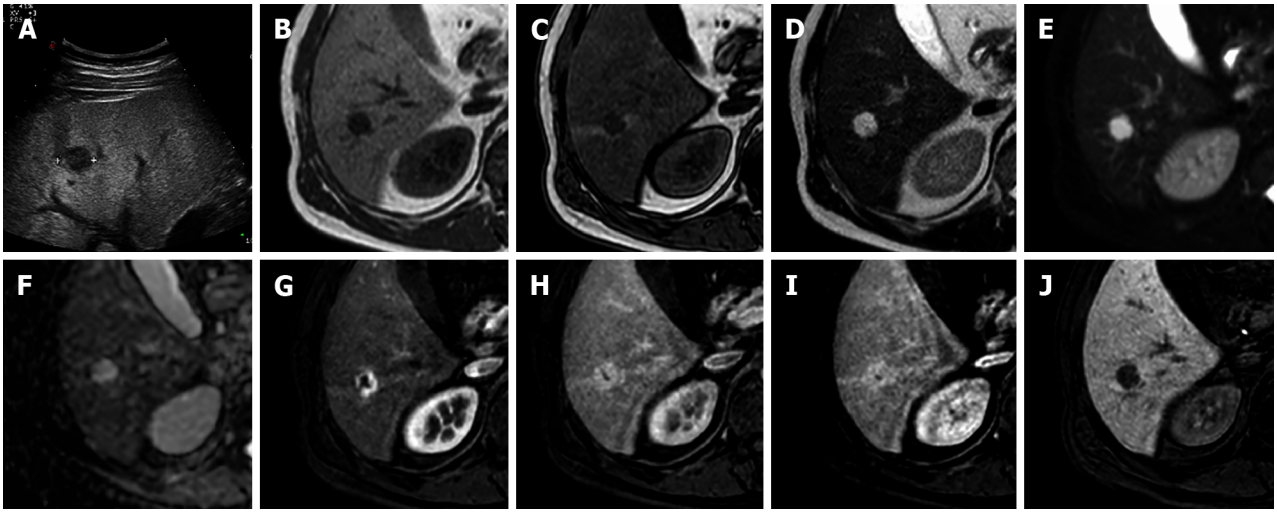

Figure 9 A 42-year-old male underwent ultrasound examination for abdominal discomfort demonstrating diffuse steatosis with a hypoechoic nodule in S6. Liver magnetic resonance imaging confirmed diffuse steatosis and a hypointense lesion on T1-weighted sequences, hyperintense on long TR sequences and on the ADC map. During dynamic study after intravenous contrast injection, the lesion presents centripetal enhancement and is hypointense on the hepatobiliary phase. These features are consistent with hemangioma. A: Liver ultrasound image; B: In-phase T1-weighted image; C: Out-of-phase T1-weighted image; D: T2-weighted image; E: T2-Spectral Attenuated Inversion Recovery; F: ADC map; G: Arterial phase magnetic resonance imaging (MRI); H: Portal venous phase MRI; I: Delayed phase MRI; J: Hepatobiliary phase MRI.

sequences, indicating intralesional fat components[46] (Figure 11).

Lesions with minimal fat content (angiomatous or myomatous types) are more difficult to characterize and are frequently classified as HCC because of their appearance on morphologic sequences and after contrast injection: They show moderate to high signal intensity on T2-weighted sequences, hypointensity on T1-weighted sequences, and early dishomogeneous contrast enhancement during contrast-enhanced dynamic studies with CEUS, CT, and MRI, with longer duration[47].

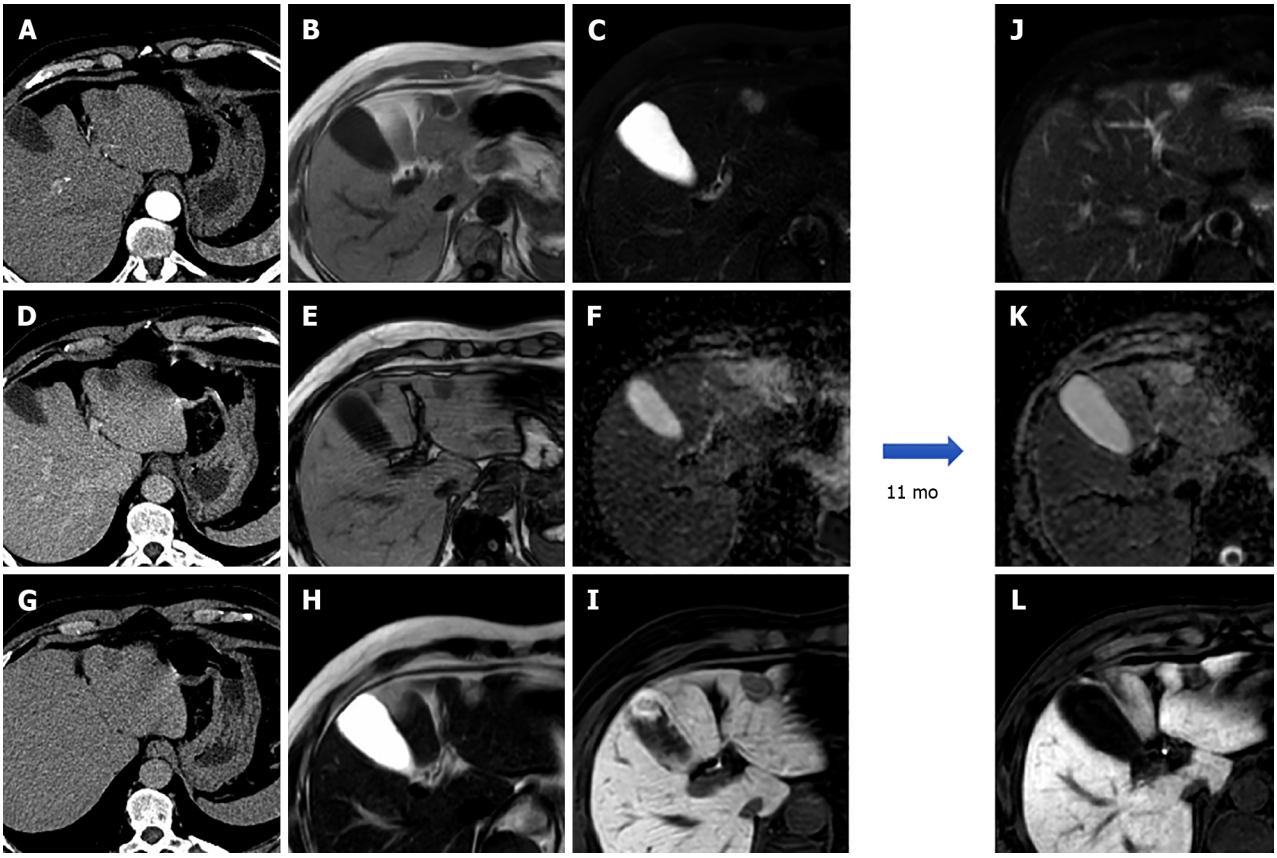

Figure 10 A 57-year-old male underwent abdominal computed tomography scan with contrast for the evaluation of a renal mass demonstrating a hepatic lesion in S3, hypodense on contrast-enhanced computed tomography. Liver magnetic resonance imaging (MRI) confirmed the presence of a nodule hypointense on T1-weighted sequences, slightly hyperintense on long TR sequences, without significant restriction on the ADC map. During dynamic study after intravenous contrast injection (gadoxetic acid), the lesion does not have significant enhancement and it appears hypointense on the hepatobiliary phase. At 11 mo follow-up, the nodule shows stable dimensions and MRI signal features. These features are consistent with fibrous hemangioma. A: Arterial phase of liver computed tomography (CT); B: In-phase T1-weighted image; C: T2-Spectral Attenuated Inversion Recovery; D: Portal venous phase of liver CT; E: Out-of-phase T1-weighted image; F: ADC map; G: Delayed phase of liver CT; H: T2-weighted image; I: Hepatobiliary phase (HBP) magnetic resonance imaging (MRI); J: T2-Spectral Attenuated Inversion Recovery; K: ADC map; L: HBP MRI.

Recent studies have established the utility of DWI-MRI with ADC values in the differential diagnosis of HAMLs and HCCs with fat components as HAMLs have higher values than HCCs with fat. Since HAML does not contain hepatocytes, it appears hypointense in the HBP[47].

Lymphangioma

Hepatic lymphangioma (HL), a malformation of the lymphatic system is a rare benign liver neoplasm. Most lymphangiomas are located outside the abdominal cavity, being less then 5% intra-abdominal, affecting primarily the small bowel mesentery, followed by the omentum, mesocolon, and retroperitoneum. Liver location is usually seen when lymphangiomas affect multiple organs, as in lymphangiomatosis. Solitary HL is rarely seen and could lead to clinical misdiagnosis[48,49].

HLs are histologically classified into capillary lymphangioma, cystic lymphangioma, and cavernous lymphangioma. They may occur at any age, with an average age of 30 years and a slightly higher incidence in females[48,50]. HL may be asymptomatic and incidentally found on cross-sectional imaging. The symptoms, if present, usually depend on compression of adjacent organs by the neoplasm [51].

Imaging appearances of HLs are various. On US they may be non-echoic, low-echoic, and mixed-echoic masses containing cystic and solid components within unicystic chamber or multichambers. On CT scanning a unilocular cystic mass shows low density and no enhancement. In some cases, it may present like a low-density mass with enhancement. For a multilocular mass with mixed components, enhancement is observed in the septum and solid parts but not in the cystic components. However, some cases described in the literature show atypical appearance, for example with wash-in and wash-out in the solid component mimicking malignancy[48,51].

MRI of HL shows a low signal on T1-weighted imaging, high signal on T2-weighted imaging, and occasionally mixed signals, related to intralesional bleeding that may occur. In the case of microcystic lymphangioma mistaken for a solid mass depending on its enhancement after intravenous injection of contrast, MRI with T2-weighted imaging may be very useful in the detection of multiple microcysts. The

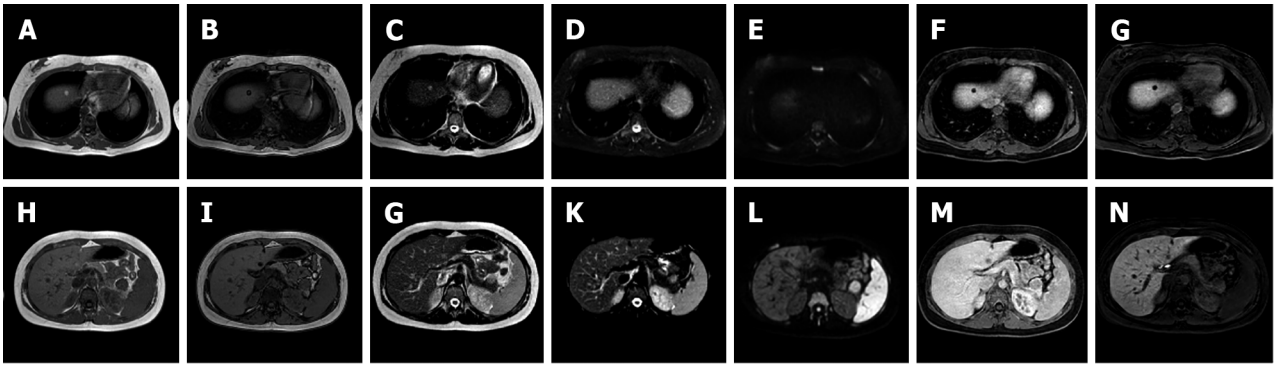

Figure 11 A 20-year-old female with tuberous sclerosis. Liver magnetic resonance imaging demonstrates a small focal lesion in S8 hyperintense on in-phase T1-weighted images, with signal loss on opposed-phase sequences with marginal india-ink artifact because of chemical shift, hyperintense on T2 turbo spin echo sequences, hypointense on T2-Spectral Attenuated Inversion Recovery (SPAIR) images, without increased signal on diffusion weighted images and hypointense in all phases during dynamic study and on the hepatobiliary phase (HBP). These features are consistent with lipoma. A second nodule in S2-S3 depicts a hyperintense signal on in-phase T1-weighted images, with signal loss on opposed-phase sequence, hyperintense on T2-TSE images, hypointense on T2-SPAIR sequences, without increased signal on diffusion weighted images. The nodule presents minimal vascularization during dynamic study and appears hypointense on HBP, such features are compatible with angiomyolipoma. A: In-phase T1-weighted image; B: Out-of-phase T1-weighted image; C: T2-weighted image; D: T2-Spectral Attenuated Inversion Recovery (SPAIR); E: High *b*-value diffusion weighted imaging; F: Portal venous phase magnetic resonance imaging (MRI); G: Hepatobiliary phase (HBP) MRI; H: In-phase T1-weighted image; I: Out-of-phase T1-weighted image; J: T2-weighted image; K: T2-SPAIR; L: High *b*-value diffusion weighted imaging; M: portal venous phase MRI; N: HBP MRI.

presentation of enhancement is similar to that of enhanced CT[48,52].

Diagnosis of lymphangioma is difficult due to a lack of typical symptoms and signs and differential diagnosis from simple cyst, BCA, BCAC, hydatid cyst, sclerosing HA, or HCC is very difficult[52]. Definitive diagnosis relied on pathological examination, that can be necessary in a cirrhotic liver with a lesion diameter less than 2 cm and if the cystic appearance is not predominant on imaging[48].

Macroscopically, the cysts resemble unilocular cysts or multilocular cysts of varying diameters with a septum, some of which have solid material or are filled with clear serous fluid, chylous fluid, and blood clots. Solid components have the microscopic appearance of fibrous hyperplasia, whilst liquid components are made up of a high number of cystic-dilated lymphatic lumens lined by simple squamous endothelium and filled with lymph. If morphology is insufficient to make a diagnosis, lymphatic markers and D2-40 (podoplanin) may be used to confirm the condition. Furthermore, endothelial markers such as von Willebrand factor, CD31, CD34, and two lymphatic markers (LYVE-1 and Prox-1) are utilized to identify lymphatic vessels from blood vessels immunohistochemically[53].

Solitary fibrous tumor

Solitary fibrous tumor (SFT) is a rare soft tissue neoplasm that originates from mesenchymal tissue. It was first reported in 1931 as an intra-thoracic mass arising from the pleura. Even if cases of SFT have been reported to originate in organs external to the thorax, hepatic location is extremely rare, accounting for less than 100 cases in the literature[54,55].

Liver SFT appears as a large, well-defined and heterogeneous single lesion that usually involves the right hepatic lobe, with a median tumor size of 17 cm[54,56]. It may occur at any age, with a prevalence in middle-aged women[56]. SFT is asymptomatic in 80% of patients. Symptoms depend on tumor effects, including pain, weight loss and nausea, and less frequently weakness, fever and hypoglycemia. Laboratory parameters are usually non-specific[57]. SFT does not reveal specific findings on imaging examination.

Ultrasonography often shows a heterogeneous mass that may be low-echoic, hyperechoic or both, with or without calcifications. On CT scan it shows early arterial enhancement with delayed venous wash-out. MRI may reveal a heterogeneous lesion, slightly hyperintense on T2-weighted sequences and hypointense on T1-weighted sequences, with a high signal on DWI[55]. In some cases reported in the literature, the imaging appearance of SFT with gadoxetic acid shows heterogeneous enhancement on arterial and portal phase images and homogeneous hypointensity on HBP[54,58].

As these findings are non-specific and mimic those of HCC or leiomyomas, the diagnosis of SFT is challenging, as these tumors have no typical symptoms and signs[55]. A definitive diagnosis relied on histopathological and immunohistochemical studies[55]. On histology, SFT is characterized by fibroblast-like spindle cells within thick collagen bundles, with patternless architecture in which hypo and hypercellular areas coexist; collagenous stroma contains hemangiopericytic vessels. Immunohistochemical reactions are usually positive for CD34 in most of cases, but the specific immunohistochemical marker of SFT is STAT6[55,59].

The biological behavior of SFT is controversial, in fact it may show malignant potential, as described in some reported cases of metastasis or recurrence[54,57,59]. England *et al*[60] in 1989 reported

malignancy criteria: the presence of intratumoral necrosis or hemorrhage, mitotic changes, pleomorphism of cellular nuclei, metastasis, large dimensions (more than 10 cm) and cellular atypia[59].

Mesenchymal hamartoma

Mesenchymal hamartoma (MH) of the liver is a rare benign tumor that originates from an anomalous development of primitive portal mesenchymal tissue, comprising predominantly of a mesenchymal stromal component and an aberrant malformed biliary structure with a small amount of hepatic lobules [61].

This neoplasm usually affects children mainly in the first 2 years with a slightly male predominance and it accounts for 8% of liver tumors in children[61-63]. Liver MH appears as a large benign multicystic/solid cystic liver mass, sometimes pedunculated, arising in the right lobe of the liver in 75% of cases[64].

MH has non-specific clinical manifestations, usually depending on the size of the tumor, which can reach up to 30 cm. Patients may present with hepatomegaly, a non-tender abdominal mass causing occlusion of the inferior vena cava or dyspnea and sometimes with fever, weight loss or vomiting. Alpha-fetoprotein is usually normal[61]. Imaging appearance depends on the cystic, solid/cystic or solid predominance of the tumor[61,64].

US often shows a complex cystic mass with internal septation[65]. On CT scan it shows a heterogeneous appearance with enhancing stromal elements and cystic components with water attenuation [64]. The stromal component may be predominant in some cases, although cystic components are more frequent and may vary in size; if the lesion is predominantly cystic it may range from a "swiss cheese appearance" if cysts are small, into a multilocular cystic lesion with septa, if the cystic components are large[61,64]. MRI appearance depends on the cystic *vs* stromal composition of the neoplasm and on the protein content of the fluid. The solid component may be hypointense to adjacent liver both on T1-weighted and T2-weighted sequences due to fibrosis. Cystic components usually show signal intensity similar to water on T2-weighted images and may have variable signal intensity on T1-weighted sequences owing to the protein content of cystic areas. After injection of gadolinium only stromal components and septa were enhanced[64].

On histology, a fibromyxoid connective background with bland spindle cells characterizes MH with branching bile ducts, thick walled vascular channels and scattered island of hepatocytes in the periphery of the lesion[66]. The biological behavior of MH is benign, although cases of malignant degeneration in undifferentiated embryonal sarcoma are described in the literature[65].

PSEUDOTUMOR

Focal fatty infiltration

Focal fatty hepatic infiltration can be easily recognized using the standard liver MRI examination, based on T1-weighted in-phase/out-of-phase and T2-weighted images, which in the latter shows a slight hypointense signal. However, in some cases, these focal areas can be misdiagnosed as focal liver lesions and they have to be assessed using dynamic contrast enhancement and HBP imaging. Considering the physiological GD-BOPTA and Gd-EOB-DTPA uptake by normal hepatocytes, the focal fatty infiltration or hypersteatosis areas have decreased contrast enhancement due to a reduced number of functioning hepatocytes or atypical vascularization. Moreover, in the case of nodular-shaped focal fatty infiltration, its differential diagnosis with a fat-containing liver lesion can be challenging. In this setting, a wedge or pyramidal shape and the lack of mass effect are useful features to distinguish a focal fatty lesion from a focal liver lesion[67] (Figure 12).

Yeom *et al*[68], by evaluating 27 focal fat deposition areas, reported a homogeneous or heterogeneous enhancement during the dynamic study, both in the arterial and portal venous phase, with hypointense signal during HBP. On the other hand, fat spared areas do not show any signal drop-off during in-phase/out-of-phase imaging and may induce increased signal intensity on HBP due to preserved or increased focal liver function[69].

Infection–liver abscess

The liver abscess, defined as a localized collection of inflammatory products caused by bacterial, fungal or parasitic agents, can be easily diagnosed with all imaging techniques, but is best with US and CT. MRI can help to distinguish between uni- and multi-focal necrotic lesions disseminated through the liver parenchyma. In this setting, the typical appearance of liver abscess during the standard MRI protocol can support the final diagnosis, based on the iso- to hyperintense appearance on T1-weighted images due to the presence of proteins, hemorrhagic foci, or inflammatory degradation products, without a significant drop-off in signal during in-phase/out-of-phase imaging. T2-weighted imaging is useful for determining not only the amount of free water inside the lesion but also to identify the slight peripheral hyperintense area due to edema or peripheral inflammation. Usually, on DWI an abscess presents a high restricted diffusion with very low values of ADC because of the high viscosity of inflammatory debris and cellularity[21] in the necrotic cavity, while no diffusion restriction is seen

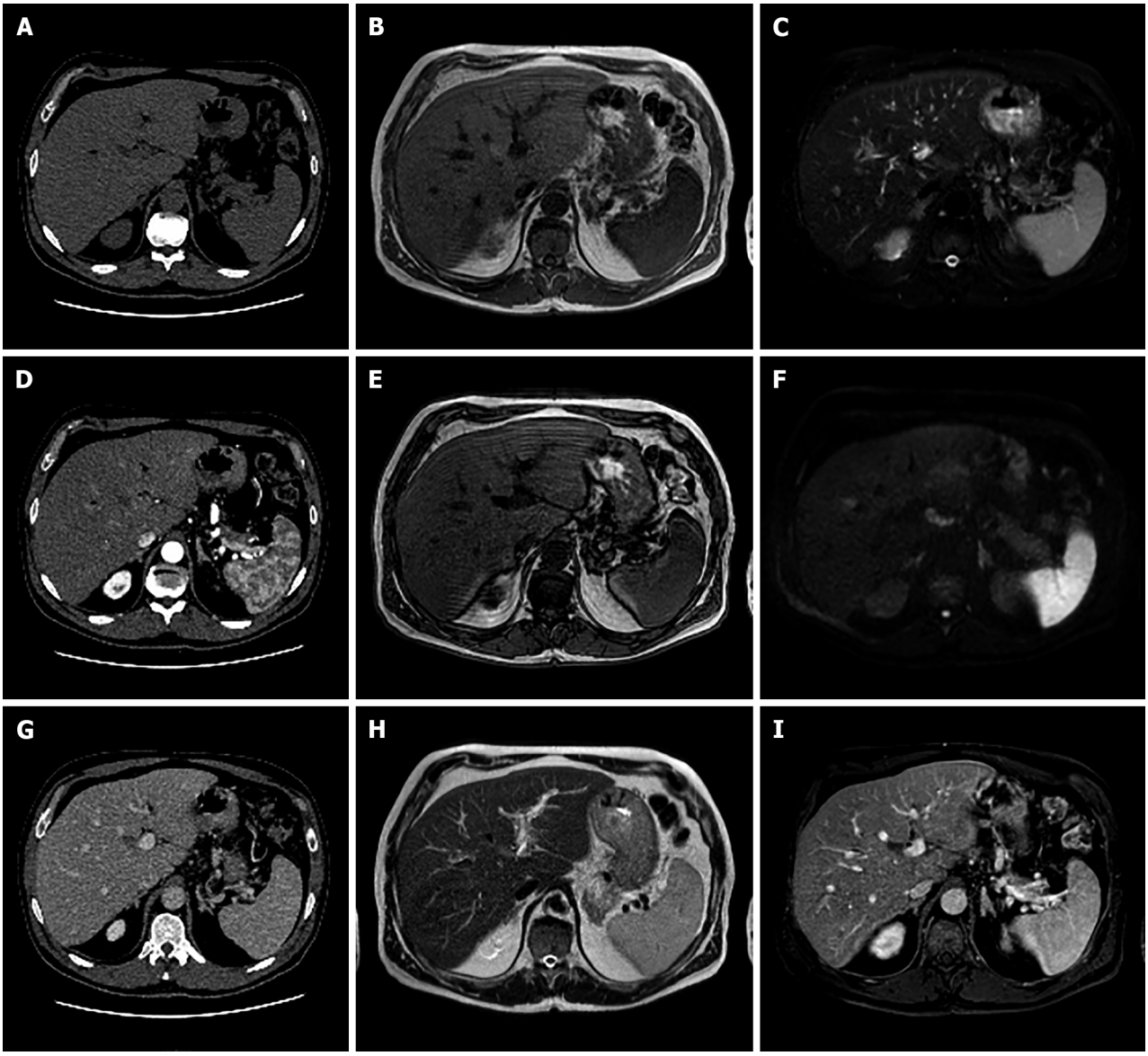

Figure 12 A 50-year-old male, unremarkable past medical history. Multiphase abdominal computed tomography study shows a hypodense liver area near the porta hepatis. Subsequent magnetic resonance imaging liver study confirmed a hilar area slightly hyperintense on T1 in-phase sequence, with signal loss on opposed-phase images, slightly hyperintense on T2-turbo spin echo images, hypointense on T2-Spectral Attenuated Inversion Recovery images, without significant increased signal on diffusion weighted images and with no contrast enhancement on dynamic study. These features are consistent with an area of focal fatty infiltration. A: Unenhanced liver computed tomography (CT); B: In-phase T1-weighted image; C: T2-Spectral Attenuated Inversion Recovery; D: Arterial phase of liver CT; E: Out-of-phase T1-weighted image; F: High *b*-value diffusion weighted imaging; G: Portal venous phase of liver CT; H: T2-weighted image; I: Portal venous phase magnetic resonance imaging.

peripherally. Furthermore, as reported by Park *et al*[70], DWI is helpful in differentiating hepatic abscess from malignant mimickers considering the higher peripheral restriction in the case of malignancy with 98% diagnostic accuracy.

During the dynamic study, liver abscess typically shows a peripheral rim enhancement, in particular on the portal-venous and delayed or transitional phase, due to the presence of a pseudocapsule and inflammatory cells. During the HBP, the core typically does not change in appearance, while the peripheral area can show an ipo- to isointense signal, due to edema or the normal function of hepatocytes, respectively (Figure 13).

Although size, shape, number, and signal intensity can help to establish a differential diagnosis, the only pathognomonic signs are the presence of air bubbles within the lesion or an air-fluid level[71], seen as a single or multiple flow-void foci. The most important differential diagnosis of liver abscess is metastasis. As mentioned above, liver metastasis can be recognized due to the different signal intensity on T2-weighted images and DWI with a slight and clear hyperintense appearance, respectively, as well as during the dynamic study, in particular with a poor peripheral ring enhancement due to the presence of pathological cells. On the portal-venous and transitional phase, solid liver metastases present an isointense signal while abscess shows a hypointense signal.

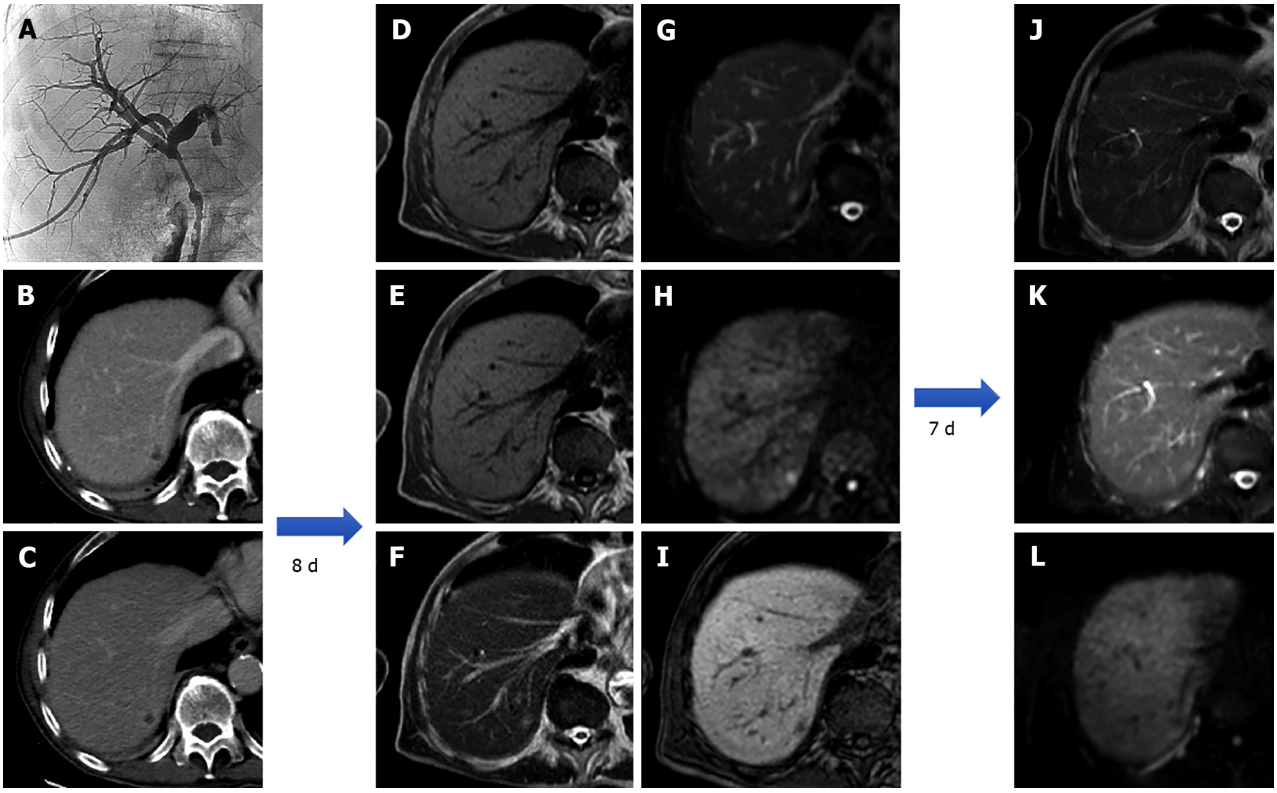

Figure 13 An 80-year-old patient with the finding of common hepatic bile duct stenosis. Cholangiography procedure with the placement of biliary drainage in which histological diagnosis of the biliary tract suggested adenocarcinoma of the main biliary duct. Following an episode of fever, increase in white blood cells (13.02 × 10^{-9}/L) and C-reactive protein (90.1 mg/L) computed tomography examination showed a small hepatic hypodensity in the 7th segment. Liver magnetic resonance imaging (MRI) performed after a few days of therapy shows a nodular lesion, hypointense on the T1-weighted sequences, slightly hyperintense in the T2 sequences, with increased signal on diffusion weighted imaging (DWI) and hypointense signal on the hepatobiliary phase. A 7-d follow-up liver MRI examination showed a further evolution of the lesion with evidence of a very slight hyperintense signal on T2 sequences and almost disappearance of the lesion itself on DWI, which confirmed the diagnosis of hepatic abscess. A: Percutaneous transhepatic cholangiography; B: Portal venous phase of liver computed tomography (CT); C: Delayed phase of liver CT; D: In-phase T1-weighted image; E: Out-of-phase T1-weighted image; F: T2-weighted image; G: T2-Spectral Attenuated Inversion Recovery (SPAIR); H: High *b*-value diffusion weighted imaging; I: Hepatobiliary phase magnetic resonance imaging; L: T2-weighted image; M: T2-SPAIR; N: high *b*-value DWI.

To date, only one study has evaluated the importance of Gd-EOB-DTPA in distinguishing between hepatic microabscesses and metastasis: Choi *et al*[72], by enrolling 72 patients, demonstrated that perilesional edema and arterial rim enhancement were maintained during the following dynamic sequences. Also the size discrepancy between T1-weighted and T2-weighted images and between T1-weighted and HBP images, allow a high diagnostic accuracy for microabscess (90.9%).

Infection-Echinococcus granulosus

Different tapeworms belonging to the *Echinococcus* genus can infect humans, both as the intermediate or definitive host. The two most important types of disease are cystic echinococcosis and alveolar echinococcosis, caused by *Echinococcus granulosus* (*E. granulosus*) and *E. multilocularis*, respectively.

This subsection will focus on *E. granulosus* considering its higher incidence and global distribution in all countries except Antarctica[73]. Sheep and pigs are definitive hosts of *E. granulosus* and typically excrete hundreds of eggs within their feces. Intermediate hosts, such as dogs, cats, or humans, can eat these eggs directly or indirectly *via* contaminated water, food, or soil[74]. The tapeworm, following the blood vessels typically stops in the liver more frequently in the right lobe, where it starts growing as a cystic lesion, with a growth rate ranging from 1-2 mm to 10 mm per year. Cysts are composed of two layers of membrane: A germinal and an outer layer. The immune system responds to the cyst by forming a capsule that during years will be completely calcified[74].

When performing MRI, it should be taken into account that cystic echinococcosis can show different imaging features according to the parasitic stage in the infected liver. In the first stage of infection, a rounded, thin-walled cyst is recognizable, showing an inhomogeneous signal in T1-weighted images, hyperintense in T2-weighted images, with only peripheral enhancement best seen on the portal-venous or delayed phases. During cystic growth, it is possible to observe the typical echinococcosis cyst features, appreciable in approximately 75% of cases: The daughter cysts within the main cyst may produce a honeycomb-like structure with a typical thin peripheral capsule, hypointense both in T1-weighted and T2-weighted images. When the daughter cysts grow, the pressure inside the whole cyst

can lead to their collapse or rupture into the biliary tree or peritoneal seeding: Under this circumstance, the internal appearance may vary according to the amount of cystic debris, especially on T2-weighted images (Figure 14). Finally, the dead cyst is composed of a solid matrix, due to the immune response manifesting with the peripheral calcified layer, from partial to complete, that can be seen in about 50% of cases[75].

Inflammatory disorder of the liver-pseudotumor

Hepatic inflammatory pseudotumor, even if rare, has an unknown etiology and can manifest itself as a consequence of different immune responses, such as infective agents, including viruses (such as Epstein-Barr virus), bacteria and fungi (nocardia and actinomycosis), primary and secondary liver tumors including hepatic lymphoma[76], and as the hepatic manifestation of immunologic disease, such as IgG4-related disease[77], inflammatory bowel disease[78], and, finally, a response to a foreign body[79].

Hepatic pseudotumor can present as single or multiple lesions with a wide range of distribution, including liver parenchyma, peri-portal spaces, and peri-biliary ducts. On unenhanced MRI it can show a hypointensity signal on T1-weighted images, without any signal drop-off during in-phase/out-of-phase imaging, with variable signal intensity on T2-weighted images strictly linked to the presence of acute edema or, if chronic, fibrosis can be present. In line with the T2 signal, the pseudotumor can show areas of signal restriction on DWI, typically peripheral, due to the high cellularity of inflammatory cells.

Considering the mixed content within the lesion, during dynamic sequences the enhancement can be different. It can mimic a CCC with peripheral enhancement during the arterial phase with a delayed central-filling, in the case of diffuse fibrotic components; heterogeneous enhancement can be easily identified in the case of acute or subacute findings, while chronic lesion variable enhancement can be present. Sometimes it can also show internal septa with delayed enhancement. On HBP imaging, pseudotumor typically manifests as a hypointense lesion due to the small number of functioning hepatocytes, surrounded by fibro-inflammatory components. The abovementioned features were confirmed by a recent study published by Ichikawa *et al*[80], in which the typical appearance of pseudotumor was demonstrated, a central hypointense signal with a relatively peripheral hyperintensity on HBP, helping the differential diagnosis with colorectal liver metastasis.

Inflammatory disorder of the liver-sarcoidosis

Even if typically considered a thoracic disease, sarcoidosis can involve all human organs, with an estimated 50% of patients with extra-thoracic manifestations[81]. Based on the underlying pathophysiology, it can manifest as hepatomegaly with possible jaundice and with manifestations of portal hypertension, even if not commonly reported. Thus, liver MRI should be performed to exclude the presence of focal liver lesions. In patients with sarcoidosis the typical changes due to liver fibrosis were the most common findings, underlining that only 25% of patients showed normal liver morphology with no radiological signs of portal hypertension. In this setting, focal hepatic and splenic nodules were seen in about 25% of subjects at presentation, demonstrating coalescing granulomas sometimes confluent and mimicking malignancy[82]. The sarcoidosis nodules are usually hypointense on T1-weighted images, without any changes on in-phase/out-of-phase imaging, while a variable signal on T2-weighted images can be seen, ranging from hypo to hyperintensity, strictly linked to the amount of edema both intra- and peri-lesional. During enhanced sequences, these lesions may show a slight and peripheral enhancement on the arterial phase while during the next sequences, including the HPB, the lesions show hypointense signals (Figure 15). Sometimes the sarcoidosis can manifest exclusively with chronic liver failure.

CONCLUSION

Liver lesions are common findings in radiologists' daily routine. They are a complex category of pathology that ranges from solitary benign lesions to primary liver cancer and liver metastases. Liver MRI is a fundamental radiological method in these patients as it allows with its multiparametric approach an optimal non-invasive tissue characterization. Liver MRI can be used to differentiate between pseudotumors and tumors, as well as benign and malignant lesions, and it can also be utilized for differential diagnosis. Although histological examination can be useful in making a definitive diagnosis, liver MRI is an important modality in the diagnosis of liver lesions with a significant impact on patient care.

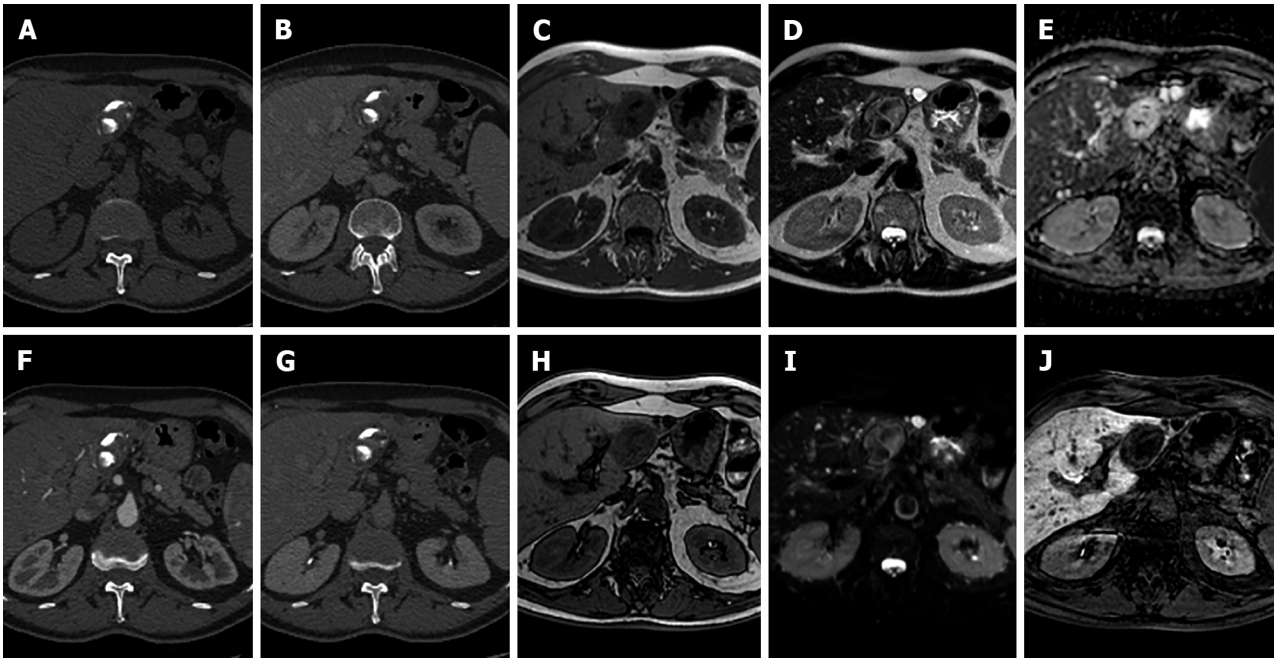

Figure 14 A 67-year-old male who underwent multiphasic abdominal computed tomography scan demonstrated a lesion with gross calcifications in S3 that did not present contrast enhancement in any phase. Liver magnetic resonance imaging confirmed a nodular formation, hypointense on T1-weighted sequences, isointense on long TR sequences with more hypointense components corresponding to calcification on computed tomography images, without significant restriction on diffusion weighted images and hypointense on the hepatobiliary phase. These features are consistent with a calcified echinococcal cyst. A: Unenhanced liver computed tomography (CT); B: Portal venous phase of liver CT; C: In-phase T1-weighted image; D: T2-weighted image; E: ADC map; F: Portal venous phase of liver CT; G: Delayed phase of liver CT; H: Out-of-phase T1-weighted image; I: T2-Spectral Attenuated Inversion Recovery; L: Hepatobiliary phase magnetic resonance imaging.

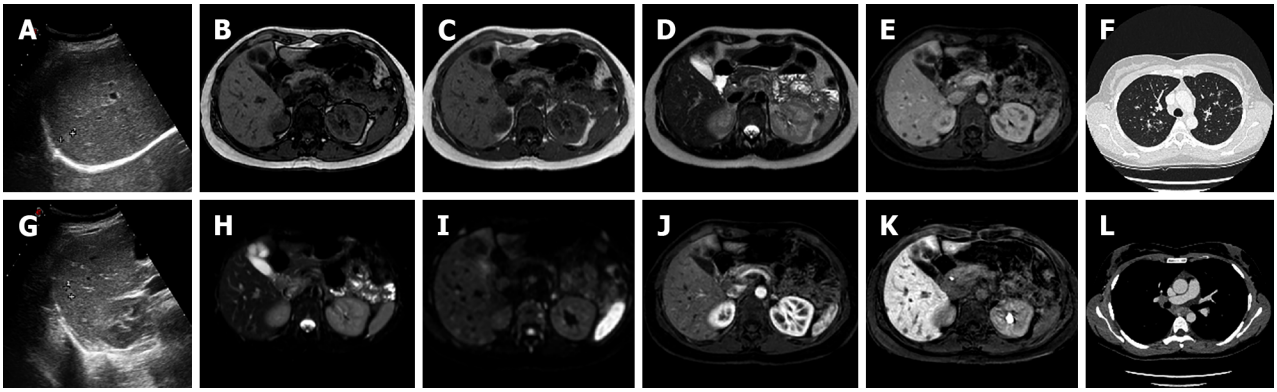

Figure 15 A 38-year-old female, smoker, polycystic ovary syndrome, previous appendicectomy. Abdominal ultrasound performed due to neutropenia demonstrated hyperechoic liver nodules. Subsequent liver magnetic resonance imaging confirmed multiple liver nodules hypointense in T1-weighted sequences slightly hyperintense on T2 sequences, with increased signal on diffusion weighted images, without contrast enhancement after dynamic study with gadoxetic. Lesions are hypointense on the hepatobiliary phase. Chest computed tomography with contrast demonstrated bilateral nodules with a perilymphatic pattern and a bigger lesion with satellite nodules (galaxy sign) on the right; increased hilar lymph nodes were present. Transbronchial biopsy demonstrated noncaseating granulomas which led to the diagnosis of sarcoidosis. A: Liver ultrasound image; B: Out-of-phase T1-weighted image; C: In-phase T1-weighted image; D: T2-turbo spin echo; E: Portal venous phase magnetic resonance imaging (MRI); F: Chest computed tomography (CT) with pulmonary parenchymal window; G: Ultrasound image; H: T2-Spectral Attenuated Inversion Recovery; I: High *b*-value diffusion weighted imaging; J: Arterial phase MRI; K: Hepatobiliary phase MRI; L: Chest CT with mediastinal window.

FOOTNOTES

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MINIREVIEWS

Pediatric acute viral hepatitis with atypical variants: Clinical dilemmas and natural history

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Abstract

Classical acute viral hepatitis (AVH) has an uncomplicated outcome. Acute liver failure has a grave prognosis. Atypical manifestations of AVH are a group of disorders that causes significant morbidity and dilemmas in children. These include prolonged cholestasis, relapsing hepatitis, ascitic form of AVH, late-onset hepatic failure (LOHF), intravascular hemolysis, and provoking an autoimmune trigger leading to autoimmune hepatitis. These entities cause significant liver dysfunction or worsening and are often difficult to differentiate from chronic liver disease (CLD). Ascitic form of AVH, LOHF, decompensated CLD and acute-on-chronic liver failure have significant overlapping features that need to be carefully dissected out. In many cases, only on long-term follow-up, these clinical entities can be separately identified. Intravascular hemolysis is usually caused by associated glucose-6-phosphate dehydrogenase deficiency. Rarely CLD such as Wilson disease and autoimmune hepatitis can also present with hemolysis in the initial presentation, which can mimic AVH with hemolysis. Identifying deviations from typical manifestations aid in avoiding unnecessary investigations, allowing focused therapy and alleviating anxiety.

Key Words: Viral; Hepatitis; Atypical; Cholestasis; Relapsing; Hemolysis; Ascites

Core tip: Acute viral hepatitis (AVH) in children can manifest with atypical features in about a quarter of children. The most common entities, such as prolonged cholestasis and relapsing hepatitis, cause liver dysfunction and are often confused with chronic liver diseases (CLDs). Similarly, ascitic form of AVH and late-onset hepatic failure are close differential diagnoses of acute-on-chronic liver failure and decompensated CLD. A combination of a thorough history, clinical findings, basic investigations and outcome on follow-up allows focused workup and management in atypical AVH.

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INTRODUCTION

Ancient Sumerians considered the liver to be the seat of the soul and jaundice to be caused by the devil. From that era, we have come a long way in understanding hepatitis. Although the transmissible nature of hepatitis was known even in the Middle Ages, discovery of the Australia antigen and subsequently the other hepatotropic viruses formed the watershed in the history of acute viral hepatitis (AVH)[1]. While the majority of AVH has a benign self-limiting course with spontaneous resolution, acute liver failure (ALF) has a fulminant pattern with poor recovery of native liver. In between the two extremes of these phenotypes lies a major subgroup of complications that are collectively known as atypical manifestations of AVH (Figure 1). These entities have a variable outcome. They include complications such as prolonged cholestatic phase, relapsing hepatitis, ascitic variant of AVH, hematological involvement (immune thrombocytopenia, intravascular hemolysis, and aplastic anemia) and extrahepatic multisystemic issues (acute kidney injury, pancreatitis, Guillain-Barré syndrome, myocarditis, and myositis)[2]. In addition late-onset hepatic failure (LOHF), a debated entity closely mimics acute-on-chronic liver failure (ACLF). Due to the overlapping presentation, it is a challenging task to distinguish AVH from underlying chronic liver disease (CLD) (Figure 2). Of all the atypical features, this review is limited to the discussion of those uncommon features of AVH that are associated with liver dysfunction or worsening.

EPIDEMIOLOGY

The prevalence of AVH is variable but atypical manifestations are encountered in 14%-22% of children [3]. Etiology-specific prevalence is 30% in hepatitis A virus (HAV), 15% in hepatitis E virus (HEV) and 3% in hepatitis B virus (HBV) infections[3]. The wide range of prevalence is possibly due to the difference in definitions used to describe atypical features in the studies. Classical AVH begins with a prodrome that may include fever, nausea, vomiting, malaise followed by jaundice that resolves within the next 2-3 wk (Figure 3)[4]. Any deviation from this classical presentation is included under the umbrella of atypical features. Prolonged cholestasis is the most common atypical feature that is seen in 11%, AVH with ascites in 7%, intravascular hemolysis in 3% and relapsing jaundice in 2%[3].

Prolonged cholestasis

Natural history: In the natural history of typical AVH, the cholestatic phase lasts for a brief period (3-4 wk)[5]. Prolonged cholestasis is an atypical manifestation that is worrisome for the caregivers, a dilemma for physicians and troublesome for symptomatic patients. Deepening jaundice, pale stools, intractable pruritus, multivitamin deficiency features, fatigue, poor quality of life, school absenteeism and psychosocial difficulties predominate in this phase. In developing countries, the problem is compounded by self-imposed dietary restrictions that perpetuate malnutrition. In the initial description of cholestasis in patients with HAV infection, symptoms lasted for > 12 wk typically with serum bilirubin > 10 mg/dL and aminotransferases < 500 IU/L[6]. Liver biopsy in patients with cholestasis showed portal inflammation rich in plasma cells, centrilobular cholestasis, ductular proliferation and cholestatic rosettes. Periportal necrosis may impede the normal bile flow contributing to cholestasis[7]. In a pediatric study of prolonged cholestasis in HAV infection, the initial liver biopsies showed extensive periportal and centrilobular fibrosis similar to chronic hepatitis. A repeat biopsy 3 mo after resolution of cholestasis in the above cohort showed complete normalization and resolution of fibrosis [8]. Children who develop this deviation from the classical presentation are older (> 10 years) and more commonly have HAV (15%) as compared to HBV (1%), HEV (9%) and coinfections (11%). Cholestasis can last up to 6 mo and 10% can also have relapsing hepatitis. Jaundice to pruritus interval is usually 20

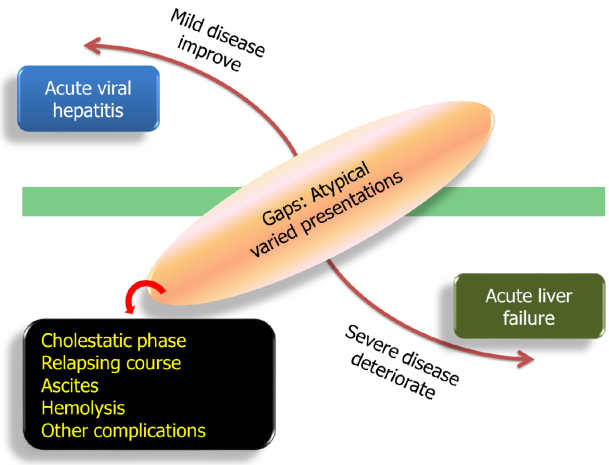

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Figure 1 Spectrum of acute viral hepatitis.

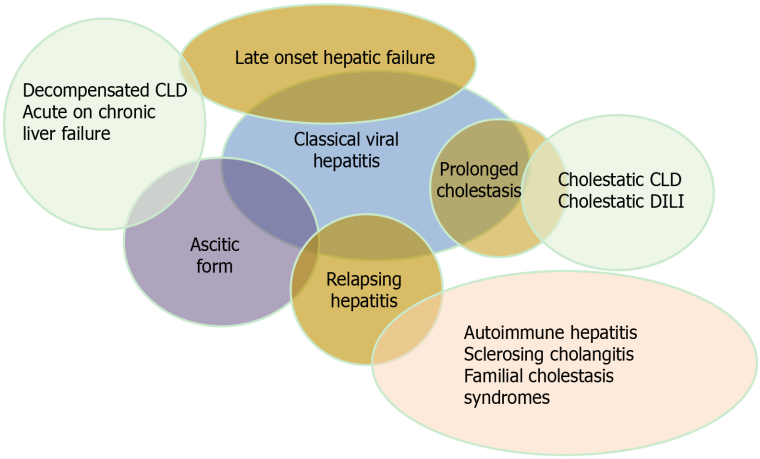

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Figure 2 Overlapping atypical manifestations of acute viral hepatitis. CLD: Chronic liver disease; DILI: Drug-induced liver injury.

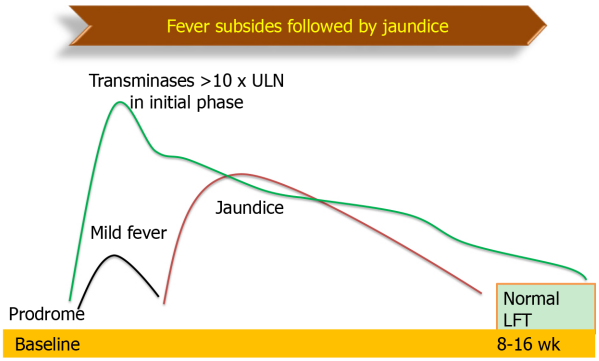

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Figure 3 Natural history of classical acute viral hepatitis. LFT: Liver function tests.

(15-30) d[3]. Figure 4 depicts the natural history of the prolonged cholestatic phase. It is postulated that HAV infection triggers polymorphisms in ATP binding cassette proteins that are involved in bile secretion[9]. It has also been observed that in prolonged cholestasis, the time taken for HAV RNA to become undetectable in the serum is 46-105 d as compared to 11-20 d in classical hepatitis[10]. These findings indirectly indicate that a complex interaction exists between genetic predisposition, immune response to HAV and prolongation of cholestasis.

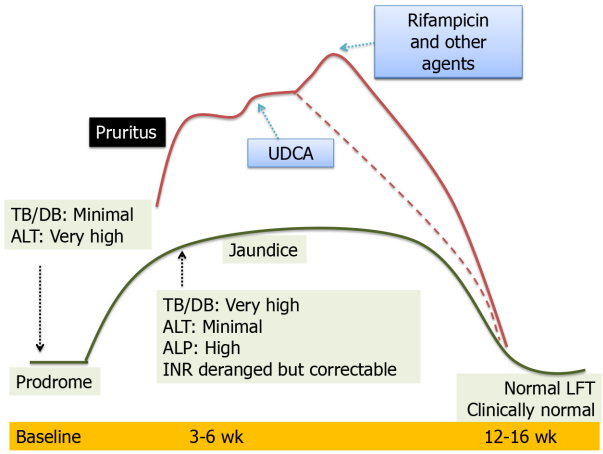

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Figure 4 Natural history of prolonged cholestasis in acute viral hepatitis. LFT: Liver function tests; UDCA: Ursodeoxycholic acid; ALT: Alanine aminotransferase; TB/DB: Tuberculosis/Disulfide bond; ALP: Alkaline phosphatase; INR: International normalized ratio.

Differential diagnosis: When a child presents for the first time with a long history of icterus (progressive or relapsing), pruritus or pale stools, the tendency would be to look for cholestatic chronic liver diseases. Intrahepatic causes of cholestasis include progressive familial intrahepatic cholestasis, sclerosing cholangitis, Alagille syndrome, cystic fibrosis, alpha-1-antitrypsin deficiency and infiltrative disorders. Extrahepatic causes of cholestasis are choledochal cyst, Caroli's disease, sclerosing cholangitis, biliary stricture, biliary compression by lymph nodes or masses and portal cavernoma cholangiopathy[11]. Clues in the clinical presentation, extrahepatic manifestations, familial clustering, features of portal hypertension and radiology distinguish the above.

Mutations in *ATP8B1*, *ABCB11* and *TJP2* present as cholestasis from early infancy with persistently low or normal -glutamyl transpeptidase (GGT) levels and liver dysfunction[12]. Hence these disorders are rarely confused for AVH with prolonged cholestasis. Mutations in *ABCB4* gene that result in a dysfunctional multidrug-resistant protein (MDR)3 may have milder phenotypes that appear for the first time as cholestasis during AVH. These disorders have a significant family history, maternal antenatal cholestasis, recurrent cholestatic pattern, biliary (intrahepatic and gall bladder) calculi and persistently high GGT levels[13]. Liver biopsy would demonstrate periportal fibrosis and ductular proliferation with absence or faint staining of MDR3 protein in immunohistochemistry[14]. Benign recurrent intrahepatic cholestasis is a milder variant in all the above disorders that have recurrent bouts of cholestasis in the second decade without progressive liver disease[15]. These bouts may overlap with AVH. Genetic analysis should be sent if clinical suspicion is strong.

Alagille syndrome is an autosomal dominant disorder due to mutation in either *JAGGED1* or *NOTCH2* genes with incomplete penetrance and variable expressivity[16]. Hepatic involvement may range from asymptomatic elevation of transaminases to decompensated CLD. The majority will have cholestasis in early infancy which can progress or resolve. A small proportion may present for the first time with late-onset cholestasis up to the age of 10 years who may mimic prolonged cholestasis of AVH [17]. Characteristic facies along with positive family history, cardiac and skeletal abnormalities, will provide clues to the underlying disease. Liver biopsy showing paucity of interlobular bile ducts and/or positive mutation will confirm the diagnosis[18].

It is known that smooth muscle actin (SMA) and antinuclear (ANA) autoantibodies, may be incidentally detected during AVH due to autoimmune phenomena[19]. Those presenting with cholestasis may be mistaken as having autoimmune sclerosing cholangitis or overlap syndrome. In those with extrahepatic autoimmunity, features of portal hypertension and biliary changes on sonography should be worked up with magnetic resonance cholangiography and/or liver biopsy[20].

Drug-induced liver injury (DILI) often complicates a regular course of AVH. A variety of complications such as a surge in transaminases, liver failure and cholestasis may occur during this period. It is important to carefully elicit a concomitant drug intake, especially herbal and alternative medicines. Liver tonics and immune boosters are marketed widely in Asia and obtained over the counter with a myth of hastening the recovery of icterus[21]. Commonly used drugs that can cause cholestasis include antibiotics like amoxicillin-clavulanic acid, trimethoprim-sulfamethoxazole, erythromycin and tricyclic antidepressants[22]. Temporal correlation with drug intake and improvement upon withdrawal supports the diagnosis of drug-induced cholestasis. However, in cases of chronic cholestasis, improvement may not be evident even after drug withdrawal and may progress to CLD[23]. In Asian children with DILI, 39% of cases were accounted for by herbal medications and the rest by antitubercular drugs, antibiotics and antiepileptics. Twenty-seven percent had a cholestatic pattern and

22% had a mixed pattern of injury based on the R factor. Fourteen percent went on to develop chronic DILI. Non-hepatocellular injury pattern was one of the factors predicting poor outcomes[24].

In a setting of prolonged cholestasis in AVH, an extensive workup including liver biopsy is generally not required. It is prudent to wait and watch for the natural resolution to take place if the liver functions are improving over 3-4 mo. Detailed workup for chronic diseases is indicated if there are significant clues in presentation, static or worsening liver dysfunction.

Treatment: Although spontaneous resolution can be seen, sequential pharmacotherapy may be required in those with significant and debilitating cholestasis-related pruritus. Ursodeoxycholic acid could aid in the resolution of cholestasis in 80% of cases. It increases the hydrophilicity of bile, induces the activity of carrier proteins for increasing biliary secretion and has antiapoptotic activity[25]. Addition of rifampicin is required in 18% of cases. Rifampicin acts as an agonist of pregnane-X-receptor, which in turn mitigates bile acid toxicity. Cholestyramine, a bile acid sequestrant is required in 4% of cases[3]. In refractory cases, opioid antagonist (naltrexone) or selective serotonin reuptake inhibitor (sertraline) is used for severe pruritus[26]. The role of steroids in prolonged cholestasis of AVH is questionable. Few case series have shown a response of refractory pruritus to prednisolone (1 mg/kg/d) for 2 wk with tapering over 4-8 wk[27]. Multivitamin supplementation is prudent.

Relapsing hepatitis

Natural history: Relapsing hepatitis is seen in 2-4% of children with AVH in whom it typically presents as a biphasic illness or rarely polyphasic relapses[6,8]. In this entity, the second phase of hepatitis occurs after the resolution of the first phase and near-normalization of liver functions. The interval period between the two hepatitic phases may last for weeks to months[28]. The second or subsequent phases may be icteric or anicteric. There may be a recurrence of prodrome. The peaks of liver enzymes may be variable or similar to the first episode. The subsequent phase may be similar, less severe or more severe than the first episode. Rarely, the relapsed phase may culminate in ALF or complicate into a cholestatic phase[29]. Figure 5 depicts the natural history of relapsing hepatitis in AVH. Since liver function tests are measured at fixed time points of follow-up, anicteric relapses may often be missed. Hence the true prevalence of relapsing hepatitis remains undermined. During the relapses, HAV RNA can be detected in the serum and IgM HAV remains positive[11]. Relapse is postulated to result from incomplete elimination of HAV in the initial phase. Recent mouse model studies have shown that anti-HAV IgA immune complexes reach the liver from the enterocytes by enterohepatic circulation. The process is continuous until IgG response is initiated. In those with inefficient IgG response, recurrent seeding of anti-HAV IgA immune complex to the liver can cause relapsing hepatitis[30]. Treatment is supportive with close monitoring for progression to ALF.

Differential diagnosis: Relapsing jaundice is the presenting feature in 33% of children with autoimmune hepatitis that can last from 6 to 24 mo. Apart from icterus, presentations are fatigue, weight loss and anorexia. Absence of acute viral markers, presence of stigmata of CLD, decompensation or features of portal hypertension warrant workup for autoimmune hepatitis[31].

Sclerosing cholangitis can present with multiple relapses and spontaneous remissions. Magnetic resonance cholangiography and workup for underlying etiology confirm the diagnosis in the majority of cases. However, about 13% of cases of primary sclerosing cholangitis have exclusive small duct involvement that can be confirmed only by liver biopsy[32]. Children with extrahepatic biliary obstruction have a prominent history of recurrent fever, cholangitis and absence of prodrome that differentiates from relapsing viral hepatitis.

Children with ATP8B1 and ABCB11 disease present with recurrent jaundice in 8% and recurrent-persistent jaundice in 23% of cases. Pruritus is a universal feature in them[33]. Recurrent jaundice in ABCB4 disease is variable, dependent on the severity of mutation and often precipitated by drugs[34, 35]. Dubin-Johnson syndrome is a rare disorder of bilirubin excretion due to mutations in *ABCC2* gene, with a characteristic black liver caused by lysosomal pigment accumulation. These patients can present with recurrent jaundice with elevated conjugated bilirubin from as early as 7 years of age[36]. Hemolytic disorders and Gilbert syndrome clinically present with recurrent jaundice; however, they have unconjugated hyperbilirubinemia[37]. These disorders have normal liver enzymes and synthetic functions.

AVH with ascites

Natural history: Ascites in children with AVH is present in 13% of cases; of which 38% are clinically detectable and have high serum ascites albumin gradient (> 1.1). Eleven percent have evidence of ascitic fluid infection (AFI). Associated pedal edema is present in 33% of cases and pleural effusion (hepatic hydrothorax) in 44%[38]. Children who develop ascites in AVH are younger, with lower serum albumin, lower total protein and higher prothrombin time, signifying greater liver dysfunction than those with uncomplicated AVH. Features of portal hypertension (esophagogastric varices, portal hypertensive gastropathy, dilated portal vein, and abdominal portosystemic collaterals) or chronicity (shrunken liver, nodular surface, irregular margins or differential lobe hypertrophy) are not seen. Diuretics are required in 44% of cases. Unlike CLD, the ascites responds rapidly and completely by 8 wk

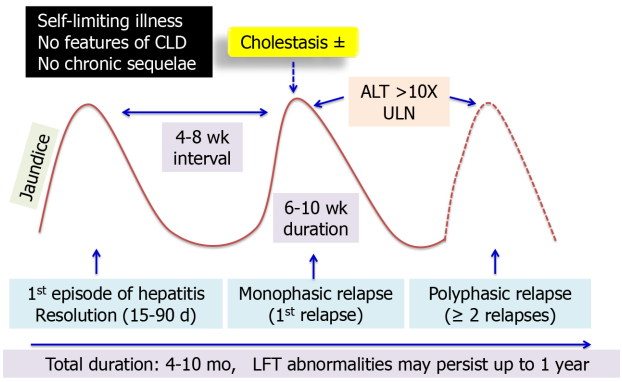

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Figure 5 Natural history of relapsing hepatitis in acute viral hepatitis. CLD: Chronic liver disease; ALT: Alanine aminotransferase; LFT: Liver function tests.

without any recurrence. The resolution is paralleled with complete improvement in liver function and normalization of liver size (appropriate for age)[38]. Figure 6 depicts the natural history of ascites in AVH. The mechanism of development of ascites was elegantly demonstrated in a study in which hepatic venous pressure gradient (HVPG) and liver histology were analyzed, which showed that liver cell dropout caused sinusoidal collapse that impeded intrahepatic blood flow resulting in portal hypertension. This finding was corroborated by the demonstration of higher HVPG (> 6mmHg) in those with ascites[39].

Differential diagnosis: Ascites in the setting of liver dysfunction needs to be carefully analyzed. In a given case, there may be significant overlap between various entities of diagnostic and therapeutic dilemmas. Differential diagnoses of ascitic form of AVH are decompensated CLD, acute on chronic liver failure (ACLF) and LOHF as summarized in Table 1. Decompensation in those with CLD is defined as ascites, encephalopathy and/or gastrointestinal bleeding. Among all admitted children with CLD, 40% have ascites[40,41]. Twenty eight percent of children with decompensated CLD have evidence of AFI [42]. According to the Asian Pacific Association for the Study of Liver Diseases (APASL), ACLF is defined as acute hepatic insult manifesting as jaundice (serum bilirubin > 5 mg/dL) and coagulopathy (INR > 1.5) complicated within 4 wk by clinically detectable ascites and/or hepatic encephalopathy in a patient with previously diagnosed or undiagnosed CLD[43]. Among the children with ACLF, 92% had ascites and 66% had hepatic encephalopathy. The majority of them presented for the first time with ACLF and CLD, and were silent until the precipitation of severe liver dysfunction. AVH was the precipitating factor in one-third of them, HAV and HEV in 35% each, HBV in 17% and Epstein-Barr virus in 11%[44]. Thus, in a child presenting with jaundice and ascites, features of portal hypertension and chronic liver disease ought to be looked for actively. Esophagogastroduodenoscopy, portal vein diameter (age-appropriate cut-off values), HVPG and liver biopsy are required in selected cases. Other supportive elements such as presence of growth failure, stigmata of CLD and their etiologies, family history of CLD, or past history of similar symptoms reinforce the suspicion of an underlying CLD.

Treatment: Restriction of dietary sodium to 2 mEq/kg/d, fluid restriction and diuretics form the mainstay of management of clinically significant ascites. Ascites in AVH rarely require aggressive management like large-volume paracentesis and albumin infusions. Diagnostic tapping of new-onset ascites in any liver disease is important for the presence of AFI. AFI needs to be treated as per the recommended antibiotic guidelines[45].

LOHF

Natural history: In the disease continuum of ALF, a subset of patients with liver dysfunction fall into the criteria where jaundice to encephalopathy interval ranges for a longer period commonly 5 to 12 wk. [46] This entity has been termed as sub-fulminant hepatic failure, sub-acute hepatic failure or LOHF. Other authors have re-defined the same entity differently in various studies with jaundice to encephalopathy interval ranging from 2 wk to 26 wk[47,48]. Most experts agree that the cut-off between jaundice and encephalopathy should be > 4 wk interval to define LOHF.[49] The definition of pediatric ALF lacks a discrete timeline thus making it difficult to recognize LOHF in children[50]. LOHF occupies a distinct position in the maze of severe liver dysfunction that is different from ALF and ACLF in terms of difference in its natural history and prognosis[51].

Of all the children with ALF, LOHF is seen in 8.3% with a median duration of illness of 53 d and an interval between jaundice and liver failure (ascites or encephalopathy) of 35 d. Ascites is the predominant sign of the failing liver seen in 94% and encephalopathy is present in 50% (advanced encephalopathy in 11%). Among all cases of LOHF, AVH is the most frequent etiology in nearly two-

Table 1 Differentiation between Ascitic form of acute viral hepatitis, decompensated chronic liver disease, acute on chronic liver failure and late onset hepatic failure

	Ascitic form of AVH[26]	Decompensated CLD[29]	ACLF[31]	LOHF[42]
Prodrome	Yes	No	Yes/No	Yes
Jaundice to ascites interval	Variable	Within first 3-4 wk	Within first 3-4 wk	After 3-4 wk
Encephalopathy	No	43%	66%	51%
Coagulopathy	No	Yes	Yes	Yes
Variceal bleeding	No	16%	14%	No
Growth failure	No	Yes	28%	No
Stigmata of liver disease	No	Yes	Yes	No
Acute insult	Yes	No	Yes	Yes
Nature of ascites	Rapidly resolving and Non-recurrent	Intractable and recurrent	Intractable and recurrent	Intractable and recurrent
Liver span	Enlarged	Enlarged or shrunken	Enlarged or shrunken	Mostly shrunken
Liver nodularity	No	Yes	Yes	No
Liver margins	Regular	Irregular	Irregular	Regular
Portal vein	Normal	May be dilated	May be dilated	Normal
Large Esophageal varices	No	May be present	May be present	No
Gastric varices	No	Yes	Yes	No
Survival with native liver	Yes	66%	61%	25%

AVH: Acute viral hepatitis; CLD: Chronic liver disease; ACLF: Acute on chronic liver failure; LOHF: Late onset hepatic failure.

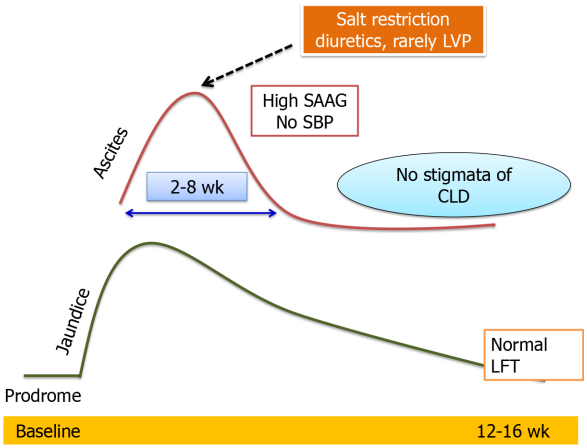

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Figure 6 Natural history of ascites in acute viral hepatitis. LVP: Levator veli palatine; SAAG: Serum-ascites albumin gradient; SBP: Systolic blood pressure; CLD: Chronic liver disease; LFT: Liver function tests.

thirds with HAV being the most common which is also the predominant etiology of AVH (64%) as well as ALF (70%) in developing countries[6,52]. In contrast HBV is the predominant etiology in adults with LOHF (32-46%)[53]. Mortality is higher in children with indeterminate etiology, hepatic encephalopathy, renal failure, infection, coagulopathy and high pediatric end-stage liver disease (PELD) score. Thus, referral for liver transplantation should be considered earlier in this group of children with LOHF. PELD score > 32 can predict mortality or the need for liver transplantation with good sensitivity. Post-mortem liver biopsy shows multiacinar massive or submassive necrosis without any features of chronicity. One-fourth of children recover spontaneously with native liver, as late as 24 mo after the onset of illness and without any evidence of CLD on follow-up[54]. This is in contrast to adults who exhibit features of chronic hepatitis in LOHF at follow-up[51]. It is imperative to recognize that LOHF is not merely old wine in a new bottle but is a distinctive entity whose natural history is yet to be

determined diligently in children.

Differential diagnosis: ACLF, decompensated CLD closely mimics LOHF. The absence of coagulopathy differentiates ascitic form of AVH from LOHF.

Treatment: Supportive treatment in lines of ALF management, maintenance of euglycemia, electrolytes, control of ascites and encephalopathy are required. Since they are at high risk of developing infections, a lower threshold has to be maintained for starting or changing antibiotics. Raised intracranial pressure is uncommon in LOHF thus, management of encephalopathy would be as per the guidelines for those with CLD[55].

Intravascular hemolysis in liver disease

Natural history: Intravascular hemolysis at presentation is encountered in 3% of children with AVH[3]. They have severe anemia, deep icterus and dark-colored urine akin to cola. Since the jaundice is accentuated, there is often a dilemma in interpretation of liver function tests and chances of overdiagnosing the condition as terminal liver disease. Liver functions show a high fraction of unconjugated bilirubin. Aspartate aminotransferase is higher than alanine aminotransferase. Lactate dehydrogenase levels is markedly elevated. Reticulocyte count is high and peripheral smear shows schistocytes and hemolytic cells. Intravascular hemolysis is further evident by demonstrating plasma hemoglobin and urine hemosiderin elevation. Underlying glucose-6-phosphate dehydrogenase (G-6-PD) deficiency is often unveiled in an episode of AVH or by drugs like vitamin K. Among those with hemolysis, there is G-6-PD deficiency in 36%, direct Coomb's test positive in 7%[3]. It is crucial to identify hemolysis early to avoid drugs that precipitate red blood cell destruction especially vitamin K which is universally administered in AVH and also to start appropriate fluid management to prevent pigment nephropathy [56]. G-6-PD levels may be falsely normal when measured during an acute episode of hemolysis due to relatively higher levels in new red blood cells. Hence, it has to be repeated after 3 mo to confirm G-6-PD deficiency, until then all contraindicated drugs have to be avoided. Figure 7 depicts the natural history of intravascular hemolysis associated with AVH.

Differential diagnosis: Hemolysis is associated with CLD in Wilson disease and autoimmune hepatitis. Acute hemolysis as the presenting feature would be there in 6.7% of cases of Wilson disease[57]. Acute release of copper from the necrosing liver cells is postulated to cause damage to the RBC membrane resulting in hemolysis, which is supported by finding very high serum and urine copper levels. Hemolysis is present in 22% of all children with Wilson disease and this proportion increases to 68% when considering those presenting as ALF[58]. Leipzig score which is used to diagnose Wilson disease in children gives weightage to Coomb's negative hemolytic anemia in addition to low ceruloplasmin, high urinary copper, high liver copper, positive copper staining on hepatocytes, Kayser-Fleisher ring, neuropsychiatric symptoms, positive family history and mutation in ATP7B gene[59]. Acute hemolysis in Wilson disease is an emergency as it is an indication to start immediate plasmapheresis to remove excess serum copper and also list for liver transplantation[60]. Autoimmune hemolytic anemia in children with autoimmune hepatitis is encountered in 35% of children. Coomb's test will be positive and needs to be treated with steroids and/or immunoglobulins[61].

Treatment: In acute intravascular hemolysis in the background of AVH, with a presumed diagnosis of G-6-PD deficiency all contraindicated drugs should be stopped. Hyper-hydration and diuretics to maintain urine output would protect from pigment nephropathy[62]. Simultaneous work-up for Wilson disease and autoimmune hepatitis ought to be done in select cases so that specific treatment is not delayed. In case of development of acute kidney injury, plasmapheresis and dialysis support may be required.

Autoimmune trigger

HAV infection itself can act as a trigger to uncover autoimmunity. In children with HAV, 63% have positive autoantibodies (60% ASMA and 3% ANA). LKM antibodies are usually not found in concomitant AVH[63]. The release of intracellular antigen during hepatocyte necrosis induces the antibody formation. The genes associated with the development of type 1 autoimmune hepatitis are located in HLA II DRB1 0301 Loci which are also reported to be present in those with persistent HAV infection. This may explain the precipitating role of HAV in autoimmune hepatitis[64]. Though autoantibodies have been demonstrated in children with HAV, the exact role of HAV in triggering AIH is not well studied in children.

Systemic complications of AVH

Other than the above atypical features, systemic complications of AVH include acute kidney injury, thrombocytopenia, acute pancreatitis, hemophagocytic lymphohistiocytosis, aplastic anemia and transverse myelitis[65].

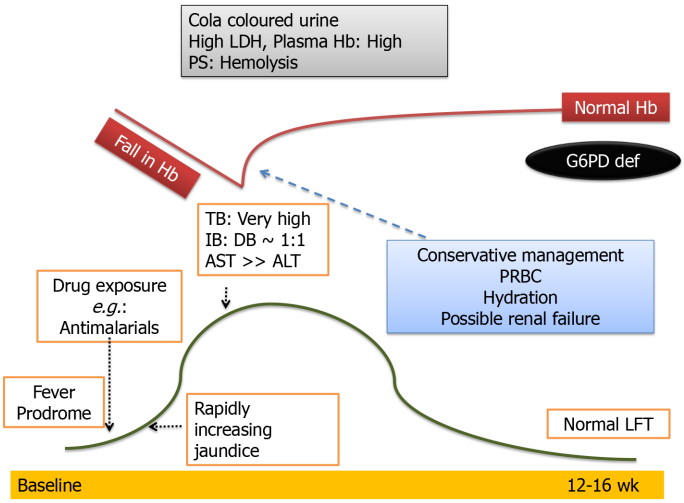

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Figure 7 Natural history of intravascular hemolysis (G6PD deficiency) in acute viral hepatitis. LDH: Lactate dehydrogenase; TB: Tuberculosis; IB: Illipe butter; DB: Disulfide bond; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PRBC: Packed red blood cells; LFT: Liver function tests.

CONCLUSION

Atypical presentations of AVH may have a myriad of presentations often mimicking an underlying CLD. Understanding the natural history of relapsing hepatitis, prolonged cholestasis, ascites and intravascular hemolysis in the setting of AVH aids in decision-making and avoiding unwarranted investigations that be may be unyielding. Invasive investigations and aggressive management must be carefully considered and indicated only when the suspicion is strong for an underlying CLD. LOHF is a distinct and aggressive variant of AVH that needs to be viewed in the lines of ALF.

FOOTNOTES

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Functions of three ubiquitin-conjugating enzyme 2 genes in hepatocellular carcinoma diagnosis and prognosis

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Abstract

BACKGROUND

Liver cancer ranks the third cause of cancer-related death worldwide. The most common type of liver cancer is hepatocellular carcinoma (HCC). The survival time for HCC patients is very limited by years due to the lack of efficient treatment, failure of early diagnosis, and poor prognosis. Ubiquitination plays an essential role in the biochemical processes of a variety of cellular functions.

AIM

To investigate three ubiquitination-associated genes in HCC.

METHODS

Herein, the expression levels of ubiquitin-conjugating enzymes 2 (UBE2) including UBE2C, UBE2T, and UBE2S in tumor samples of HCC patients and non-tumor controls at the Cancer Genome Atlas (TCGA) database, was comprehensively analyzed. The relationship of *UBE2* gene expression level with cancer stage, prognostic outcome, and TP53 mutant status was studied.

RESULTS

Our results showed that *UBE2C*, *UBE2T*, and *UBE2S* genes were overexpressed in HCC samples compared to non-tumor tissues. Dependent on the cancer progression stage, three *UBE2* genes showed higher expression in tumor tissues at all four stages compared to non-tumor control samples. Furthermore, a significantly higher expression of these genes was found in stage 2 and stage 3 cancers compared to stage 1 cancer. Additionally, overexpression of those genes was negatively associated with prognostic outcome and overall survival time. Patients with TP53 mutation showed a higher expression level of three *UBE2* genes, indicating an association between UBE2 expression with p53 function.

CONCLUSION
In summary, this study shed light on the potential roles of UBE2C, UBE2T, UBE2S on diagnostic and prognostic biomarkers for HCC. Moreover, based on our findings, it is appealing to further explore the correlation of those genes with TP53 mutation in HCC and the related mechanisms.

Key Words: Hepatocellular carcinoma; Ubiquitin-conjugating enzyme 2; UBE2C; UBE2T; UBE2S; TP53 mutant; Biomarker; Diagnostic; Prognostics

Core Tip: Liver cancer ranks the third cause of cancer-related death worldwide. The most common type of liver cancer is hepatocellular carcinoma (HCC). Lack of effective treatment options and early diagnostic biomarkers results in a short survival time of HCC patients. Ubiquitination plays an essential role in the biochemical processes in cells. In this study, using bioinformatic analysis of the online TCGA database we found that three ubiquitin-conjugating enzyme 2 (UBE2) genes were overexpressed in HCC samples compared to normal samples in a stage-dependent manner, including *UBE2C*, *UBE2T*, and *UBE2S*. Additionally, overexpression of those genes was negatively associated with prognostic outcomes and overall survival times. Patients with TP53 mutation showed a higher level of expression of three *UBE2* genes, indicating an association between UBE2 expression with p53 function. This study shed light on the potential roles of *UBE2C*, *UBE2T*, and *UBE2S* on diagnostic and prognostic biomarkers for HCC, as well as the therapeutic strategy.

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INTRODUCTION

Liver hepatocellular carcinoma (HCC, or LIHC) is the most common type of primary liver cancer, which is the third most common cause of cancer-related death worldwide[1,2]. Hepatitis viral infections, abuse of alcohol, liver fibrosis, and cirrhosis are the major factors that cause liver cancer. HCC is closely associated with many metabolic diseases, such as non-alcoholic fatty liver disease (NAFLD), diabetes, obesity, cardiovascular diseases[3]. Surgical operation is a curative treatment for early-stage of liver cancer[4]. However, most HCC cases were found at the late stage due to a lack of effective diagnostic biomarkers, which are not suitable for surgical procedures[5]. Therefore, early diagnostic and exploration of novel treatment options are urgently needed. The mechanism-based investigation both on the genetic and molecular levels is necessary to further facilitate the exploration of diagnosis and treatment.

Ubiquitin is a highly conserved regulatory protein in all eukaryotic organisms, and it is covalently tagged to proteins, severing as a signal for further proteasome degradation[6]. Ubiquitination is an essential biochemical process, which contributes to a variety of cellular functions, such as cell signaling pathway regulation, cell death, protein degradation, innate and adaptive immune response. Due to the significant role of ubiquitination in cell survival and death, it is closely associated with host health and disease[7,8]. When exploring the top overexpressed genes of E2 ubiquitin-conjugating enzymes (UBE2) in HCC patient tumor samples from the Cancer Genome Atlas (TCGA) database, UBE2C, UBE2T, and UBE2S were ranked as top 4, top 8, and top 31, respectively. Those highly expressed genes draw our attention in exploring their roles in cancers, specifically for HCC. Therefore, in this study, we focused on the investigation of highly expressed UBE2C, UBE2T, UBE2S as potential biomarkers for HCC diagnosis and prognosis, as well as their expression levels at different cancer stages.

UBE2 is a large enzyme family that plays a fundamental role in the second step of ubiquitination that connects the first step of ubiquitin activation by UBE1 enzyme and together with the third step ubiquitin-protein ligation *via* UBE3 enzyme to conduct the complex ubiquitination process[7]. UBE2C protein, encoded by gene *UBE2C*, was reported to exacerbate cell apoptosis[9] and contribute to chromosome mis-segregation during the formation of tumors[10]. Overexpression of gene *UBE2C* has been found in tumor cells of HCC patients compared to noncancerous liver cells in 62 out of the studied 65 clinical cases[11]. Most recently, another study also showed that UBE2C can promote cancer cell growth and migration[12].

UBE2T can bind to Fanconi anemia complementation group L and meditate the monoubiquitinating of Fanconi anemia complementation group D2 (FANCD2), a critical process of regulation associated with damaged-DNA repair in the Fanconi anemia pathway[13,14]. It was also reported that UBE2T played an important role in the carcinogenesis in different cancer types[15-18], such as human breast cancer cells[17,18], lung cancer[19], gastric cancer[20], *etc*.

UBE2S serves as the key component on the degradation of anaphase-promoting complex/cyclosome substrate *via* mitosis, in which process UBE2C is also involved[21]. In breast cancer cells, UBE2S deficiency can suppress their migration, invasion, and growth *via* disruption of actin cytoskeleton and focal adhesion[22]. In addition, silencing UBE2S reduced cell proliferation and colony formation of lung cancer cells, resulting in cell apoptosis[23]. In HCC, UBE2S was upregulated and showed oncogenic activity by increasing p53 ubiquitination[24]. Overall, these three genes play pivotal roles in cancer cell progression and invasion, indicating the potential as biomarkers for HCC.

However, the bioinformatic-based systematic analysis of UBE2C, UBE2T, and UBE2S in liver cancers from clinical patients have not been reported. Herein, this study carried a comprehensive bioinformatic-based analysis of clinical data from the online database to illustrate the significant roles of three *UBE2* genes in HCC, by analyzing their gene expression levels between non-tumor and tumor tissues, their association with cancer progression stage, and their prognostic values, and by investigating their expression in different cell types, association with TP53 mutation status, co-expression efficiency, involved signaling pathways, and associated proteins in interaction networks.

MATERIALS AND METHODS

Ethics statement

All the data for this study originated and was generated from the online open resource database and published literature.

TCGA database

The RNA-seq data and LIHC/HCC patient clinic information were originated and generated from TCGA. (TCGA research network: https://www.cancer.gov/tcga). The genomic alterations for UBE2C, UBE2T, UBE2S in different liver cancer types were analyzed using cBioPortal (https://www.cbioportal.org/)[25,26]. A survival heatmap was generated using GEPIA[27].

UALCAN

The expression level of the genes of interest on pan-cancer was analyzed using UALCAN web-based tool. The quired gene expression level in pan-cancer was analyzed using Student's *t*-test to compare the normal and tumor group with $P < 0.05$ as significant differential expression. Heatmap was generated to display the RNA-seq data using the median value of expression Log2 (TPM+1). Analysis of gene expression level on HCC cancer progression stages, expression level on TP53 mutant status and non-TP53 mutant status was performed using online resources UALCAN[28].

Human Protein Atlas Database

The analysis of RNA expression in different cell types and the expression location of *UBE2* genes in three different cells including epidermoid carcinoma cell line A-431, human osteosarcoma cell line U-2 OS, and human glioblastoma cell line U-251 MG were explored using the Human Protein Atlas database (Human Protein Atlas, https://www.proteinatlas.org, Protein Atlas version 21.0), an online public resource for the investigation of protein-coding genes of variable cancers in cell and tissue samples[29].

STRING database for PPI network

In this study, the STRING online tool[30] was used to show the interaction of functional enrichment of the generated network with queried input: UBE2C, UBE2T, UBE2S, and TP53. (Default threshold for interaction specificity score > 0.4 was defined as significant). The functional property was generated and summarized based on the online information and literature.

Statistical analysis

The survival curve was generated using Kaplan Meier-plotter that was commonly used for assessment of the gene expression on survival from a large database such as TCGA samples. The significance of survival impact was measured by a log-rank test. A log-rank $P < 0.05$ was set for statistically significant cut-off.

Expression of genes in tumor samples and normal samples using Student's *t*-test (considering unequal variance) for comparison between different groups. A *P* value less than 0.05 is considered statistically significant.

Overall survival heat map of the patients across multiple cancer types was generated with the input of 95% confidence interval and the calculation of the hazard ratio based on the Cox PH model. Pearson's

correlation coefficient (r) was used to plot the co-expression between quired input paired genes.

RESULTS

Expression levels of UBE2C, UBE2T, and UBE2S in pan-cancers

As shown in Figure 1, three UBE2 family members including UBE2C, UBE2T, and UBE2S were highly expressed in most tumor samples compared to the corresponding normal (non-tumor) samples. A formula of log2 (TPM+1) was used to calculate the *UBE2* gene expression level, where TPM is transcripts per million. The case numbers for each cancer were included in the figure. The expression level among normal and tumor tissues are heterogeneous, with relatively high expression in both normal and tumors, such as colon and rectum adenocarcinoma (COAD and READ), skin cutaneous melanoma (SKCM), and thymoma (THYM). For HCC (or LIHC), UBE2C showed a high expression level in tumor samples (n = 371) compared with normal samples (n = 50), with the mean value of the log2 (TPM + 1) of 4.2 for tumor samples and 0.7 for normal samples. UBE2T also showed an increased expression level in tumor samples with the mean value of log2 (TPM + 1), compared to 0.6 for normal samples. Similarly, a higher expression level was shown for UBE2S, with the value of the log2 (TPM + 1) of 4 for tumor samples and the value of log2 (TPM + 1) of 2 for normal samples.

Expression level in liver cancer and normal samples

Similarly, the distribution of the expression *UBE2C*, *UBE2T*, and *UBE2S* indicated that they were expressed in HCC tumor samples compared to normal samples (Figure 2A-C), indicating the potential as a diagnostic biomarker for HCC. Furthermore, the expression of each gene at different tumor stages was analyzed to investigate the association of gene expression with the tumor progression stage. The results demonstrated that along with the progression of cancer, the expression patterns of genes *UBE2C*, *UBE2T*, and *UBE2S* kept increasing from stage 1 to stage 3 (Figure 2D-F). There was a pike level at stage 3, while at stage 4, the expression level significantly decreased for *UBE2C* and *UBE2S* compared to that from stage 2 and stage 3, even though it was increased compared to that in normal tissue (Figure 2D and F). In addition, the expression of *UBE2T* was also decreased at stage 4 of HCC, but without significant change compared to that in stages 2 and 3, which might be impacted by the sample size (Figure 2E). Even though, the significantly increased expression level of those genes in HCC stage 1, 2, and 3 when compared to normal samples or compared between stage 1 and stage 2&3, which suggests that it is valuable for further exploring them as potential biomarkers or key genes mediating HCC progression.

Relationship between UBE2 expression and the survival of HCC patients

To explore the prognostic value of these gene expression levels in HCC patients, the survival outcome of patients, the expression of *UBE2* genes, and the survival curve were analyzed. Remarkably, high expression levels of *UBE2C*, *UBE2T*, and *UBE2S* were associated with a negative prognostic outcome in HCC patients. The patients with overexpression of *UBE2C* (n = 91) showed significantly less survival time compared to the patients with low or medium expression levels (n = 274) (Figure 3A, $P < 0.0001$). For *UBE2T*, 277 patients with low or medium expression levels showed significantly higher survival days compared to the patients (n = 88) with high expression levels (Figure 3B, $P < 0.0001$). Similar results were also found for *UBE2S*, a significantly shorten survival time was associated with the higher *UBE2S* expression level (n = 89) compared with longer survival patients with low or medium expression levels of UBE2S (n = 276) (Figure 3C, $P < 0.0001$).

In addition, with the analysis of prognostic markers for pan-cancers, the results indicated that a higher expression level of *UBE2C*, *UBE2T*, and *UBE2S* was associated with the poor prognostic outcome for most of the cancers, such as adrenocortical carcinoma (ACC), kidney chromophobe (KICH), brain lower grade glioma (LGG) (Figure 3D).

Genetic mutations in UBE2 genes in HCC

We further explored the underlying mechanisms of three *UBE2* genes in HCC development and progression. A total of 1238 samples were analyzed using the cBioPortal from the TCGA pan-cancer database for genetic alterations including mutations, structural variants, and copy number alteration of three *UBE2* genes. The OncoPrint results analyzed from cBioPortal showed the queried genes genomic alteration frequency from TCGA studied HCC samples is 2.1% (26 out of 1238). Among them, the genetic alteration occurred on genes *UBE2T* and *UBE2S*, and no alteration was found for *UBE2C* (Figure 4A). Then, the genetic alteration based on liver cancer subtypes was further analyzed. Among 1238 samples, 369 samples were HCC and 712 samples were hepatobiliary cancer. The genetic alteration was mostly found in HCC and hepatobiliary cancer. For HCC, the overall genetic alteration frequency of genes *UBE2T* and *UBE2S* was 6.23% (23 out of 369 samples) that largely resulted from amplification (5.96%, 22 cases, red color) and less from mutation (0.27%, 1 case, green color) out of 369 cases. For hepatobiliary cancer, the overall genetic alteration frequency of genes *UBE2T* and *UBE2S* was 0.28% in total 712 cases with 1 case amplification (0.14%) and 1 case mutation (0.14%) (Figure 4B). Specifically, for

	UBE2C		UBE2T		UBE2S		Sample number (*n*)	
	N	C	N	C	N	C	N	C
BLCA	3.1	7.5	3.1	5.4	4.4	6.1	19	408
BRCA	2.3	6.2	2.5	5.4	2.9	4.9	114	1097
CESC	1.5	7.6	2.1	6.1	3.6	6.3	3	305
CHOL	0.4	4.9	0.5	4.2	2.0	4.4	9	36
COAD	5.5	7.5	4.1	5.7	4.0	5.8	41	286
ESCA	2.7	7.2	2.1	5.6	3.1	5.8	11	184
GBM	0.5	6.5	4.2	5.4	5.0	5.6	5	156
HNSC	4.9	6.9	3.6	5.1	3.6	5.3	44	520
KICH	0.9	3.0	2.1	2.0	3.7	4.2	25	67
KIRC	1.0	3.1	2.0	2.6	2.9	3.2	72	533
KIRP	0.7	2.5	1.8	2.9	3.2	3.5	32	290
LIHC	0.7	4.2	0.6	3.6	2.0	4.0	50	371
LUAD	2.2	6.0	1.9	5.1	3.2	4.6	59	515
LUSC	2.3	7.1	2.1	5.8	3.4	6.1	52	503
PAAD	3.8	5.3	2.9	4.1	3.7	4.6	4	178
PRAD	1.4	2.9	2.3	2.8	3.7	4.3	52	497
PCPG	1.1	2.6	2.3	4.2	4.3	5.4	3	179
READ	5.6	7.6	4.6	5.6	4.4	6.1	10	166
SARC	2.7	6.8	3.0	5.4	3.0	5.3	2	260
SKCM	6.4	6.6	5.3	5.3	5.5	6.0	1	472
THCA	1.1	2.4	2.2	2.8	3.7	3.5	59	505
THYM	6.9	7.2	5.4	5.7	5.9	6.1	2	120
STAD	4.8	7.2	3.2	5.6	3.7	5.4	34	415
UCEC	2.0	7.0	2.2	5.4	4.0	6.1	35	546

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Figure 1 Gene expression levels of UBE2C, UBE2T, and UBE2S in pan-cancer with normal samples and tumor samples (the Cancer Genome Atlas database). N: Normal samples; C: Cancer samples. Color-coded only based on each cancer type. Red: higher expression level; Blue: lower expression level when compared tumor samples with normal samples in a particular cancer type. The number represents the median value of expression Log2 (transcript count per million (TPM)+1). The total sample number for the corresponding cancer type was listed on the right side of the figure (N: total sample number of normal samples. C: total sample number of tumor samples) Abbreviations: BLCA: Bladder urothelial carcinoma; BRCA: Breast invasive carcinoma; CESC: Cervical squamous cell carcinoma; CHOL: Cholangiocarcinoma; COAD: Colon adenocarcinoma; ESCA: Esophageal carcinoma; GBM: Glioblastoma multiforme; HNSC: Head and Neck squamous cell carcinoma; KICH: Kidney Choromophobe; KIRC: Kidney renal clear cell carcinoma; KIRP: Kidney renal papillary cell carcinoma; LIHC (HCC): Liver hepatocellular carcinoma; LUAD: Lung adenocarcinoma; LUSC: Lung squamous cell carcinoma; PAAD: Pancreatic adenocarcinoma; PRAD: Prostate adenocarcinoma; PCPG: Pheochromocytoma and Paraganglioma; READ: Rectum adenocarcinoma; SARC: Sarcoma; SKCM: Skin Cutaneous Melanoma; THCA: Thyroid carcinoma; UCS: Uterine Carcinosarcoma; THYM: Thymoma; STAD: Somach adenocarcinoma; UCEC: Uterine Corpus Endometrial Carcinoma)

gene *UBE2T*, the only genetic alteration type is amplification which was found in 21 out of 369 cases of HCC samples, while no mutation had occurred for *UBE2T* (Figure 4C). For *UBE2S*, genetic alteration occurred in 2 cases out of 369 cases (0.54%) in HCC, including 1 case of mutation (0.27%) and 1 case of amplification (0.27%). Similarly, genetic alteration of *UBE2S* occurred in 2 cases out of 712 cases (0.28%) in hepatobiliary cancer, including 1 case mutation (0.14%) and 1 case amplification (0.14%) (Figure 4D). Overall, the most of genetic alteration occurred in HCC samples with 23 out of 369 samples (6.23%), which was mainly from gene *UBE2T*.

RNA and protein expression patterns

Single-cell RNA sequencing (scRNA-seq) revealed three clusters c-6 (B-cells), c-15 (T-cells), and c-16 (Erythroid cells) exhibited high mRNA expression of all three *UBE2C*, *UBE2T*, and *UBE2S* (Figure 5A-

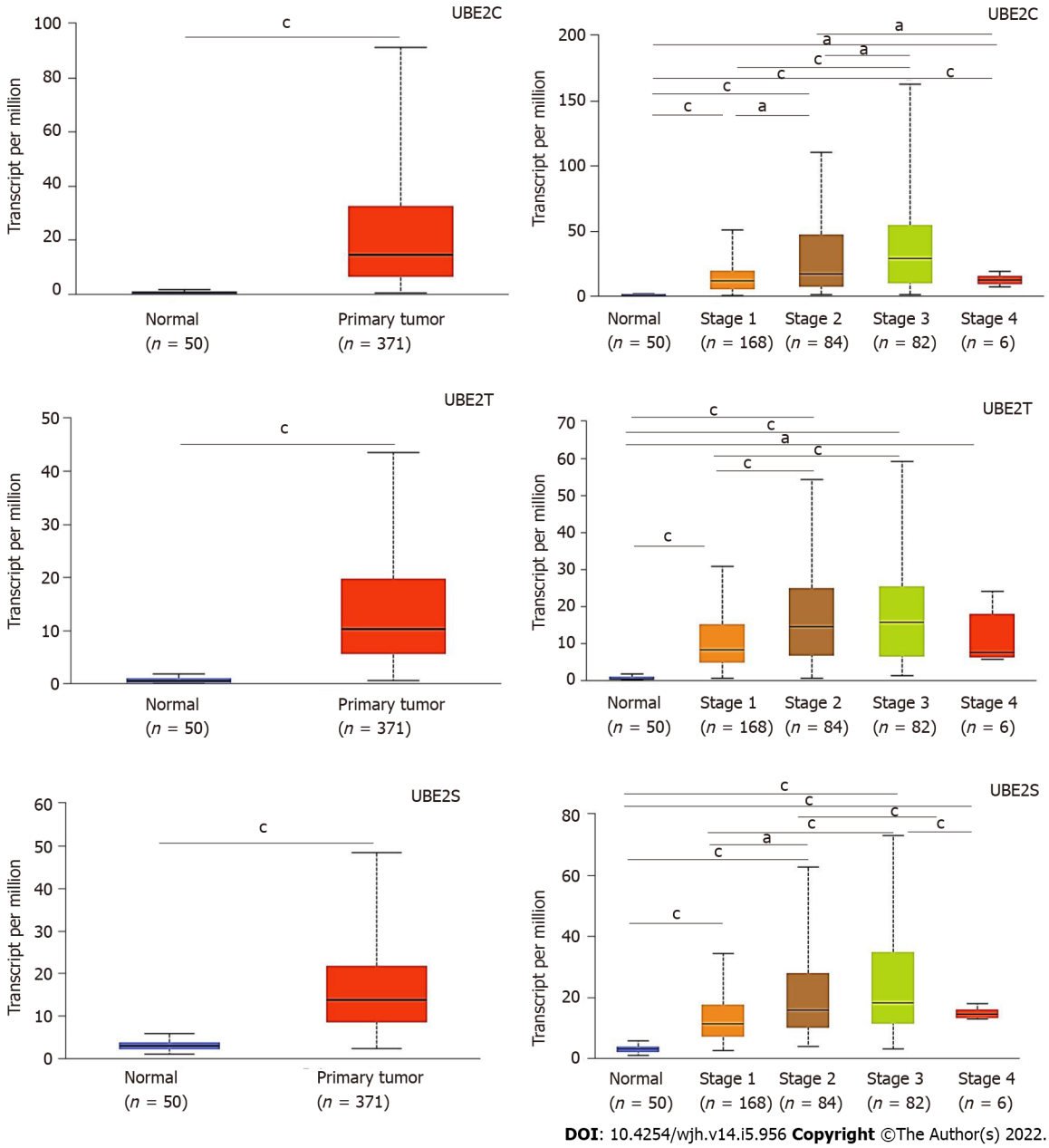

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Figure 2 UBE2C, UBE2T, and UBE2S expression levels in hepatocellular carcinoma based on normal and tumor sample types and based on cancer stages (the Cancer Genome Atlas database). A: Expression of UBE2C in hepatocellular carcinoma (HCC) based on sample types; B: Expression of UBE2T in HCC based on sample types; C: Expression of UBE2S in HCC based on sample types; D: Expression of UBE2C in HCC based on individual cancer stages; E: Expression of UBE2T in HCC based on individual cancer stages; F: Expression of UBE2S in HCC based on individual cancer stages ($^aP < 0.05$; $^cP < 0.001$).

G). In addition, the protein expression location in three cell lines including A-431, U-2 OS, and U-251 MG (Human Protein Atlas, https://www.proteinatlas.org) indicated that UBE2C was mainly expressed in the cytosol and plasma membrane, UBE2T was mainly expressed in nucleoli or nucleoplasm, and UBE2S was highly expressed in the cytosol and plasma membrane and less expressed in nucleoli (Figure 6). Furthermore, the co-expression relationship of these genes was analyzed, since all three genes *UBE2C*, *UBE2T*, and *UBE2S* were expressed by the same clusters in HCC. The correlation expression of pair-wise genes was analyzed using Pearson methods (Figure 7A, 7B, 7C). Results showed there was a co-expression between two paired genes ($r = 0.83$ UBE2C-UBE2S; $r = 0.76$ UBE2C-UBE2T; $r = 0.76$ UBE2S-UBE2T). In addition, the analysis of co-expression of their proteins also showed that there was a correlation among three proteins (Figure 7D), indicating a co-expressing pattern.

TP53 mutation impacts the expression of *UBE2S*, *UBE2T*, and *UBE2S* genes

Notably, the expression levels of all three genes *UBE2C*, *UBE2T*, and *UBE2S* were significantly higher in *TP53* mutant samples compared with both normal samples and non-*TP53* mutant samples (Figure 7E-7G). However, the correlation or causation between the higher expression level of *UBE2C*, *UBE2T*,

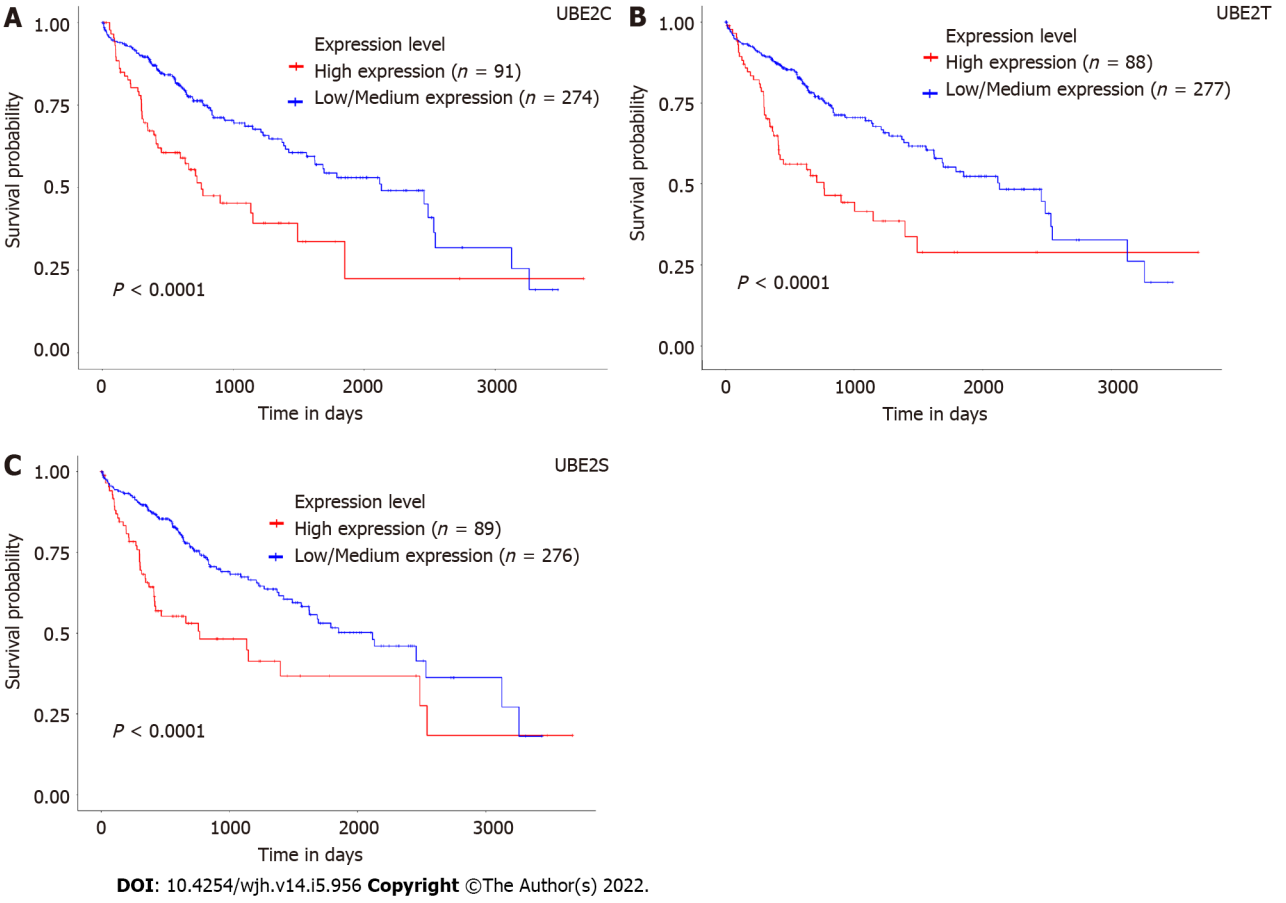

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Figure 3 Association of expression levels of UBE2C, UBE2T, and UBE2S with prognostic outcomes of hepatocellular carcinoma patients.
A: Survival of patients with a high expression level of UBE2C compared with patients with low/medium expression level; B: Survival of patients with a high expression level of UBE2T compared with patients with a low/medium expression level; C: Survival of patients with a high expression level of UBE2S compared with patients with low/medium expression level; D: Overall survival heat map of patients across multiple cancer types. Red color represents higher risk on survival and blue color represents lower risk. The frame indicated the significant unfavorable (red) and favorable (blue) prognostic outcome. (Calculation of the hazards ratio based on Cox PH Model, 95% Confidence Interval) (ACC: Adrenocortical carcinoma; LGG: Brain lower grade glioma; DBLC: Lymphoid Neoplasm Diffuse Large B-cell Lymphoma; MESO: Mesothelioma; OV: Ovarian serous cystadenocarcinoma; TGCT: Testicular germ cell tumors; UVM: Uveal melanoma).

UBE2S, and *TP53* mutation is unknown. This finding of a comprehensive analysis of clinical patient samples was supported by several studies. For example, overexpression of *UBE2C* in patients with endometrial cancer was associated with cancer progression and recurrence, by increasing endometrial cancer cell proliferation, migration, invasion, as well as the process of epithelial-mesenchymal transition through inhibition of p53 expression[31]. UBE2T can enhance the ubiquitination of p53 in HCC cells[32, 33]. Similarly, UBE2S also can promote the ubiquitination of p53 and mediate its protein degradation in HCC cells[24]. Therefore, recovering or enhancing p53 function can partially attenuate UBE2 genes-induced malignant phenotypes of tumor cells.

Protein-protein interaction network

To further investigate the association among three genes *UBE2S*, *UBE2T*, *UBE2S*, and *TP53* function, we generated the protein-protein interaction network using their proteins and *TP53*. The most closely associated proteins related to these protein interactions were analyzed using STRING, shown in Figure 7H. The functional annotation and associated signaling pathways of the queried genes were summarized in Table 1.

DISCUSSION

The early diagnostic of HCC or LIHC is critically important for cancer treatment and selection of therapeutic methods[34-36]. In addition, a better understanding of the development and progression of the cancer stage is helpful to choose the therapy that can result in a good outcome. In this study, with clinical data analysis, we found that the expression of *UBE2C*, *UBE2T*, and *UBE2S* was increased in tumor tissue compared to normal tissue, and their expression was associated with the procession of

Table 1 Functional enrichment in the queried network (query input UBE2C, UBE2T, UBE2S, TP53)

UBE2 genes			Biological process
	UBE2T	UBE2S	Protein K27-linked ubiquitination
	UBE2T	UBE2S	Protein K29-linked ubiquitination
UBE2C		UBE2S	Free ubiquitin chain polymerization
	UBE2T	UBE2S	Protein K6-linked ubiquitination
UBE2C		UBE2S	Exit from mitosis
UBE2C		UBE2S	Positive regulation of ubiquitin protein ligase activity
UBE2C	UBE2T	UBE2S	Protein K11-linked ubiquitination
UBE2C	UBE2T		Protein K48-linked ubiquitination
	UBE2T	UBE2S	Protein K63-linked ubiquitination
UBE2C		UBE2S	Anaphase-promoting complex-dependent catabolic proceed
UBE2			**Cellular component and uniport annotated keywords**
UBE2C		UBE2S	Anaphase-promoting complex
UBE2C	UBE2T	UBE2S	Nucleoplasm
UBE2C	UBE2T	UBE2S	ATP-binding
UBE2C		UBE2S	Cell cycle
UBE2C	UBE2T	UBE2S	Transferase
UBE2C	UBE2T	UBE2S	Ubl conjugation
UBE2			**Reactome pathways**
UBE2C		UBE2S	Aberrant regulation of mitotic exit in cancer due to RB1 defects
UBE2C		UBE2S	APC-Cdc20 mediated degradation of Nek2A
UBE2C		UBE2S	APC/C:Cdc20 mediated degradation of Cyclin B
UBE2C		UBE2S	APC/C: Cdh1 mediated degradation of Cdc20 and other APC/C
UBE2C		UBE2S	Cell cycle checkpoints
UBE2C		UBE2S	Cell cycle, mitotic
UBE2C		UBE2S	Cellular responses to stress
UBE2C		UBE2S	Cellular Senescence
UBE2C		UBE2S	Conversion from APC/C: Cdc20 to APC/C: Cdh1 in late anaphase
UBE2C		UBE2S	Generic transcription pathway
UBE2C		UBE2S	Inactivation of APC/C *via* direct inhibition of the APC/C compel
UBE2C		UBE2S	Phosphorylation of the APC/C
UBE2C	UBE2T	UBE2S	Post-translational protein modification
UBE2C	UBE2T	UBE2S	Synthesis of active ubiquitin: roles of E1 and E2 enzymes
UBE2C		UBE2S	Synthesis of DNA
UBE2C		UBE2S	Transcriptional regulation by VENTX

UBE2: Ubiquitin-conjugating enzyme 2; APC/C: Anaphase-promoting complex/cyclosome; VENTX: Ventral-expressed homeobox (VENT)-like homeobox protein 2.

cancer stage from stage 1 to stage 3, although there was a decrease at last stage (stage 4). These results suggest there is a potential to use those genes as biomarkers to assist the diagnosis of HCC at the early-stage point. What's more, the results also showed that there was a significantly increased expression level of those genes at stage 2 and stage 3 compared with stage 1 during HCC progression. This may shed light on the potential usage of those genes as biomarkers to better predict the cancer progression stage.

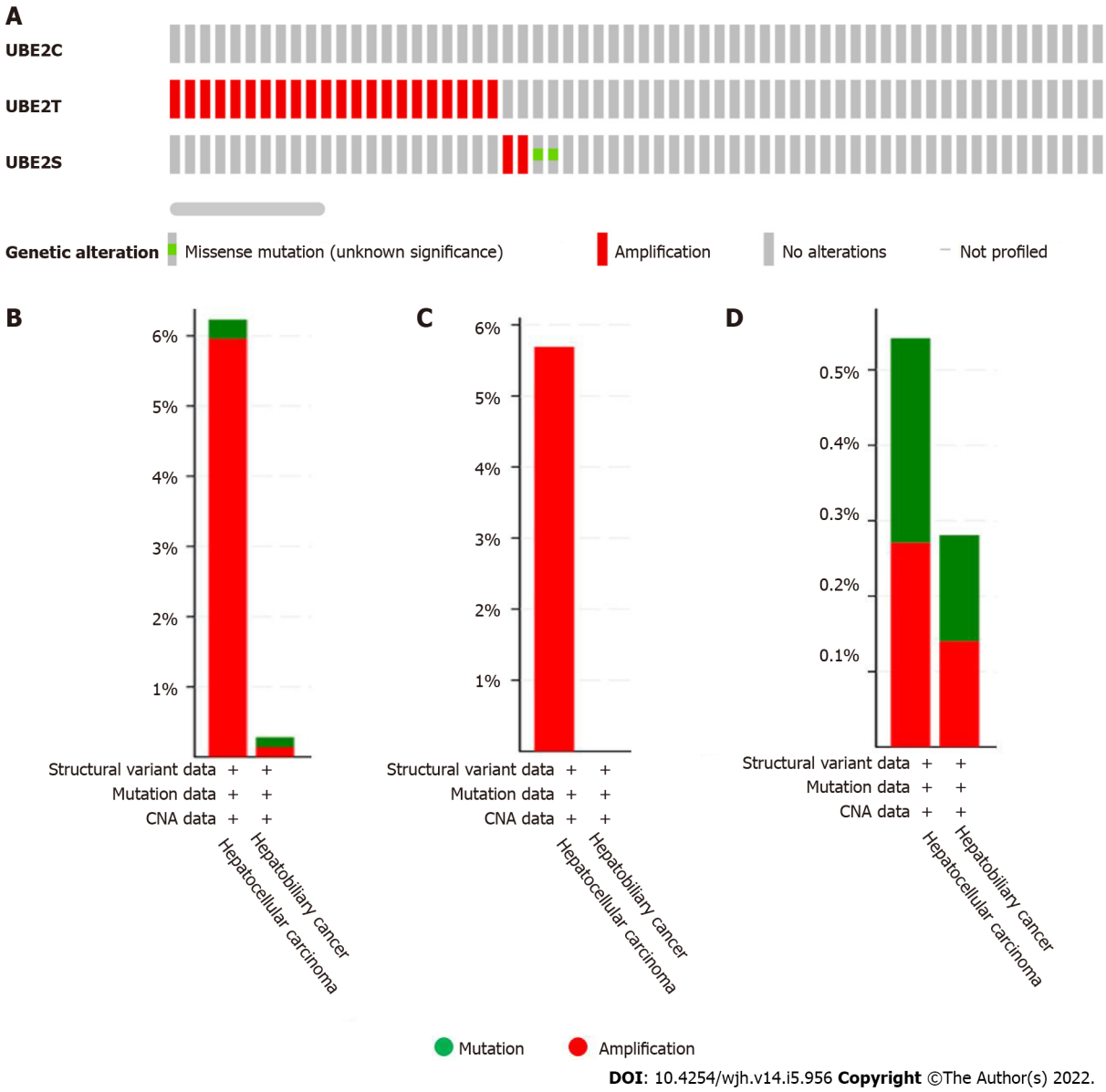

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Figure 4 The genetic alteration occurred in UBE2C, UBE2T, and UBE2S in liver cancer (1238 samples from the Cancer Genome Atlas). A: Genetic alteration occurred in UBE2C, UBE2T, and UBE2S in HCC; B: UBE2T and UBE2S, overall alteration frequency 6.23% (23/369 cases) in hepatocellular carcinoma including 22 cases implication and 1 case of mutation. In hepatobiliary cancer type, the alteration frequency is 0.28 (2/712) with 1 case of amplification and 1 case of mutation; C: UBE2T genetic alteration frequency 5.69% (21/369 cases) categorized by cancer type; D: UBE2S genetic alteration frequency 0.54% (2/369 cases) in HCC (1 case amplification and 1 case mutation); In hepatobiliary cancer, UBE2S alteration frequency is 0.28% (2/712 cases) (1 case amplification and 1 case mutation).

UBE2 family members play a role in the development and prognosis of cancers[37,38], such as ovarian cancer. It has been shown that the mRNA expression of UBE2A in liver cancer cell lines (*e.g.*, HepG2 and Huh-7) was significantly higher compared to that in normal liver cancer line HL-7702. Meanwhile, UBE2A mRNA and protein were highly expressed in HCC tumor tissues than those in the adjacent normal tissues[39]. In HCC, qPCR data showed that the expression of UBE2S was significantly increased in HCC samples compared to non-tumor liver tissues[40]. Another study showed that the expression of UBE2T mRNA and protein was significantly increased in HCC tissues compared to adjacent non-tumor tissues. A molecular mechanism study showed that UBE2T can suppress the G2/M transition of hepatoma cells by regulating cyclin B1 and cyclin-dependent kinase 1 expression[41]. A recent study showed that the expression of UBE2T can be regulated by microRNA miR-212-5p, and overexpression of UBE2T can promote HCC cell proliferation and migration[42]. In this study, we have demonstrated that overexpression of UBE2C, UBE2T, and UBE2S was associated with poor prognosis and shorter survival time. These results indicate the gene expression levels of three genes might be useful to assist to predict the outcome of HCC. Remarkably, our analysis revealed that HCC tumors with *TP53* mutant status exhibited significantly higher expression levels of those genes compared with *TP53* non-mutant status in tumor samples. This finding shows the potential correlation between the

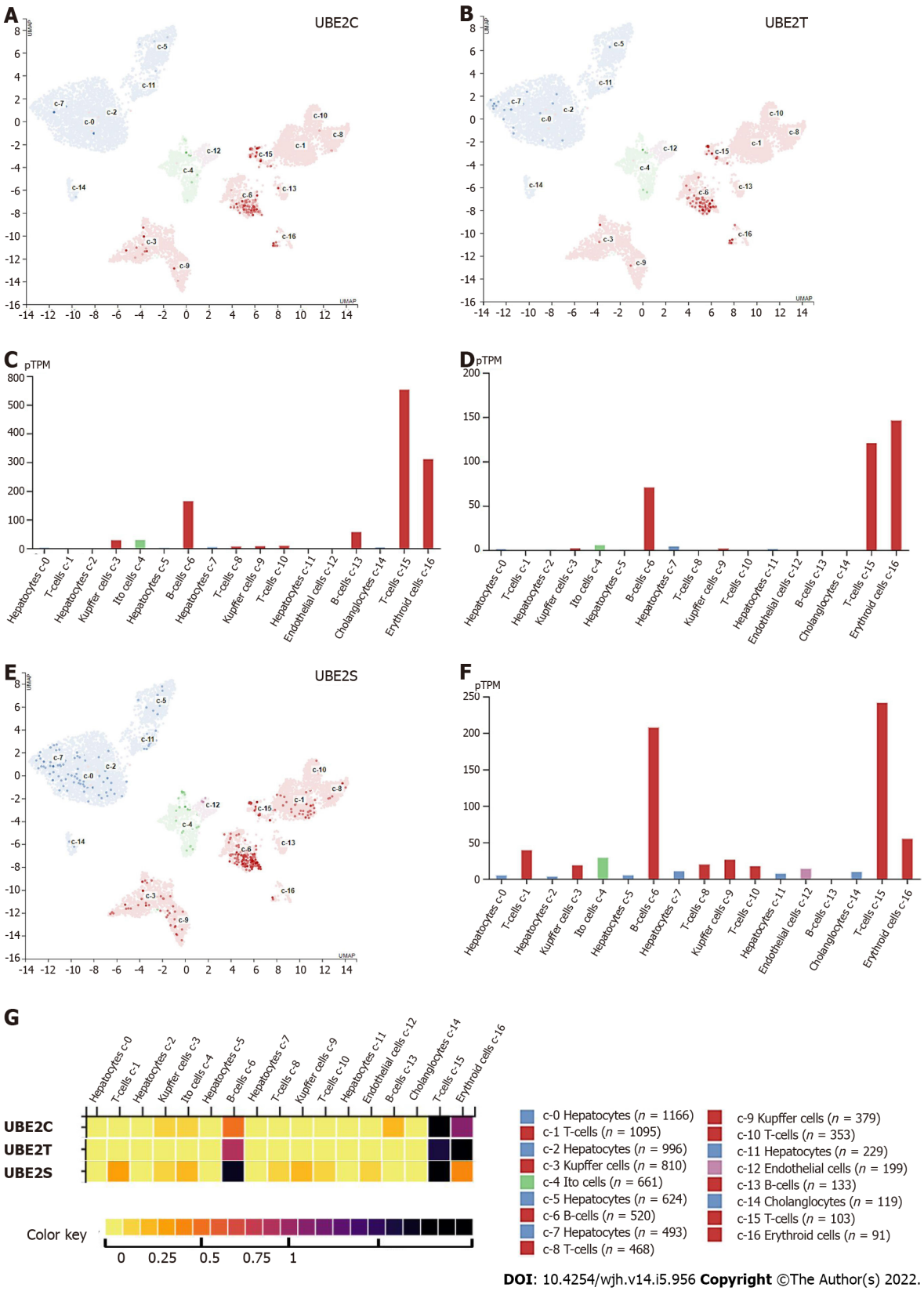

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Figure 5 UBE2C, UBE2T, and UBE2S mRNA expression in different cell types. A: UBE2C expressed genes in each cell cluster of scRNA-seq data; B: Bar plot of the transcript abundance of UBE2C in different cell types based on the scRNA-seq data. The level of UBE2C mRNA was represented by the mean pTPM (Protein-coding transcripts per million); C: UBE2T expressed genes in each cell cluster; D: Bar plot of the transcript abundance of UBE2T in different cell types based on the scRNA-seq data. The level of UBE2T mRNA was represented by the mean pTPM; E: UBE2S expressed genes in each cell cluster; F: Bar plot of the transcript abundance of UBE2S in different cell types based on the scRNA-seq data. The level of UBE2S mRNA was represented by the mean pTPM (Colored according to cell type group); G: Heat map of the expression level of UBE2C, UBE2T, and UBE2S. (Color key from 0-1 represent low–high expression).

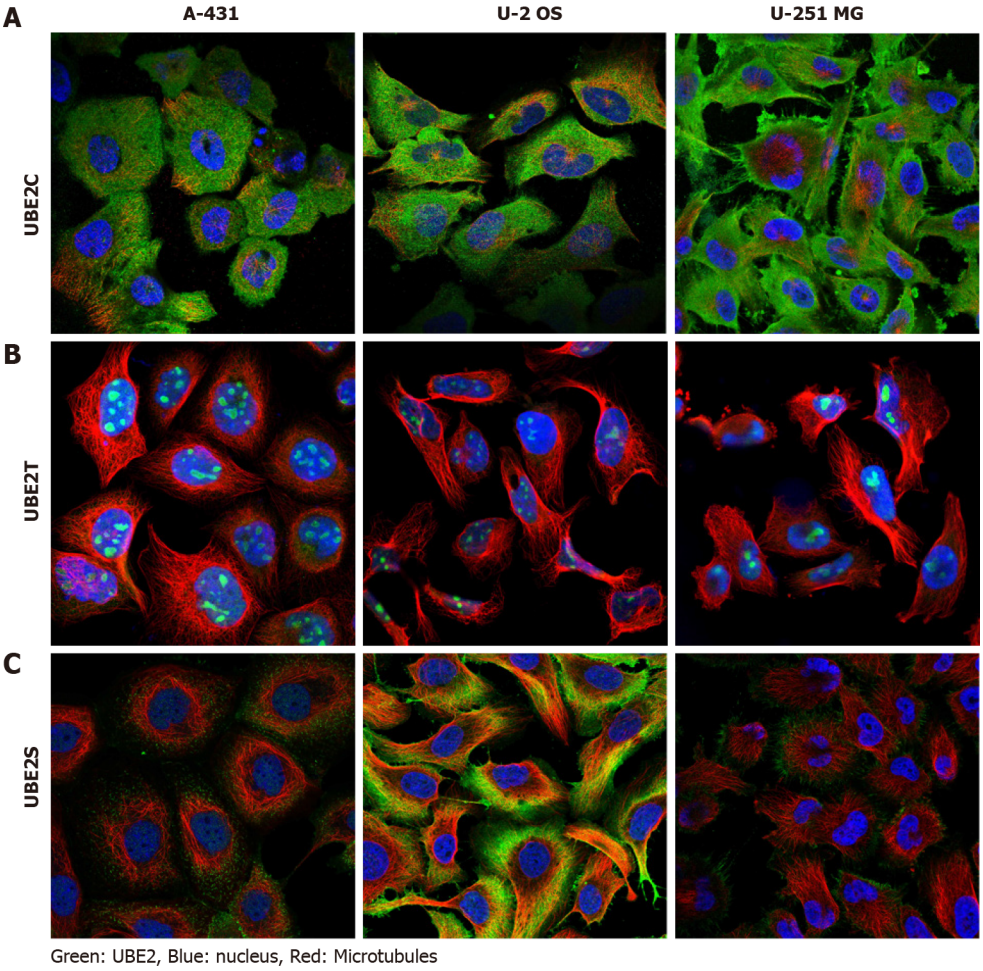

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Figure 6 UBE2C, UBE2T, and UBE2S protein expression in cell lines. A: UBE2C was mainly expressed in cytosol and plasma membrane; B: UBE2T was mainly expressed in nucleoli or nucleoplasm; C: UBE2S was highly expressed in cytosol and plasma membrane and less expressed in nucleoli.

overexpression of investigated genes and *TP53* mutation status, as well as their contribution to HCC progression. A further mechanistic study needs to be investigated in the field.

From the therapeutic and treatment standpoint, considering the significant roles of UBE2C[43,44], UBE2T[33,45], and UBE2S[46,47] in the ubiquitination process, which contributes to the cellular function and their close association with tumor cell's function, UBE2C, UBE2T, and UBE2S could be used as a diagnostic biomarker to assist the diagnosis and prediction of the progression of HCC as mentioned above. Most importantly, the causation of the higher level of UBE2 expression, as well as the contributing effect of those highly expressed *UBE2* genes on the disease outcome should be thoroughly investigated for further exploration of the effective therapeutic strategy discovery.

Further studies from following aspects, such as (1) Identification of causing factors of UBE2 overexpression; (2) investigation of the underlying mechanism on overexpression of *UBE2* genes causing disease severity and poor survival outcome of patients; (3) exploration of the associated therapeutic targets of UBE2; (4) the roles of co-expressed genes from the analysis of protein-protein network in HCC; and (5) the relationship of p53 mutation with UBE2 expression; will be studied in the future research to better understand the role of three *UBE2* genes in liver cancer.

CONCLUSION

This bioinformatics study sheds light on the important roles of UBE2C, UBE2T, UBE2S for HCC diagnostic and prognostic as potential biomarkers. In addition, it is appealing to further explore the correlation of those genes with TP53 mutation in HCC and the related mechanisms.

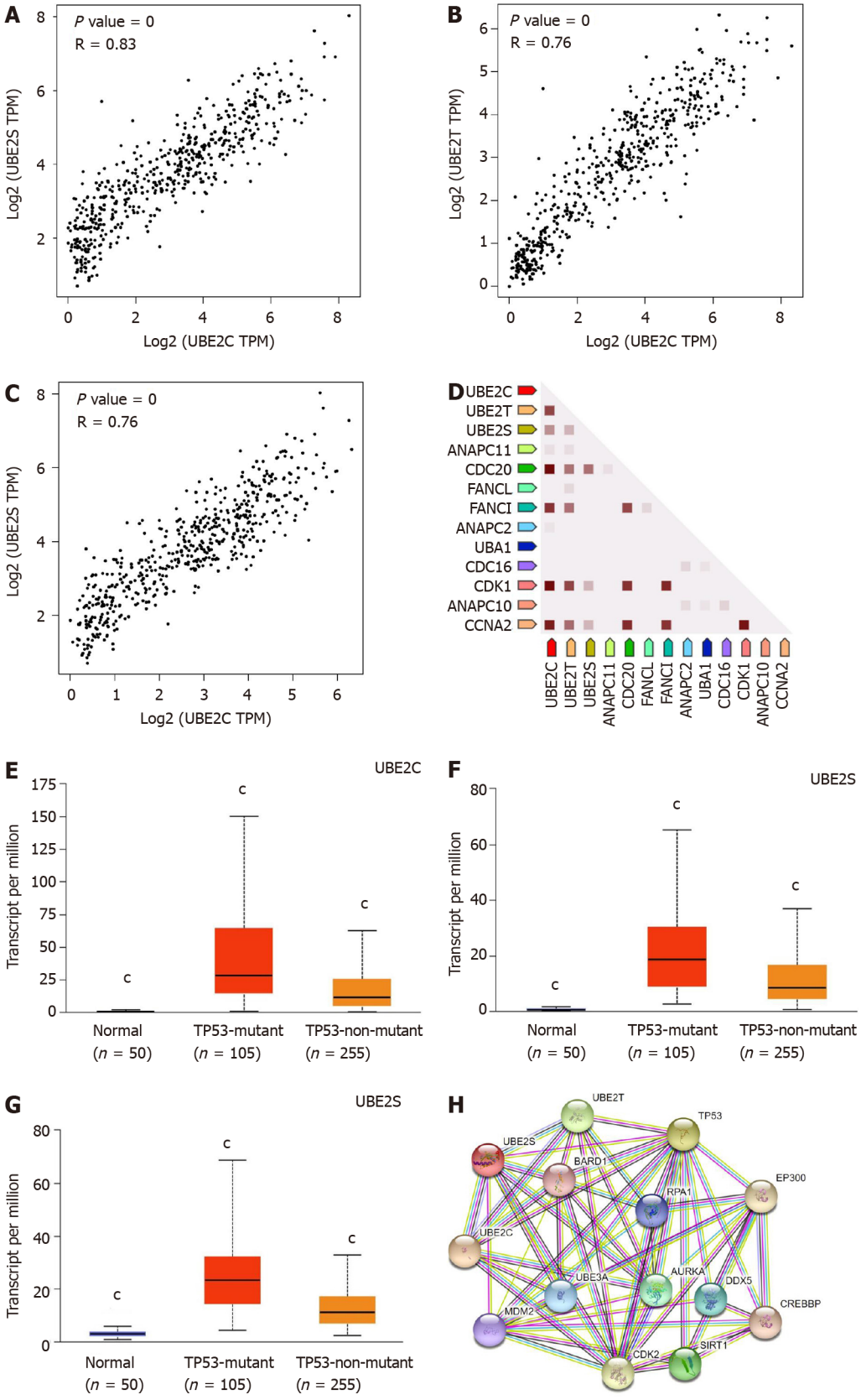

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Figure 7 UBE2C, UBE2T, and UBE2S expression in homo sapiens and protein-protein interaction network. A: Co-expression of UBE2C and UBE2S; B: Co-expression of UBE2C and UBE2T; C: Co-expression of UBE2T and UBE2S; D: Co-expression of UBE2C, UBE2T, and UBE2S predicts functional association. In the triangle matrices above, the intensity of the color indicates the level of confidence that two proteins are functionally associated in Homo sapiens. Figures E-G showed the expression of UBE2C, UBE2T, and UBE2S in HCC based on TP53 mutation status. TCGA samples for analysis; E: UBE2C expression in normal, HCC with TP53 mutation, and HCC non-TP53 mutation samples; F: UBE2T expression in normal, HCC with TP53 mutation, and HCC non-TP53 mutation samples; G: UBE2S expression in normal, HCC with TP53 mutation, and HCC non-TP53 mutation samples. (°*P* value < 0.001 Compared with other two groups); H:

Protein-protein interaction of functional enrichment of queried network using query input of UBE2C, UBE2T, UBE2S, and TP53.

ARTICLE HIGHLIGHTS

Research background

The expression of three ubiquitin-conjugating enzymes 2 (UBE2) including UBE2C, UBE2T, and UBE2S was significantly increased in HCC samples compared to non-tumor tissues.

Research motivation

To explore potential diagnostic and prognostic markers for HCC.

Research objectives

To identify the potential of UBE2C, UBE2T, and UBE2S as potential biomarkers as HCC.

Research methods

Online database was analyzed with different bioinformatic tools.

Research results

Our data showed that *UBE2C*, *UBE2T*, and *UBE2S* genes were overexpressed in hepatocellular carcinoma (HCC) samples compared to non-tumor tissues. Dependent on the cancer progression stage, three *UBE2* genes showed higher expression in tumor tissues at all four stages compared to non-tumor control samples. Furthermore, a significantly higher expression of these genes was found in stage 2 and stage 3 cancers compared to stage 1 cancer. Additionally, overexpression of those genes was negatively associated with prognostic outcome and overall survival time. Patients with TP53 mutation showed a higher expression level of three *UBE2* genes, indicating an association between UBE2 expression with p53 function.

Research conclusions

This bioinformatics study sheds light on the important roles of UBE2C, UBE2T, UBE2S for HCC diagnostic and prognostic as potential biomarkers. In addition, it is appealing to further explore the correlation of those genes with TP53 mutation in HCC and the related mechanisms.

Research perspectives

Further studies from following aspects, such as (1) Identification of causing factors of UBE2 overexpression; (2) investigation of the underlying mechanism on overexpression of *UBE2* genes causing disease severity and poor survival outcome of patients; (3) exploration of the associated therapeutic targets of UBE2; (4) the roles of co-expressed genes from the analysis of protein-protein network in HCC; and (5) the relationship of p53 mutation with UBE2 expression; will be studied in the future research to better understand the role of three *UBE2* genes in liver cancer.

FOOTNOTES

Author contributions: Zhang CY and Yang M conceived the idea for this study and collected and analyzed the data, wrote, finalized the manuscript letter, and contributed equally; All authors approved the submitted version and published version.

Institutional review board statement: This study was performed without animal and human studies.

Institutional animal care and use committee statement: This study was performed without animal and human studies.

Conflict-of-interest statement: Both authors declared that there was no conflict of interest with the content of this study.

Data sharing statement: All the data analyzed in this study originated from publicly available The Cancer Genome Atlas database (TCGA Research Network: https://www.cancer.gov/tcga).

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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ORIGINAL ARTICLE

Case Control Study

Innovations in education: A prospective study of storytelling narratives to enhance hepatitis C virus knowledge among substance users

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Abstract

BACKGROUND

Even though substance users have the highest hepatitis C virus (HCV) burden, many lack knowledge about the infection. Lack of knowledge is an important obstacle to pursuing HCV care. Although printed materials are conventionally utilized to disseminate HCV-related knowledge, narrative story-telling videos may be an alternative. Data are extremely limited, however, in the ability of storytelling videos to increase HCV knowledge among substance users. In this study, we hypothesized that a story-telling narrative video would increase substance user's immediate and 1-month HCV-related knowledge compared to a printed format.

AIM

To assess immediate and 1-month HCV-related knowledge retention among substance users comparing education delivered *via* a storytelling narrative video compared to a printed format.

METHODS

We conducted a prospective matched, case-control study among substance users actively prescribed buprenorphine enrolled from two sites. The intervention site received the video and the control site, the brochure. Participants (n = 176) were matched on age, gender, and race. We obtained extensive patient and stakeholder input on the video's design, validated the video's content, and developed a recruitment plan to guide participant enrollment. Knowledge was assessed by administration of a 25-item instrument immediately before, immediately after, or one month after the intervention. Data were analyzed using nonparametric and

generalized linear mixed-effects models.

RESULTS

We recruited a total of 176 substance users, 90 and 86 individuals, from each site, respectively. One-month follow up occurred in 92% and 94% of enrollees in the control and intervention groups, respectively. In comparison with the pre-intervention scores, immediate knowledge recall increased significantly for both the intervention ($P < 0.0001$) and control ($P < 0.0001$) groups. Multivariate modeling revealed a significant improvement in HCV-related knowledge and retention ($P = 0.033$) among participants who viewed the storytelling video.

CONCLUSION

Storytelling narratives emphasizing HCV education appear to be an effective method to increase HCV-related knowledge among substance users. They should become an educational cornerstone to promote HCV management among this population.

Key Words: Hepatitis C virus education; Hepatitis C Virus; Hepatitis C virus knowledge; Persons with opioid use disorder; Decision-making in healthcare

Core Tip: Despite high hepatitis C virus (HCV) burden, people with opioid use disorder (PWOUD) frequently lack knowledge about HCV. Printed brochures are the conventional method of HCV knowledge dissemination, although storytelling narrative videos have attributes that suggest they may be more effective. In this study, we assessed HCV knowledge improvement among PWOUD comparing a storytelling narrative video to a written brochure. Among 176 PWOUD, we found that immediate HCV-related knowledge recall was significantly increased by both methods. Multivariate modeling revealed a significant improvement in HCV-related knowledge and retention among intervention participants. In conclusion, storytelling narratives effectively improve HCV-related knowledge among PWOUD.

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INTRODUCTION

Hepatitis C virus (HCV) is an extremely common chronic blood-borne virus pathogen and is principally transmitted through injection drug use[1]. HCV is a leading cause of morbidity and mortality among persons with opioid use disorder (PWOUD) reaching as high as 80% with an annual incidence ranging from 16% to 42%[2,3]. Untreated HCV can progress to cirrhosis, end-stage liver disease and hepatocellular carcinoma. Recently, direct acting antivirals (DAAs) have revolutionized HCV treatment. DAAs are all oral medications, administered for 2 to 3 mo, with nearly universal efficacy and minimal side effects[4]. In fact, recent data have documented the importance of simultaneous treatment of opioid use disorder and HCV; treatment for both entities results in improved treatment adherence and a reduction in substance use[5,6]. Given the importance and burden of HCV, the US Preventative Services Task Force recently revised HCV screening recommendations[7]. Currently, all individuals aged 18 to 79 years should undergo a one-time HCV antibody test with more frequent assessments among high-risk individuals, such as PWOUD. In addition, HCV elimination by 2030 has been promulgated in the United States and globally[8,9]. To achieve HCV elimination objectives, however, we need to address HCV knowledge gaps among PWOUD. Only fifty percent of HCV-infected PWOUD know their infection status, and many underestimate the urgent need for HCV treatment[10,11]. Furthermore, substance users have recently been shown to have low to moderate health literacy levels[12-14], and health literacy is an extremely important predictor of health status[15]. Thus, interventions to increase HCV-related knowledge targeted to PWOUD are required to achieve HCV elimination objectives[16].

Lack of consideration of health literacy status often complicates patient-provider communication especially among low health literacy populations. In terms of written material, a typical method of health knowledge dissemination, readability at an appropriate grade level is an important consideration. One study found that internet-derived liver disease information is written at a higher readability score than is recommended, an important potential source of misinformation among liver disease patients[17]. In the area of HCV, several studies, which largely utilized in-person delivery of

didactic sessions, have illustrated that educational interventions can improve HCV knowledge[18-22], screening[23,24], and linkage-to-care[25,26]. These methods, however, can be burdensome in terms of cost, resources, and time requirements. Alternatively, computerized health platforms appear to facilitate health education dissemination ubiquitously and at a reduced cost compared to in-person lectures[27-30]. While a few studies have assessed their educational value, these platforms largely disseminate facts or schematics about HCV or liver diseases using an instructional or didactic framework. While informative, these knowledge dissemination methods may not resonate with substance users.

Narrative videos, defined as "first-person stories about experiences with illness and its personal consequences", have been shown to be more persuasive at behavioral change than written narratives or didactic lectures[31,32]. Although data are limited, narratives that address healthcare issues may be a particularly effective method to increase knowledge among PWOUD. A systematic review concluded that videos detailing story-based messages were more persuasive than non-narrative videos[32]. To improve HCV-related knowledge dissemination to substance users, we sought to develop and to validate an educational intervention targeted to PWOUD utilizing narratives presented in a video format. Virtual communication strategies, such as videos, are easily conductive to storytelling and can employ an expanded repertoire of communication styles in comparison with printed material. As storytelling is a particularly effective pedagogical method amongst PWOUD, our objective was to assess whether a video would improve PWOUD's immediate information recall and one-month knowledge retention compared with a written brochure.

MATERIALS AND METHODS

Study design

We conducted a prospective case-control study to assess immediate and longer-term knowledge retention comparing two different educational delivery methods. The control arm received an HCV educational brochure, and in the intervention arm, participants viewed a twelve-minute educational video, which contained the same information as was included in the brochure. Participants in each arm also completed the same knowledge assessments at the same time points: prior to reading the brochure or viewing the video, immediately following the interventions (*i.e.*, brochure or video), and within 30 +/- 14 d of the initial time point.

Participants were recruited according to a recruitment plan (see below) between January and October, 2020 from community health organizations that provide treatment services, specifically buprenorphine, for opioid use disorder. Each site is under the auspices of New York State Office of Addiction Services and Supports (OASAS) and has adopted a harm reduction approach, which refers to policies and programs designed to reduce drug-use associated harm; the goal is harm prevention from drug use rather than prevention of drug use itself[33,34].

Participant inclusion criteria included individuals at least 18 years old, who were currently prescribed buprenorphine, and who spoke English as their primary language.

Patient consent statement

The study was approved by the University at Buffalo and the Catholic Health Service Institutional Review Boards, and all patients provided informed consent prior to participating in any study related activities.

Video, brochure, and knowledge assessments

Brochure: As the American Liver Foundation is a principal source for patient-oriented printed materials and handouts on a variety of liver diseases, we utilized their instructional brochure for information about HCV (Supplementary material).

Video: Patients provided the initial concept of a video and enabled us to identify important HCV-related knowledge gaps in the areas of diagnosis, pathogenesis, transmission, screening, treatment, and long-term infection consequences. We subsequently reviewed and expanded the identified topics with opioid treatment program staff. Staff also described knowledge dissemination methods for PWOUD, such as emphasizing that visuals are extremely important and that human stories are "priceless", consistent with prior literature[35]. The narrative within the video contains a storyline with a beginning, middle and an end where the storyline conflict is brought to conclusion[36]. Based upon acquired feedback, we incorporated visualization strategies into the video, such as emphasizing main points in words or captions strategically situated within the image frame, to underscore the important pedagogical value.

We next reviewed the brochure to ensure that the same information would be presented in both educational formats and subsequently engaged a professional screenwriter to develop the video script. Multiple iterations of the script were reviewed to ensure the accuracy of the educational information. After filming and editing, a draft version of the video was reviewed and finalized by the study team and content validity was assessed as described below. The final video is available at: https://drive.

google.com/file/d/1mrJbXpRl7YrqbhoPH3sUphOTz2TNgc0e/view?usp=sharing.

Knowledge assessment design and assessment of content validity: Details related to the design of the knowledge assessment and the assessment of its content validity are described in the Supplementary material.

Recruitment plan development

We matched participants recruited from each site on age, gender, and race to control for potentially unmeasured confounding factors. We initially evaluated the demographics of all individuals from both sites. After considering the inclusion criteria and those with complete demographic information, the intervention site had 1647 potential participants and the control site had 526 potential participants. The two sites were randomly assigned to either the intervention or the control.

We next used gender, age and race to perform 1:1 exact matching of individuals from the two sites utilizing the "Match" function (R package, version 3.5.3)[37]. Given the paucity of nonwhite individuals, potential participants were categorized into two groups: white and other races. Age was also classified into two groups: age ≤ 39 or > 39 years. The algorithm identified 7928 exactly matched pairs in which 508 pairs were unique individuals from the control site. According to the distribution of matched individuals, each site was requested to recruit 90 participants according to the proportion in each category (See Supplementary material for sample size calculation and Supplementary Table 3 for the recruitment plan).

Recruitment

Site staff initially identified potentially eligible participants on the clinical schedule and then assessed potential participant's interest in study participation. If interest was affirmed, research staff next obtained informed consent. Afterwards, we verified participant demographics, and they completed the pre-test assessment. Participants then read the brochure or watched the video, and they promptly completed the knowledge assessment a second time (*i.e.*, post-test assessment). Thirty days afterwards, we requested that participants complete the third knowledge assessment (1 mo post-test assessment). We compensated participants $40 for completion of all assessments. After the COVID-19 pandemic, we recruited approximately 20% of participants *via* telephone.

Statistical analysis

We performed complete-case analysis since less than 5% of the data were missing. We initially calculated the participants' response scores obtained on each knowledge assessment; each correctly answered item garnered one credit. The improvement in knowledge attributable to the intervention was defined as the difference in scores between post- and pre- educational intervention assessments. We used the Wilcoxon signed-rank test to compare each participants' pre- and post-educational intervention scores[38]. We applied the Wilcoxon rank sum test to compare the improvements in participants' scores between post- and pre-educational intervention tests between the two sites. All significance tests were performed by using R version 3.5.3[37].

Our outcome variable is the number of correct answers provided by each participant. To incorporate the time effect into our analysis and since the outcome data are counts, we used a generalized linear mixed-effects model. The fixed effects include gender, race, educational intervention, timepoint (post-education and one-month follow-up) and clinic by visit interaction with all variables measured on a categorical scale, and we incorporated age in the model. Race was categorized into two levels (white *vs* other races), considering the small sample size of the nonwhite population. We modeled the pre-education test scores through model inclusion as the baseline (BL) value, $X_{BL,i}$, associated with the i^{th} study participant to adjust for differences in participants' educational levels and other potential unmeasured confounders that might have affected baseline scores. The random effects corresponded to unknown subject effects and was captured the within-subject variability. The outcome data were assumed to be Poisson distributed and the ln (log link with base e) was used in the generalized linear mixed-effects model (SAS Version 9.4, SAS Corporation, Cary, NC).

RESULTS

Demographic characteristics

We recruited 90 and 86 participants from the control and intervention sites, respectively, according to recruitment plan specifications. Participant demographics are illustrated in Table 1.

Participant questionnaire completion

We requested that each participant complete a knowledge assessment immediately preceding and upon conclusion of the educational intervention. They also completed the same assessment one-month after the intervention to investigate longer-term knowledge retention. The proposed and actual numbers of study participants who were recruited and their response rates, as assessed by completion of the pre-,

Table 1 Demographic characteristics of study participants

Intervention	Brochure	Video
n	90	86
Age		
Median (IQR)	41 (18)	40(18)
Mean (SD)	43 (11)	41(12)
Gender		
Female	55 (61%)	51 (59%)
Male	35 (39%)	35 (41%)
Race		
White	76 (84%)	74 (86%)
Other races	14 (16%)	12 (14%)

IQR: Interquartile ratio; SD: Standard deviation.

post-, and one-month follow up assessments, are illustrated (Supplementary Table 3).

In terms of response rates, all 90 participants in the control and 86 participants in the intervention arms completed both the pre- and post-educational intervention assessments. In terms of one-month follow-up response rates, 7 control and 5 intervention participants did not complete the assessment.

Descriptive and graphical analysis

Pre- and post-educational intervention results: For participants who observed the video, post-educational intervention scores improved significantly in comparison with pre-intervention scores (the value of the Wilcoxon signed-rank test is V = 2848.5, $P < 0.0001$). For control participants, significant differences between the post- and pre-intervention scores are also observed (V = 2816, $P < 0.0001$).

Improvement in educational intervention results: We also illustrate the improvements in the scores comparing the post- and pre-intervention assessments graphically (Figure 1). The significant improvement (W = 4817 and $P = 0.0047$) in test scores comparing each intervention is shown[39]. The improvement in test scores was greater among participants in the intervention arm as compared to the control arm.

One-month education retention results: The raw mean of participants' scores on pre- and post-educational intervention as well as on the one-month follow up assessments are illustrated (Figure 2). We observed substantial improvements from the pre-intervention to the post-intervention assessments in both clinics accompanied by a slight reduction in the scores obtained on the one-month follow-up assessments. The intervention site was observed to have a relatively higher improvement in the post-intervention assessment and improved knowledge retention on the one-month follow-up assessment.

Impact of COVID-19

Due to COVID-19-related restrictions, some of the intervention site participants were recruited *via* phone. Data indicate that pre-education scores are approximately similar comparing participants recruited in-person with those *via* phone (Table 2).

Multivariate modeling results

We utilized a generalized mixed-effects model to model the data. Table 3 shows the type III tests results for fixed effects, which utilizes the exact F tests. Modeling results revealed that the type of educational intervention, specifically the video, had a significant effect on the improvement in HCV-related knowledge and retention ($P = 0.0326$) as compared to the brochure (Table 3). The age, gender, race, and the interaction term of the intervention and time point are not significant. The overall time point effect is significant ($P = 0.0004$), and within each clinic, the least square mean differences between one-month follow up and post-education test scores are -0.0634 ($P = 0.0357$) and-0.0908 ($P = 0.0033$) for the video and brochure, respectively, which indicates a 0.0634 decrease in test score on the one-month follow-up compared to the post-intervention assessment for the video group and 0.0908 decrease in the brochure group. The baseline score, *i.e.*, the number of correct responses prior to any educational intervention, has a significant impact on the post-intervention and one-month follow-up test scores with $P < 0.0001$. We refer to this variable as the baseline score; its inclusion in the model adjusts for any pre-existing knowledge differences that might exist between clinics.

Table 2 Descriptive statistics associated with pre-education scores of individuals recruited on site and those recruited *via* phone

Pre-education score	Recruitment method		Intervention site total (*n* = 86)
	On-site (*n* = 50)	Via phone (*n* = 36)	
Mean (SD)	19.3 (2.27)	18.6 (2.51)	19.0 (2.38)
Median (Min, Max)	19.0 ([15, 24])	18.5 ([13, 24])	19.0 ([13, 24])

SD: Standard deviation.

Table 3 Results of type III tests of fixed effects

Effect	F value	*P* value
Age	0.92	0.3378
Gender	0	0.9710
Race[1]	2.62	0.1072
Educational intervention[2]	4.65	0.0326
Time point[3]	13.01	0.0004
Educational intervention × time point	0.41	0.5219
Baseline score (Pre-education score)	36.22	< 0.0001

[1]Race is categorized into to two levels: white and other races.
[2]Educational intervention indicates either the intervention or the control group.
[3]Time point indicates the post-education or one-month follow up assessment.

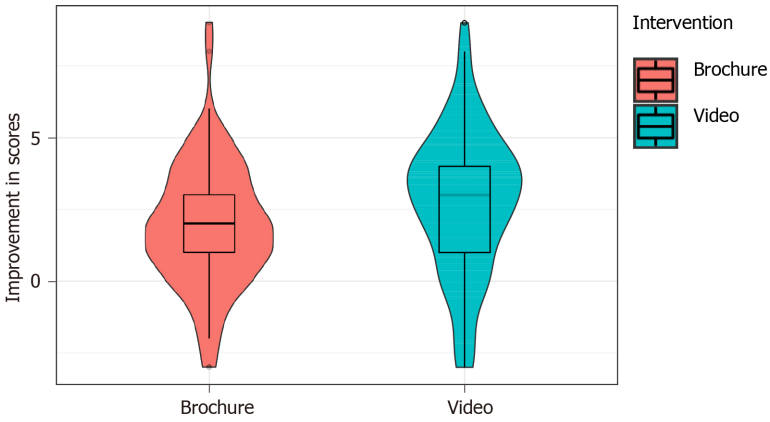

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Figure 1 The distribution of improvement in scores calculated as the pre-intervention subtracted from the post-intervention for both interventions are illustrated in the violin plot wrapping a boxplot. The box extends from the 25th to the 75th percentile with lines extending outward depicting the smallest value within 1.5 times the interquartile range (IQR) below the 25th percentile and largest value within 1.5 times the IQR above the 75th percentile. The dark line in the middle of the box illustrates the median values. Peach indicates the control site (brochure) and blue (video) the intervention site. The violin displays the density plot of the values, where the width indicates the frequency.

DISCUSSION

In this study, we developed and validated an HCV educational narrative video targeted to PWOUD. Each narrative emphasizes HCV-related knowledge, and we utilized technological enhancements to underscore important points. Utilizing a case-control design, we subsequently enrolled PWOUD on buprenorphine from two separate sites, one received the intervention and the other was a control site. Although both the video and the printed formats increased immediate recall and retention of HCV-related knowledge, gains were significantly greater amongst the participants who viewed the video.

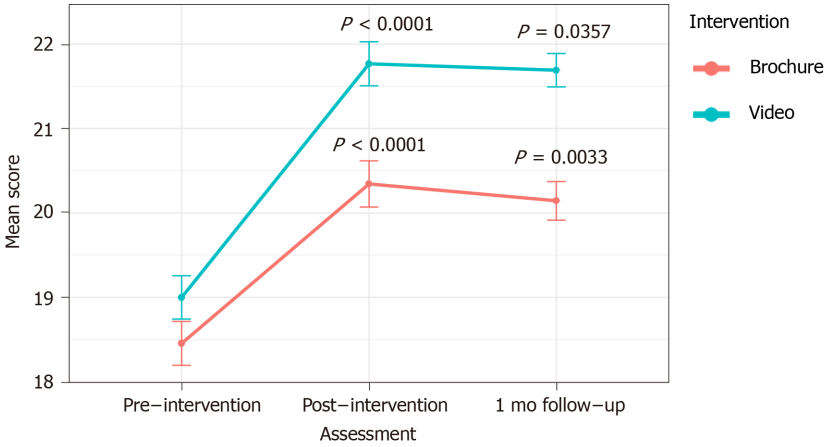

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Figure 2 Illustrated are mean scores for the assessments obtained pre- and post- as well as after one month after the video (blue) and brochure (red/peach) educational interventions.

Substance users have a method for information spread amongst themselves, referred to as the "peer pipeline", that compromised our ability to randomize at the patient level[40,41]. To minimize bias between the two sites, we matched participants on demographic factors including age, gender, and race. Several attributes between the sites were similar, including staffing ratios, treatment philosophy, and therapeutic approaches, since both sites are under the direction of the same state agency (OASAS), which regulates and funds medications for opioid use disorder (MOUD) dispensed in New York State.

Education about HCV and the benefits of DAAs is essential to promote PWOUD pursuit of HCV treatment and is required to achieve HCV elimination objectives[8,9]. Low to moderate health literacy in PWOUD and misinformation concerning basic facets of liver disease among many liver disease patients are important considerations in the design of educational interventions[12-14,42]. While several prior studies have shown knowledge increases after HCV education, these studies enrolled different populations; some recruited hospital-based clinic patients while others recruited PWOUD[2,20,22,25,28]. In a study conducted in PWOUD, no differences were identified in viral hepatitis-related knowledge between those undergoing a motivational interviewing style compared with standard counseling[20]. Another study that reported low health literacy among PWOUD illustrated that personalized educational communication techniques result in increased knowledge[13].

Addressing HCV-related knowledge gaps requires innovative and targeted educational approaches. Storytelling narrative videos are a potential alternative to conventional in- person instructional lectures, although the latter has been evaluated in most HCV-related published work to date[27-30,43]. One study used an I-pad platform to deliver educational instruction about HCV[30]. Another study developed a didactic video about liver disease, and a third developed a short didactic video message designed to persuade PWOUD to pursue HCV surveillance[27,28]. Additionally, Hochstatter *et al*. identified no differences in HCV testing uptake between intervention and control participants who utilized HCV-related educational content and functionality added to an m-health substance use disorder relapse prevention platform[29,43]. mHealth systems developed to date designed to educate PWOUD about HCV appear to largely utilize a didactic approach complemented by linkage to community and government-based resources as opposed to narrative stories.

Multiple, as opposed to single, interventions have shown promise to improve health information comprehension by low literacy populations[44,45]. Multimodal presentations, combined with storytelling narratives, allow for participant transportation into the narrative, defined as "the audience's immersion in the imagined world"[36,46]. The degree of the viewer's emotional engagement with the narrative and the identification with the protagonist are important determinants of narrative's persuasiveness for behavioral modification[47,48]. Storytelling is used extensively to promote persuasiveness in healthcare[31,49,50], and video narratives have been shown to be more persuasive at health promotion than written narratives or instructional or didactic videos[32,51]. Future investigation should evaluate whether different educational modalities will lead to improvements in PWOUD pursuit of HCV management.

Increasing PWOUD pursuit of HCV diagnosis and treatment can accrue significant benefits. Besides improvements in patient-reported outcomes, such as fatigue[52], an HCV cure decreases the risk of progression to cirrhosis and hepatocellular carcinoma[53]. Furthermore, as alcohol intake has been shown to be an independent predictor of hepatic cirrhosis and mortality in HCV-infected individuals [54], alcohol cessation education is required for all HCV-infected individuals.

Extensive stakeholder input on the intervention design, the comparison group, the construct validation, and study design are investigative strengths. Additionally, we minimized potential bias by

conducting the study in geographically separated sites and through matching participants on available demographics. We were limited by the inability to conduct a randomized trial given the research infrastructure present, the available resources, and the existence of the peer pipeline. We were, unfortunately, unable to collect information on participant's education levels or on their substance use treatment course. To address these limitations, we included the baseline questionnaire responses as a dependent variable in the multivariate modeling. The inclusion of the pre-education test score controls for differences, for example, in education levels between the two sites.

HCV-related education is critical to promote PWOUD screening and linkage-to-care. PWOUD have the highest HCV prevalence and incidence, and highly-effective DAAs decrease HCV-associated morbidity and mortality[4]. HCV treatment has also been shown to diminish or stabilize substance use, to improve retention in OUD treatment and to increase medication adherence[6,55]. MOUD also reduces risk of HCV reinfection[56]. To maximize the effectiveness of HCV educational interventions targeted to PWOUD, future investigation should evaluate knowledge dissemination and engagement among PWOUD comparing educational formats (*i.e.*, storytelling narratives vs. instructional/didactic formats) that incorporate multimodal technologies.

CONCLUSION

Education improves HCV-related knowledge among PWOUD. Storytelling narratives significantly improve knowledge retention compared to written instructional brochures. They should become a cornerstone to promote HCV knowledge among PWOUD.

ARTICLE HIGHLIGHTS

Research background

Despite high hepatitis C virus (HCV) incidence and prevalence, many substance users lack basic knowledge about HCV infection and its consequences.

Research motivation

HCV education targeted to persons with opioid use disorder (PWOUD) has largely utilized conventional written approaches through brochures and pamphlets. Innovative approaches, such as storytelling narrative videos, may be more appealing resulting in increased knowledge ascertainment and retention. Data are limited though on the ability of narrative videos to increase knowledge among substance users.

Research objectives

To assess immediate and 1 mo HCV knowledge recall and retention among substance users comparing education delivered *via* a storytelling narrative video to a written brochure.

Research methods

PWOUD on medication for opioid use disorder were prospectively enrolled from two sites, one site reviewed the written brochure and the other site viewed the video. Participants (n = 176), matched on age, race, and gender, completed the same knowledge assessments immediately prior to and upon completion of reading the brochure or watching the video. The same assessment was completed one month later to evaluate longer-term knowledge retention. We utilized generalized linear mixed-effects models to analyze the data.

Research results

We observed that both interventions significantly increased immediate and longer-term HCV-related knowledge. Multivariate modeling revealed significant improvements in HCV-related knowledge and retention (P = 0.033) among participants who viewed the storytelling narrative video.

Research conclusions

Storytelling narrative videos appear to be an effective strategy to increase HCV-related knowledge among PWOUD. Whether videos are an effective method to change behavior requires further investigation.

Research perspectives

Given their ability to include multimodal techniques and their ease of distribution, storytelling narrative videos may do well as an educational cornerstone to disseminate HCV-related knowledge to vulnerable populations, such as PWOUD, as well as to the general public.

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FOOTNOTES

Author contributions: Talal AH conceived of the study, obtained funding, supervised data collection, wrote and revised the manuscript; Markatou M obtained funding, designed the study and supervised the analysis, wrote and revised the manuscript; Ding Y designed the study and performed the analysis, wrote and revised the manuscript; all authors have read and approve the final manuscript.

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ORIGINAL ARTICLE

Retrospective Study

Impact of utilization of hepatitis C positive organs in liver transplant: Analysis of united network for organ sharing database

Amaninder Dhaliwal, Banreet Dhindsa, Daryl Ramai, Harlan Sayles, Saurabh Chandan, Rajani Rangray

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Abstract

BACKGROUND

The utility of hepatitis C virus (HCV) organs has increased after the Food and Drug Administration approval of direct acting anti-viral (DAA) medications for the HCV treatment. The efficacy of DAA in treating HCV is nearly 100%.

AIM

To analyze the United Network for Organ Sharing (UNOS) database to compare the survival rates between the hepatitis C positive donors and negative recipients and hepatitis C negative donors and recipients.

METHODS

We analyzed the adult patients in UNOS database who underwent deceased donor liver transplant from January 2014 to December 2017. The primary endpoint was to compare the survival rates among the four groups with different hepatitis C donor and recipient status: (Group 1) Both donor and recipient negative for HCV (Group 2) Negative donor and positive recipient for HCV (Group 3) Positive donor and negative recipient for HCV (Group 4) Both positive donor and recipient for HCV. SAS 9.4 software was used for the data analysis.

Kaplan Meier log rank test was used to analyze the estimated survival rates among the four groups.

RESULTS

A total of 24512 patients were included: Group 1: 16436, Group 2: 6174, Group 3: 253 and Group 4: 1649. The 1-year (Group 1: 91.8%, Group 2: 92.12%, Group 3: 87%, Group 4: 92.8%), 2-year (Group 1: 88.4%, Group 2: 88.1%, Group 3: 84.3%, Group 4: 87.5%), 3-year (Group 1: 84.9%, Group 2: 84.3%, Group 3: 75.9%, Group 4: 83.2%) survival rates showed no statistical significance among the four groups. Kaplan Meier log rank test did not show any statistical significance difference in the estimated survival rates between Group 3 *vs* all the other groups.

CONCLUSION

The survival rates in hepatitis C positive donors and negative recipients are similar as compared to both hepatitis C negative donors and recipients. This could be due to the use of DAA therapy with cure rates of nearly 100%. This study supports the use of hepatitis C positive organs in the selected group of recipients with and without HCV infection. Further long-term studies are needed to further validate these findings.

Key Words: Hepatitis C; Liver transplant; Survival; United Network for Organ Sharing; Direct acting anti-viral

Core Tip: Due to the limited availability of donor organs and high mortality rate on the transplant waiting list, newer strategies are needed. Use of direct acting anti-viral agents have led to high success rates for hepatitis C virus (HCV) treatment. Our study shows, the survival rates in hepatitis C positive donors and negative recipients are similar as compared to both hepatitis C negative donors and recipients. This study supports the use of hepatitis C positive organs in the selected group of recipients with and without HCV infection.

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INTRODUCTION

In the United States, there has been increase in the number of cases in need for liver transplantations (LT) in the last decade while the availability of organs is unchanged[1]. The major contributing factor to this is limited availability of donor liver. Due to persistence of viable organ shortage in United States, it is of utmost importance that all transplantable organs are utilized to their maximum potential[2]. With the advent of direct acting anti-viral (DAA) therapy, the rate of cure of hepatitis C virus (HCV) has increased dramatically. This has been reflected by nonalcoholic fatty liver disease and alcoholic liver disease overtaking HCV as the leading cause for LT in the United States[1,2]. The rates of HCV-positive waitlisted patients and HCV-positive LT recipients have decreased by 8.2% and 7.6% respectively between 2006 and 2016[1].

Prior to approval of DAA therapy, recurrence of HCV after LT was the most common cause of graft failure and reduced recipient survival in those for were HCV positive as compared to HCV negative patients[3,4]. This recurrence of HCV greatly influenced the allocation of HCV positive donors leading to severe under-utilization of these organs, especially in HCV negative recipients[5-8]. Development of newer generation DAAs have resulted in interferon free regimens with high sustained virologic response (SVR) rates post LT[9-12].

These newer generation DAAs have high potency and low adverse event rates leading to increase in inclination for utilizing HCV positive donor organs, including those with high viral load. However, concerns exist regarding these medications as 5% of the patients have failure to achieve SVR after undergoing DAA therapy. This puts the patients at risk for developing chronic HCV or cholestatic hepatitis with increased possibility of need of re-transplantation.

The aim of this study is perform a comparative analysis on odds of survival between the HCV positive donors and negative recipients as compared to HCV negative donors and recipients, using United Network for Organ Sharing (UNOS) database.

MATERIALS AND METHODS

We obtained data from the UNOS registry which contains data on all transplantations in the United States. Analysis was limited to records from April 1, 2014 or later, where both recipient and donor were at least 18 years or age, and HCV status was recorded for both recipient and donor. Some recipients appeared in the data set multiple times, but analysis for this study was limited to the first transplant recorded for each patient using a data element which recorded the number of previous transplants for each recipient. The remaining observations were classified into four groups based upon the HCV status of both donors and recipients: (Group 1) both donor and recipient negative, (Group 2) negative donor and positive recipient, (Group 3) positive donor and negative recipient, and (Group 4) both donor and recipient positive.

Descriptive statistics for each of the four groups, including demographic and clinical characteristics for both recipients and donors and causes of death were calculated using means and standard deviations for continuous measures and counts and percentages for categorical measures. The primary outcome was overall survival time with death indicated using the composite death indicator and censoring for those who did not die during the study period occurring at the date of last patient follow-up with the latest patient follow-up taking place on September 7, 2018. Note that all surviving patients were not censored at this date, this is just the latest date a patient was observed. Comparisons of overall survival between groups were made using log-rank tests and estimates of group survival at various time points following transplant. All analysis was completed using SAS v9.4 (The SAS Institute, Cary, NC).

RESULTS

We included a total of 24512 transplants in our analysis. Group 1 and 2 formed the majority of the transplants with 67.05% and 25.19% respectively. Group 3 consisted of 1.03% of transplants with 253 patients undergoing positive donor and negative recipient transplants while the remaining 6.73% were in Group 4. The percentages of males in the four groups were 62.87, 75.92, 66.80 and 76.53, respectively. The mean age (years) of recipients was comparable: Group 1 (54.66 ± 11.63), Group 2 (59.08 ± 6.82), Group 3 (56.13 ± 10.9) and Group 4 (58.92 ± 6.76). The mean age of donors was comparable as well: Group 1 (42.72 ± 15.60), Group 2 (42.96 ± 15.09), Group 3 (40.00 ± 12.41) and Group 4 (37.71 ± 11.82).

Cold ischemia times (hours) were similar in all the groups Group 1 (6.07 ± 2.23), Group 2 (6.20 ± 2.46), Group 3 (6.45 ± 2.03) and Group 4 (6.15 ± 2.51). The mean body mass index of the recipients were evenly matched as well: Group 1 (29.1 ± 6.12), Group 2 (28.67 ± 5.38), Group 3 (28.92 ± 6.08) and Group 4 (28.92 ± 6.08). The mean model for end-stage liver disease (MELD) scores were also similar in all groups: Group 1 (25.22 ± 10.86), Group 2 (19.41 ± 11.26), Group 3 (21.74 ± 8.39) and Group 4 (18.24 ± 7.95).

Anoxia was the most common cause of death (37.89%) followed by stroke (31.31%) and head trauma (28.4%). Majority of the transplants in all groups were whole LT: Group 1 (99.05%), Group 2 (99.14%), Group 3 (99.60%) and Group 4 (99.94%). These demographics can be seen in Tables 1 and 2. The most common primary diagnosis at listing was alcoholic cirrhosis/acute alcoholic hepatitis (26.26%) followed by HCV cirrhosis (20.92%), non-alcoholic steatohepatitis cirrhosis (15.36%) and hepatoma (12.28%). These can be seen in Table 3.

A log-rank test for survival differences between the four groups did not show any significance (P = 0.46). Observation of survival rates at 1-year (Group 1: 91.81%, Group 2: 92.13%, Group 3: 87.01%, Group 4: 92.89%), 2-year (Group 1: 88.4%, Group 2: 88.1%, Group 3: 84.3%, Group 4: 87.5%), 3-year (Group 1: 84.9%, Group 2: 84.3%, Group 3: 75.9%, Group 4: 83.2%) found that survival rates for Group 3 were lower at each point than the other three groups, which were all relatively close together. However, a second long-rank test comparing Group 3 *vs* all the other groups was also not significant (P = 0.11, Figure 1).

DISCUSSION

Despite the increasing number of LT over the years, the need for organ donors continues to outpace the availability of organs with estimated waitlist mortality of 20%[13].

In the United States, since 2000, the increased mortality related to the catastrophic opioid epidemic presented an opportunity to recruit more organ donors. However, the enthusiasm was curtailed by a relatively higher prevalence of HCV when compared to the general population[14]. Initially, the organs

Table 1 Demographics of different transplanted groups

	Mean age-donor (yr)	Mean age-recipient (yr)	Males (%)	BMI-recipient	Cold ischemia time	MELD
Group 1	42.72 ± 15.60	54.66 ± 11.63	62.87	29.1 ± 6.12	6.07 ± 2.23	25.22 ± 10.86
Group 2	42.96 ± 15.09	59.08 ± 6.82	75.92	28.67 ± 5.38	6.20 ± 2.46	19.41 ± 11.26
Group 3	40.00 ± 12.41	56.13 ± 10.9	66.8	28.92 ± 6.08	6.45 ± 2.03	21.74 ± 8.39
Group 4	37.71 ± 11.82	58.92 ± 6.76	76.53	28.92 ± 6.08	6.15 ± 2.51	18.24 ± 7.95

Group 1: Both donor and recipient negative for hepatitis C virus (HCV); Group 2: Negative donor and positive recipient for HCV; Group 3: Positive donor and negative recipient for HCV; and Group 4: Both positive donor and recipient for HCV. BMI: Body mass index; MELD: Model for end-stage liver disease.

Table 2 Cause of donor death and type of liver transplant in different groups

	Donor cause of death					Type of liver procedure		
	Anoxia	Stroke	Head trauma	CNS tumor	Other	Whole	Partial	Split
Group 1	5832	5360	4844	64	336	16280	5	151
Group 2	2196	2007	1826	25	120	6121	2	51
Group 3	182	33	33	0	5	252	0	1
Group 4	1079	276	259	0	35	1648	1	0

Group 1: Both donor and recipient negative for hepatitis C virus (HCV); Group 2: Negative donor and positive recipient for HCV; Group 3: Positive donor and negative recipient for HCV; and Group 4: Both positive donor and recipient for HCV. CNS: Central nervous system; HCV: Hepatitis C virus.

Table 3 Diagnosis of transplant recipients at the time of listing

Primary diagnosis at listing	No. of patients	Percent
Alcoholic cirrhosis/acute alcoholic hepatitis	6434	26.25
Hepatitis C cirrhosis	5128	20.92
Non-alcoholic steatohepatitis cirrhosis	3764	15.36
Hepatoma	3010	12.28
Cryptogenic (idiopathic) cirrhosis	1082	4.41
Primary sclerosing cholangitis	941	3.84
Acute hepatic necrosis	696	2.84
Autoimmune cirrhosis	650	2.65
Primary biliary cirrhosis	582	2.37

from HCV+ donors were primarily reserved for HCV+ recipients or those suffering from fulminant hepatic failure[15]. But there is still a reticence to use organs from HCV+ donors in HCV- recipients because of clinical and ethical considerations.

One of the biggest barriers to use HCV+ donor organs is the concern for increased risk of post-transplant HCV transmission. Another concern with the use of HCV+ donor liver grafts is because of the limited literature on post-transplantation outcomes. Lai *et al*[16] studying 99 recipients of HCV+ donor liver grafts demonstrated significantly higher unadjusted 1-year and 3-year rates of advanced fibrosis for recipients of HCV+ donor grafts (14% and 48%) *vs* HCV- donor grafts (7% and 33%, $P = 0.01$) [16]. Khapra *et al*[17] studying 29 recipients of HCV+ donor liver grafts showed significantly more fibrosis and a faster rate of progression compared with recipients of HCV- donor liver grafts[17]. Interestingly, when the analysis was stratified to mean donor age, both the studies showed poorer outcomes with older donors of age greater than 45 years and 50 years respectively[16,17]. But it must be noted that these studies were done in an era when antiviral therapy was initiated at later stages of fibrosis and there was low response to these regimens. At this time, more studies with longer follow up are needed to see if similar rates of fibrosis progression occur with early administration of antiviral

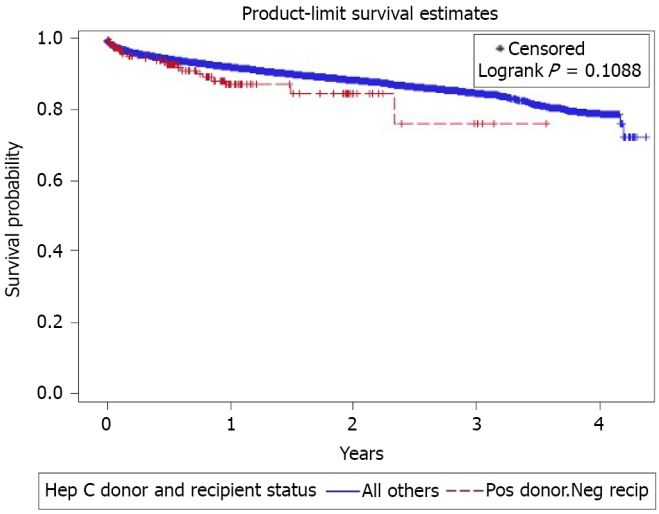

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Figure 1 Kaplan Meier curve showing survival analysis between group 3 and other groups.

therapy post-transplantation.

In terms of long-term graft and patient survival with HCV+ organs, the current literature is controversial depending on the type and success rate of HCV treatment regimens. With the advent of DAAs, recent studies from single-center experiences and large population-based databases such as UNOS and scientific registry of transplant recipients studying recipients with HCV+ and HCV- liver donors showed similar outcomes between the groups which are encouraging[18-20]. But it must be highlighted as there is no standardized protocol currently, these studies included recipients initiated on preemptive or prophylactic or both the treatment regimens.

With increasing wait-list mortality, higher mortality in patients with higher MELD (> 35 scores), and newer DAAs medications reaching clinical efficacy of nearly 96% it may be worth considering offering HCV+ donor organs to selected HCV- recipients where the risk-benefit outweighs the ethical considerations[21].

The data included in our study is from a large population-based study from an accepted UNOS database which includes a heterogeneous population from across the United States. Our study demonstrates that there is no statistically significant difference in the survival rates in HCV positive donors and negative recipients and HCV negative donors and recipients. In contrast to prior studies, which looked at the short-term outcomes such as survival rate at discharge, rejection rate prior to discharge, and 1-year mortality: Ours is the first study that shows that the survival rates were comparable in all group up to 3 years. Furthermore, the mean MELD score of the recipients in the group 3 was 21, suggesting that HCV+ donors can be used in recipients with high MELD score. Moreover, HCV positive organs were utilized in complicated cases with prolonged ischemia times and blood loss, showing tolerance to ischemia. This study supports the feasibility of expanding the general donor pool *via* the utilization of HCV positive organs for both HCV positive and negative recipients.

Due to the limitations of the data, we could not evaluate the reduction in time from listing to transplant, we can conclude that addition of HCV positive donor should decrease the organ shortage and reduce morbidity and mortality of all patients on the transplant list.

Although the outcomes of using hepatitis C positive donors are encouraging, there are some limitations of our study. The number of patients in group 3 were less, 253 out of total 24512 patient (1.03%). Even though we had estimates of survival rates up to 3 years (and longer for a small set of patients), long-term outcomes are still unknown. Additionally, it is still not clear whether preemptive or prophylactic HCV regimens are needed, and the length of HCV surveillance needed post-LT. Though the mean age of the donors were comparable in all groups, the medical co-morbidities of donors were unknown. In contrast to the study by Ballarin *et al*[22] this study was limited by the paucity of data on viral load and graft histology of both donors and recipients which may influence post LT outcomes. We also did not have information regarding the treatment status of HCV positive donors.

CONCLUSION

In summary, due to the mismatch in need and availability of donor organs and high mortality rate on the transplant waiting list, newer strategies are needed. DAA regimens have proved to be highly effective to treat HCV, even post-transplant and immunosuppressed patients. Effectiveness of these

regimens can be exemplified by similar survival rates of HCV positive donor and negative recipients in comparison to HCV negative donors and recipients. Our study shows that HCV positive organs can be transplanted to recipients, irrespective of their HCV status. However, more studies are needed to confirm our findings.

ARTICLE HIGHLIGHTS

Research background

Due to the mismatch in need and availability of donor organs and high mortality rate on the transplant waiting list, newer strategies are needed. In the era prior to direct acting anti-virals (DAAs), recurrence of hepatitis C virus (HCV) in post liver transplantations (LT) patients leading to decreased graft survival had greatly influenced the under-utilization of these organs, especially in HCV negative recipients. With the high sustained virologic response rate with DAAs both pre and post LT, this pool of organs can be utilized leading to more organ availability and decreased in mortality rate on transplant.

Research motivation

There is limited data evaluating outcomes of hepatitis C positive donor organs to HCV negative recipients in LT.

Research objectives

The aim of this study is perform a comparative analysis on odds of survival between the HCV positive donors and negative recipients as compared to HCV negative donors and recipients United Network for Organ Sharing (UNOS) database.

Research methods

We included patients in UNOS database who underwent deceased donor LT over a period of three year. Data analysis was performed using SAS 9.4 software. Survival rates amongst groups were analyzed with help of Kaplan Meier log rank test.

Research results

Our study shows similar chance of survival of recipient, irrespective of HCV status of the donor.

Research conclusions

Our study shows that HCV positive organs can be transplanted to recipients, irrespective of their HCV status due to the advent of DAA regimen.

Research perspectives

More studies are needed to confirm findings of this study.

FOOTNOTES

Author contributions: Dhaliwal A analysis and interpretation of data, drafting the article; Dhindsa B drafting the article; Ramai D acquisition of data; Sayles H analysis and interpretation of data; Chandan S critical revision; Rangray R critical revision, final approval.

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ORIGINAL ARTICLE

Retrospective Study

Angle of covered self-expandable metallic stents after placement is a risk factor for recurrent biliary obstruction

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Abstract

BACKGROUND

Studies have shown that covered self-expandable metallic stents (CSEMS) with a low axial forces after placement can cause early recurrent biliary obstruction (RBO) due to precipitating sludge formation.

AIM

To ascertain whether the angle of CSEMS after placement is a risk factor for RBO in unresectable distal malignant biliary obstruction (MBO).

METHODS

Between January 2010 and March 2019, 261 consecutive patients underwent self-expandable metallic stent insertion by endoscopic retrograde cholangiopancreatography at our facility, and 87 patients were included in this study. We evaluated the risk factors for RBO, including the angle of CSEMS after placement as the primary outcome. We measured the obtuse angle of CSEMS after placement on an abdominal radiograph using the SYNAPSE PACS system. We also evaluated technical and functional success, adverse events, time to RBO (TRBO), non-RBO rate, survival time, cause of RBO, and reintervention procedure as secondary outcomes.

RESULTS

We divided the patients into two cohorts based on the presence or absence of RBO. The angle of CSEMS after placement (*per* 1° and *per* 10°) was evaluated using the multivariate Cox proportional hazard analysis, which was an

independent risk factor for RBO in unresectable distal MBO [hazard ratio, 0.97 and 0.71; 95% confidence interval (CI): 0.94-0.99 and 0.54-0.92; P = 0.01 and 0.01, respectively]. For early diagnosis of RBO, the cut-off value of the angle of CSEMS after placement using the receiver operating characteristic curve was 130° [sensitivity, 50.0%; specificity 85.5%; area under the curve 0.70 (95%CI: 0.57-0.84)]. TRBO in the < 130° angle group was significantly shorter than that in the ≥ 130° angle group (P < 0.01).

CONCLUSION

This study suggests that the angle of the CSEMS after placement for unresectable distal MBO is a risk factor for RBO. These novel results provide pertinent information for future stent management.

Key Words: Covered self-expandable metallic stents; Recurrent biliary obstruction; Malignant biliary obstruction; Endoscopic retrograde cholangiopancreatography; Angle; Axial force

Core Tip: We aimed to assess whether the angle of covered self-expandable metallic stents (CSEMS) after placement is a risk factor for recurrent biliary obstruction (RBO) in patients with unresectable distal malignant biliary obstruction. We included 87 patients in this study and divided them into two cohorts. We found that the angle of the CSEMS after placement was an independent risk factor for RBO. Furthermore, we demonstrated that the cut-off value of the angle of CSEMS after placement was 130° and that time to RBO in the < 130° group was significantly shorter than that in the ≥ 130° group.

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INTRODUCTION

Covered self-expandable metallic stents (SEMS) are widely used for managing unresectable distal malignant biliary obstruction (MBO)[1-4]. However, recurrent biliary obstruction (RBO), which constitutes a major problem in patients with MBO, prevents the continuation of treatment, and patients experience a poor quality of life. Covered self-expandable metallic stents (CSEMS) are expected to prolong patency in patients with unresectable distal MBO by preventing tumor ingrowth or epithelial hyperplasia[5,6]. Additionally, improving the conformability of SEMS in the bile duct reduces the risk of migration[6]. In a recent study, sludge formation and food impaction were identified as the major causes of RBO[7].

Recently, SEMS with low axial forces (AFs) have been used frequently to improve compatibility with the bile duct, with increasing concerns that they are likely to cause early RBO as a result of sludge formation and food impaction[8]. AF represents the stent-straightening force, and it has been shown that CSEMS with a high AF have a lower frequency of sludge formation and food impaction than those with a low AF[9].

Therefore, we hypothesized that the time to RBO (TRBO) in CSEMS with a low AF would be short. This study focused on the angle of the CSEMS after placement to investigate the risk factors for RBO due to CSEMS in unresectable distal MBO.

MATERIALS AND METHODS

Patients and study design

This retrospective cohort study was conducted at the Department of Gastroenterology, Osaka City University Graduate School of Medicine, Japan. Consecutive patients who underwent SEMS placement using endoscopic retrograde cholangiopancreatography (ERCP) between January 2010 and March 2019, with follow-up until September 2019, were enrolled. Next, patients who underwent CSEMS placement were included. The exclusion criteria were as follows: (1) Postoperative patients (Billroth II, Roux-en-Y

reconstruction, *etc.*); (2) Patients with hilar biliary stricture; (3) Patients with placement of multiple SEMS; (4) Patients with benign distal biliary stricture; (5) Duplicated patients; (6) Patients with absence of abdominal radiograph; (7) Patients who had undergone percutaneous transhepatic biliary drainage, or biliary or duodenal metallic stenting; (8) Resectable distal MBO; (9) Patients with unknown treatment details; and (10) Early removal of SEMS due to cholecystitis and pancreatitis. Most cases could be pathologically evaluated, while other cases were diagnosed using radiographic imaging.

Ethical consideration

This study was conducted in compliance with the principles outlined in the Declaration of Helsinki. The study protocol was approved by the ethics committee of Osaka City University Graduate School of Medicine (No. 2020-022). All patients were given the opportunity to opt out of this study on our website's homepage.

Main outcome

The primary outcome was risk factors for RBO in unresectable distal MBO, including assessment of the angle of the CSEMS after placement. The secondary outcomes included the evaluation of technical and functional success, adverse events, TRBO, non-RBO rate, survival time, cause of RBO, and reintervention procedure.

Endoscopic procedure

All procedures were performed using a side-viewing duodenoscope (JF240, JF260V, TJF240, TJF260V; Olympus Optical Co, Tokyo, Japan) under conscious sedation with intravenous midazolam (3–10 mg), supplemented with pentazocine (15 mg) as required. These sedative drugs were re-administered as required during the procedure. Prophylactic antibiotics such as ulinastatin and nafamostat mesylate were administered to almost all patients to prevent cholangitis and pancreatitis. After selective cannulation of the bile duct using a 0.035- or 0.025-inch guidewire (Hydra Jagwire; Boston Scientific Corporation, Marlborough, United States or VisiGlide 2; TERUMO CORPORATION, Tokyo, Japan), routine cholangiography using a cannula (ERCP catheter; MTW Endoskopie, Wesel, Germany) or sphincterotome (Single-Use Sphincterotome V; OLYMPUS MEDICAL SYSTEMS Corp., Tokyo, Japan) was performed. Sphincterotomy was performed at the discretion of the endoscopist using an electrosurgical generator (ICC 200; ERBE Elektromedizin GmbH, Tübingen, Germany). The endoscopist decided which device to use during the procedure. All patients were hospitalized for at least 72 h after the procedure. Serum amylase levels were measured 4 and 24 h after ERCP. Abdominal computed tomography (CT) was performed if required.

CSEMS

Biliary drainage was performed using partially covered and fully covered SEMS. The WallFlex Biliary RX Stent (Boston Scientific Corporation, Marlborough, MA, United States) was used as the braided-type partial CSEMS. The braided-type full CSEMS employed in this study comprised a WallFlex Biliary RX Stent (Boston Scientific Corporation, Marlborough, United States), HANAROSTENT Biliary Full Cover Lasso (M. I. Tech Co., Ltd., Seoul, Korea), BONA-SHIMSTENT Covered with Lasso or BONASTENT M-intraductal (Sewoon Medical Inc., Seoul, Korea), Niti-S biliary silicone covered stent (Taewoong Medical, Seoul, Korea), and EGIS Biliary Stent (S&G Biotech Inc., Yongin-si, Korea). The laser-cut type CSEMS used was the X-Suit NIR Covered Biliary Metallic Stent (Medinol Ltd, Jerusalem, Israel).

Follow-up

Review outpatient visits based on clinical symptoms, blood tests, abdominal radiographs, and/or CT scans were performed every 3 mo until September 2019. Data were retrospectively collected from the medical records at the latest follow-up. Patients who were lost to follow-up, underwent any further surgery, or died without RBO were treated as censored cases at the time of last follow-up, operation, or death, respectively.

Definitions

RBO and TRBO: According to the TOKYO criteria[10], RBO was defined as a composite endpoint of either occlusion or migration of the CSEMS. TRBO was defined as the time from the placement of CSEMS to occurrence of RBO. Stent occlusion was defined as the presence of elevated liver enzyme levels compared with baseline values, accompanied by biliary dilation on imaging studies or endoscopic findings suggesting it. Stent migration was diagnosed when reintervention revealed a completely or partially migrated CSEMS as the cause of RBO.

Technical success and functional success: Based on the Tokyo criteria recommendations, technical success was defined as successful deployment of the CSEMS in the intended location with sufficient coverage of the stricture, and functional success was defined as a 50% decrease in or normalization of the bilirubin level within 14 d after placement of the CSEMS.

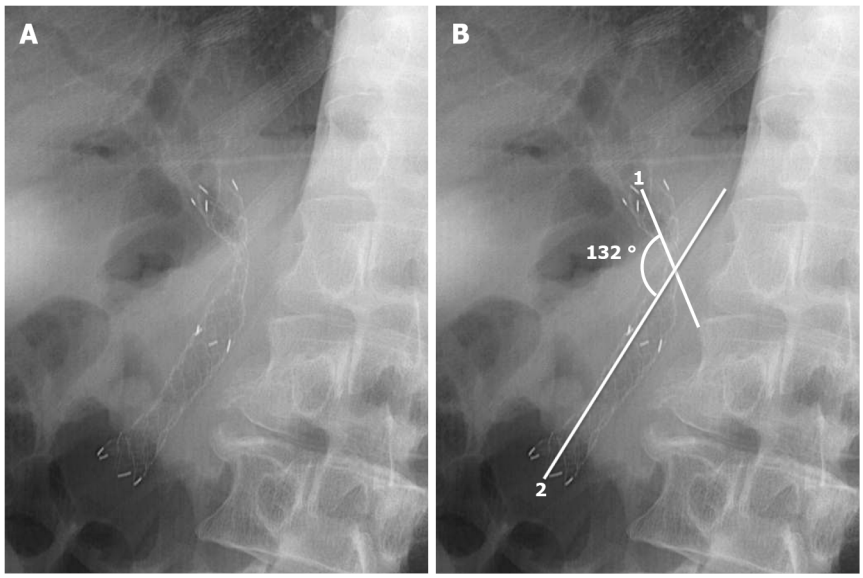

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Figure 1 Measurement of the angle of covered self-expandable metallic stents after placement on abdominal radiograph. A: Covered self-expandable metallic stent (CSEMS) after placement; B: Measurement of the obtuse angle of CSEMS after the placement. 1: The lines extending from the proximal point to the narrowest point; 2: The lines extending from the distal point to the narrowest point. The obtuse angle of CSEMS after the placement between line 1 and line 2 was 132°. CSEMS: Covered self-expandable metallic stent.

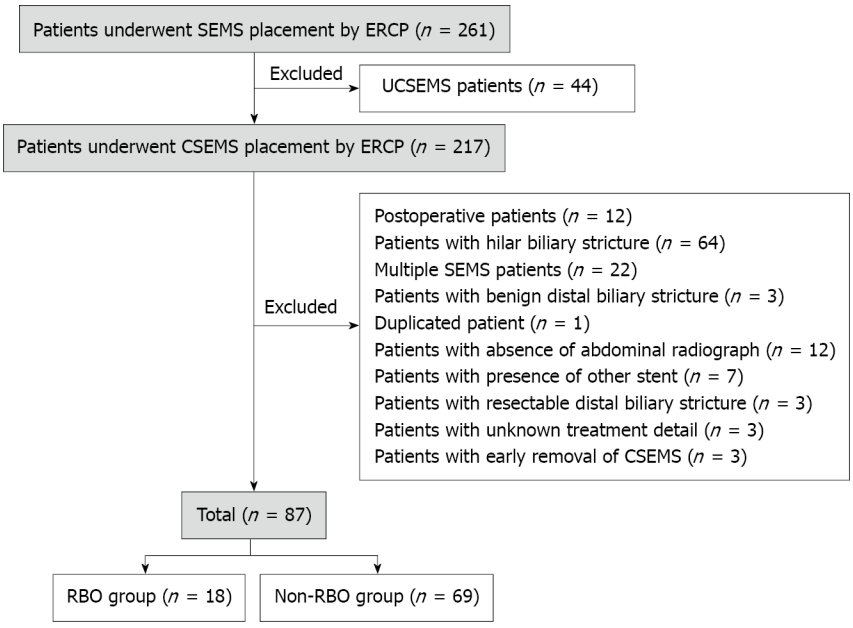

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Figure 2 Diagram of study design. SEMS: Self-expandable metallic stents; ERCP: Endoscopic retrograde cholangiopancreatography; CSEMS: Covered self-expandable metallic stents; UCSEMS: Uncovered self-expandable metallic stents; MBO: Malignant biliary obstruction; RBO: Recurrent biliary obstruction.

Distal MBO: In this study, distal MBO was defined as a malignant biliary stricture situated ≥ 2 cm from the communication of the bilateral hepatic ducts.

Adverse events: Adverse events were graded according to the American Society of Gastrointestinal Endoscopy lexicon guidelines[11].

Measurement of the angle of CSEMS after placement: The angle of the CSEMS was retrospectively measured on abdominal radiographs obtained in the standing position using a medical imaging and information management system (SYNAPSE PACS SYSTEM; FUJIFILM Corporation, Tokyo, Japan). As previously reported[12], the angle of CSEMS was defined as the angle between the lines extending from

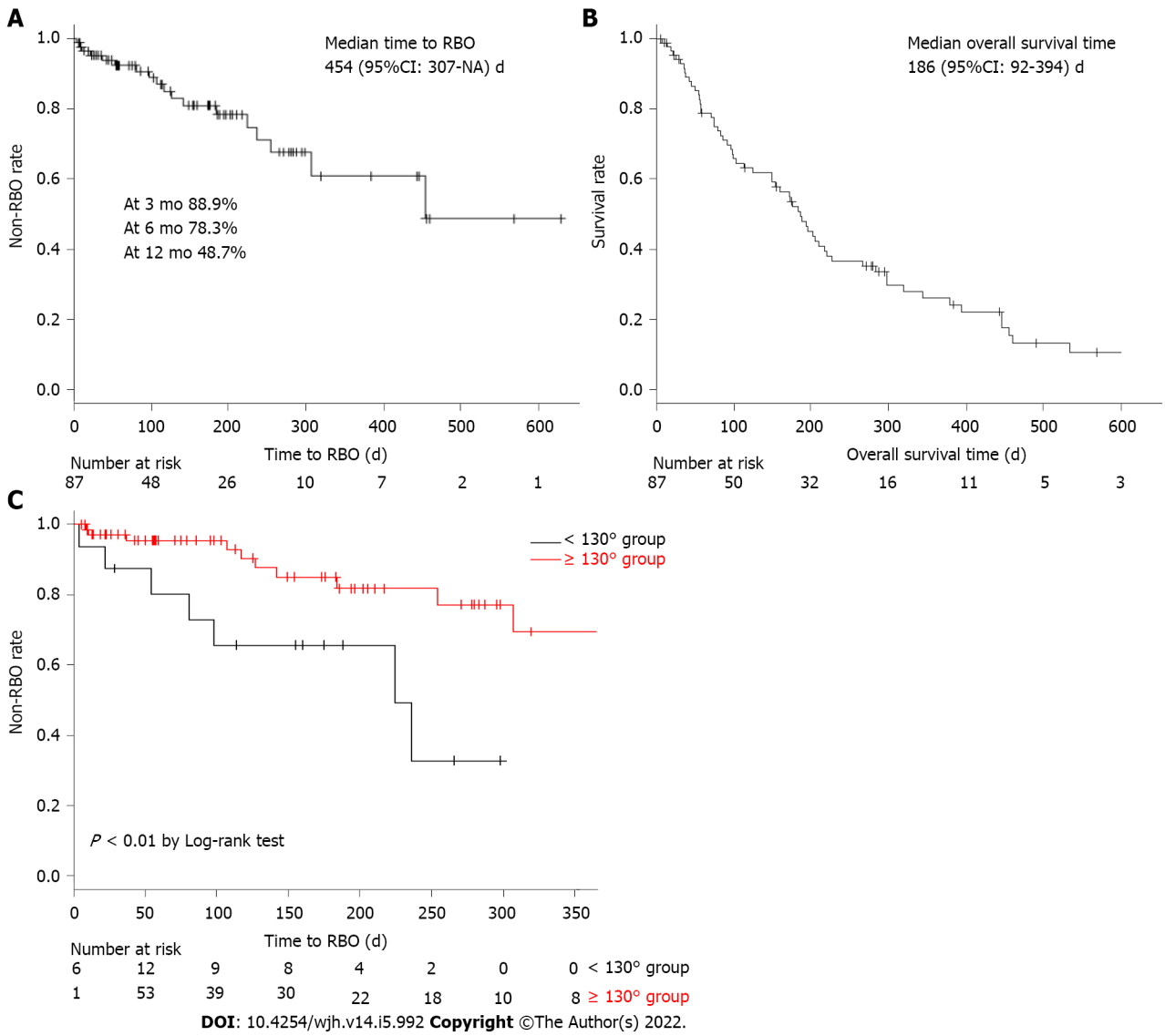

Figure 3 Long-term results of covered self-expandable metallic stents in unresectable distal malignant biliary obstruction. A: Non-recurrent biliary obstruction (RBO) rate and median time to recurrent biliary obstruction (TRBO); B: Overall survival rate; C: Comparison of TRBO in the < 130° group and ≤ 130°group. RBO: Recurrent biliary obstruction; TRBO: Time to recurrent biliary obstruction; NA: Not available; CSEMS: Covered self-expandable metallic stents; MBO: Malignant biliary obstruction; CI: Confidence interval

the proximal and distal points to the narrowest point in the CSEMS (Figure 1). The obtuse angle of the CSEMS was evaluated after 2 or more days, and not immediately after placement because the CSEMS was not fully expanded and was unstable in position.

Statistical analysis

Medians and interquartile ranges (IQRs) are used for continuous variables, while percentages and counts are used for categorical variables. Categorical variables were evaluated using the chi-squared or Fisher's exact test, and continuous variables were evaluated using the Mann-Whitney *U* test. Using the Cox proportional hazard model, the risk of RBO following CSEMS placement was estimated by calculating the hazard ratio (HR) and 95% confidence interval (CI). TRBO, non-RBO, and survival time were assessed using the Kaplan-Meier method. Using C-statistics, the model was evaluated for reliability using the Hosmer-Lemeshow test for goodness-of-fit and for validity using receiver operating characteristic (ROC) curves. The Pearson's correlation test was used to evaluate the consistency of the angle of the CSEMS after placement by two people using a previously reported method[13]. Statistical analyses were performed using IBM SPSS software, version 23.0 for Windows (IBM Corporation, NY, United States) and R software version 2.4.3 (R Foundation for Statistical Computing, Vienna, Austria). All statistical tests were two-sided, and a P value < 0.05 was considered statistically significant.

RESULTS

Baseline characteristics of the patients and treatments

A total of 261 patients were enrolled in this study. Forty-four patients (62 sessions) who were treated with uncovered SEMS were excluded. Among the remaining 217 patients (246 sessions), 12 (13 sessions) had previously undergone surgery, 64 (79 sessions) underwent SEMS placement for hepatic hilar biliary stricture, 22 (24 sessions) underwent the placement of multiple SEMS, 3 (5 sessions) had benign biliary stricture, 1 (10 sessions) had duplication of data, 12 had absent abdominal radiographs, 7 had other stents, 3 had resectable distal MBO, and 3 were unknown, and in 3 patients, CSEMS were removed early due to adverse events. Finally, 87 eligible patients were included who underwent initial CSEMS placement for unresectable distal MBO (Figure 2).

The baseline characteristics of the patients and the treatments implemented are shown in Table 1. Pancreatic cancer was the predominant primary disease, resulting in distal MBO (66.7%), with the most common clinical stage being IV (86.2%). Forty-eight patients (55.2%) received chemotherapy and 11 patients (12.6%) received radiotherapy. Endoscopic sphincterotomy was performed in 60 (69.0 %) patients. The median of the angle of CSEMS after the placement was 146.0° (IQR: 134.5-156.5).

Short-term results and adverse events

CSEMS were successfully deployed in 87 patients (100.0%), and functional success was observed in 72 patients (82.8%) (Table 2). The overall adverse event rate was 10.3%. The incidence rate of severe acute pancreatitis was 1.1%, and all patients with pancreatitis were managed conservatively. No adverse events associated with cholangitis (including non-occlusion cholangitis), cholecystitis, or other complications (bleeding, perforation, *etc.*) were observed.

Long-term results and reintervention

The median TRBO was found to be 454 [95%CI: 307-not available (NA)] d, during a median follow-up period of 117.0 (IQR: 47.5-220.5) d. The non-RBO rates at 3, 6, and 12 mo after CSEMS placement were 88.9%, 78.3%, and 48.7%, respectively (Figure 3A). The median overall survival time was 186 (95%CI: 92-394) d (Figure 3B). The number of patients with RBO after CSEMS placement was 18 (20.7%), and the most common cause of RBO was sludge formation and food impaction (11 cases, 61.1%) (Table 2). Except for tumor overgrowth, the incidence of RBO due to stent occlusion and migration was 72.2%. In 17 of 18 patients with CSEMS, reintervention for RBO was required and performed successfully. The procedures during reintervention included additional CSEMS replacement in nine patients, plastic stent placement in three, and no additional placement in five.

Risk factors for RBO following CSEMS placement

We divided the patients into two cohorts: 18 in the RBO group and 69 in the non-RBO group. The baseline characteristics were similar except for the angle of the CSEMS after placement, which differed significantly between the two groups ($P = 0.01$) (Table 3). The angle of CSEMS after placement (*per* 1° and *per* 10°) was a risk factor for RBO based on the univariate Cox proportional hazard analysis (HR, 0.96; 95%CI, 0.93-0.99; $P < 0.01$; HR: 0.67; 95%CI: 0.51-0.87; $P < 0.01$, respectively). In addition to the angle of the CSEMS after placement, we selected the American Society of Anesthesiologists Physical Status and chemotherapy that could potentially be a confounding factor using clinical knowledge[14]. In the multivariate Cox proportional hazard analysis, only the angle of CSEMS after placement (*per* 1° and *per* 10°) was significantly associated with shorter duration of RBO (HR: 0.97; 95%CI: 0.94-0.99; $P = 0.01$; HR: 0.71; 95%CI: 0.54-0.92; $P = 0.01$, respectively) (Table 4). The angle of CSEMS after placement was an independent risk factor for RBO in unresectable distal MBO.

Evaluation of the current study and the obtuse angle of CSEMS after placement

We evaluated the accuracy of the current study using the ROC curve. For early RBO diagnosis, the angle of 130° had a sensitivity of 50.0% and specificity of 85.5%, and the ROC analysis showed an area under the curve of 0.70 (95%CI: 0.57-0.84) (Figure 4). When comparing the groups based on the angle of CSEMS after placement, TRBO in the < 130° angle group was significantly shorter than that in the ≥ 130° angle group ($P < 0.01$) (Figure 3C).

Furthermore, we used the Pearson's correlation test to evaluate the consistency of the obtuse angle of CSEMS after placement. A random number table was created for 87 patients. Of these, 20 patients were randomly sampled. The angle of the CSEMS after placement was evaluated by two board-certified fellows (K.T. and H.M.) of the Japan Gastroenterological Endoscopy Society. A significant positive correlation was observed for the angle of the CSEMS after placement ($r = 0.92$; 95%CI: 0.81-0.97; $P < 0.01$) (Figure 5).

Table 1 Baseline characteristics of patients and treatment

Baseline characteristic		*n* = 87
Age (IQR, yr)		63.5 (26.0-68.0)
Sex, *n* (%)	Male	55 (63.2)
	Female	32 (36.8)
ASA-PS, *n* (%)	1	24 (27.6)
	2	44 (50.6)
	3	19 (21.8)
Primary disease, *n* (%)	Pancreatic cancer	58 (66.7)
	Bile duct cancer	9 (10.3)
	Gallbladder cancer	2 (2.3)
	Ampullary cancer	2 (2.3)
	Duodenal cancer	2 (2.3)
	Other cancers	14 (16.1)
Clinical stage, *n* (%)	II	3 (3.5)
	III	9 (10.3)
	IV	75 (86.2)
Chemotherapy, *n* (%)	Yes	48 (55.2)
Radiotherapy, *n* (%)	Yes	11 (12.6)
Total bilirubin level (IQR, mg/dL)		3.8 (2.1-7.7)
Length of stricture (IQR, mm)		31.5 (21.8-39.3)
Total procedure time (IQR, min)		32.0 (24.5-48.5)
EST, *n* (%)	Yes	60 (69.0)
CSEMS type (1), *n* (%)	Partially	38 (43.7)
	Fully	49 (56.3)
CSEMS type (2), *n* (%)	Laser-cut	6 (6.9)
	Braided	81(93.1)
Length of CSEMS (cm), *n* (%)	4	3 (3.5)
	5	1 (1.1)
	6	44 (50.6)
	7	10 (11.5)
	8	29 (33.3)
Diameter of CSEMS (mm), *n* (%)	6	3 (3.5)
	8	3 (3.5)
	10	81 (93.0)
Angle of CSEMS (IQR, °)		146.0 (134.5-156.5)

Other cancers include cancers of the stomach, colon, small intestine, liver, kidney, breast, and cervix, leiomyosarcoma, and malignant melanoma. ASA-PS: American Society of Anesthesiologist Physical Status classification; EST: Endoscopic sphincterotomy; CSEMS: Covered self-expandable metallic stents; IQR: Interquartile range.

DISCUSSION

We found that the angle of the CSEMS after placement was a risk factor for RBO in unresectable distal MBO. In addition, our study demonstrated that the cut-off value of the angle of CSEMS after placement for RBO was 130° and that TRBO in the group with an angle < 130° was significantly shorter than that in

Table 2 Short- and long-term results, adverse events, cause of recurrent biliary obstruction, and reintervention

Classification		*n* (%)
		***n* = 87**
Technical success		87 (100.0)
Functional success		72 (82.8)
Adverse event		9 (10.3)
	Pancreatitis	9 (10.3)
	Severe	1 (1.2)
	Moderate	5 (5.7)
	Mild	3 (3.4)
	Cholangitis	0 (0.0)
	Cholecystitis	0 (0.0)
	Other complications	0 (0.0)
		***n* = 18**
RBO		18 (100.0)
	Sludge formation or food impaction	11 (61.1)
	Tumor overgrowth	5 (27.8)
	Migration	2 (11.1)
	Tumor ingrowth	0 (0.0)
Reintervention		18 (100.0)
	Metallic stent	9 (50.0)
	Sweep	5 (27.8)
	Plastic stent	3 (16.7)
	Not success	1 (5.5)

Other complications include bleeding, perforation, *etc*. RBO: Recurrent biliary obstruction; TRBO: Time to recurrent biliary obstruction; IQR: Interquartile range.

the group with an angle ≥ 130°. This is the first report to demonstrate a new and quantitative risk factor for RBO in CSEMS. We believe that our results are easy for everyone to replicate and provide important information for the management of CSEMS.

A CSEMS angle of < 130° is a risk factor for early RBO. This result suggests that CSEMS with a low AF cause sludge formation, food impaction, and stent migration, which is supported by the results of several previous studies[6,15,16]. First, the placement of a CSEMS with a large diameter across the papilla causes loss of sphincter dysfunction, resulting in duodenal-biliary reflux to the bile duct because of the pressure gradient caused by food or duodenal contents. These results suggest that disruption of the sphincter mechanism may be the most important etiological factor in the development of cholangitis after metallic stent placement for MBO[16]. In addition, previous reports have suggested that food debris is an etiologic factor for acute cholangitis and warned that occlusion might be caused by reflux of duodenal contents[17,18]. CSEMS with a low AF decrease the flow velocity and increase the resistance to bile juice. Therefore, a CSEMS angle of < 130° easily causes sludge formation and food impaction and induces early RBO. Additionally, increased outflow pressure of bile juice leads to an elevated risk of stent migration[19]. In our study, the incidence of RBO in the CSEMS angle < 130° group was inclined to be higher than that in the ≥ 130° group (43.8% *vs* 15.5%) (Table 5). Isayama *et al*[8] also demonstrated that a well-bent SEMS with a low AF after placement led to RBO as a result of sludge formation and food impaction[8], which supports our results. However, unlike this previous report, we proposed a versatile index and used abdominal radiography. We believe that the measurement of the CSEMS angle in our results is simple.

Our results may have an influence on the stent management after CSEMS placement. Until now, patients with unfavorable prognoses have not been considered for CSEMS replacement. However, it is widely accepted that CSEMS are exchanged when stent occlusion and migration occur. There are no definitive guidelines or literature concerning the management after CSEMS placement. However, this

Table 3 Baseline characteristics of patients and treatment (recurrent biliary obstruction group *vs* non-recurrent biliary obstruction group)

		RBO group (*n* = 18)	Non-RBO group (*n* = 69)	*P* value
Age (IQR, yr)		66.5 (61-77)	69 (63.5-77)	0.63
Sex, *n* (%)	Female	5 (27.8)	27 (39.1)	0.42
	Male	13 (72.2)	42 (60.9)	
ASA-PS, *n* (%)	1	2 (11.1)	22 (31.9)	0.07
	2	9 (50.0)	35 (50.7)	
	3	7 (38.9)	12 (17.4)	
Primary disease, *n* (%)	Other cancers	8 (44.4)	21 (30.4)	0.28
	Pancreatic cancer	10 (55.6)	48 (69.6)	
Clinical stage, *n* (%)	II	1 (5.6)	2 (2.9)	0.08
	III	4 (22.2)	4 (5.8)	
	IV	13 (72.2)	63 (89.9)	
Chemotherapy, *n* (%)	No	8 (44.4)	31 (44.9)	1.00
	Yes	10 (55.6)	38 (55.1)	
Radiotherapy, *n* (%)	No	17 (94.4)	59 (85.5)	0.45
	Yes	1 (5.6)	10 (14.5)	
Total bilirubin level (continuous, mg/dL)		2.7 (1.1-6.7)	4.4 (2.3-9.0)	0.07
Length of stricture (IQR, mm)		34.9 (25.9-47.2)	30.9 (21.4-37.8)	0.13
Total procedure time (IQR, min)		35.5 (25-50.8)	32.0 (24-47)	0.65
EST, *n* (%)	No	5 (27.8)	22 (31.9)	1.00
	Yes	13 (72.2)	47 (68.1)	
CSEMS type (1), *n* (%)	Partially	9 (50.0)	29 (42.0)	0.60
	Fully	9 (50.0)	40 (58.0)	
CSEMS type (2), *n* (%)	Laser-cut	1 (5.6)	5 (7.2)	1.00
	Braided	17 (94.4)	64 (92.8)	
Length of CSEMS, *n* (%)	≤ 6 cm	8 (44.4)	40 (58.0)	0.55
	7 cm	3 (16.7)	7 (10.2)	
	8 cm	7 (38.9)	22 (31.9)	
Diameter of CSEMS, *n* (%)	6 mm	0 (0.0)	3 (4.4)	0.58
	8 mm	1 (5.6)	2 (2.9)	
	10 mm	17 (94.4)	64 (92.8)	
Angle of CSEMS (IQR, °)		135.5 (114.8-149.3)	149.0 (138.5-158)	0.01

ASA-PS: American Society of Anesthesiologist Physical Status classification; EST: Endoscopic sphincterotomy; CSEMS: Covered self-expandable metallic stents; IQR: Interquartile range; RBO: Recurrent biliary obstruction.

scenario has changed in recent years. With the advent of effective drugs and radiation therapy, patient prognosis has improved[20]. For this reason, stenting has shifted from being used for palliative care to maintaining the overall health status of patients undergoing antitumor therapy as part of multidisciplinary treatment. Therefore, long-term maintenance without stent dysfunction is recommended. However, recent studies have reported that the non-RBO rate at 6 mo was 63%-91% for full CSEMS[6,21,22], and that there was a need for replacement of CSEMS before the patients died.

In our study, among patients who had RBO due to CSEMS in the < 130° angle group, 86% had elevated liver enzyme levels according to the latest laboratory data before the occurrence of RBO compared with previous laboratory data, and all patients were asymptomatic (Table 5). Thus, a potentially high risk of RBO might be considered if the CSEMS angle is < 130° and liver enzyme levels

Table 4 Risk factors for recurrent biliary obstruction following covered self-expandable metallic stent placement by Cox proportional hazards analysis

				Univariate analysis		Multivariate analysis	
		n	Case (%)	Unadjusted HR (95%CI)	*P* value	Adjusted HR (95%CI)	*P* value
Age (continuous, yr)		87	18 (20.7)	1.00 (0.95-1.04)	0.91		
Sex	Female	32	5 (15.6)	1.00			
	Male	55	13 (23.6)	1.44 (0.51-4.05)	0.49		
ASA-PS	1	24	2 (8.3)	1.00			
	2	44	9 (20.5)	2.59 (0.56-12.00)	0.23		
	3	19	7 (36.8)	5.12 (1.06-24.81)	0.04	1.76 (0.85-3.62)	0.13
Primary disease	Others	29	8 (27.6)	1.00			
	Pancreatic cancer	58	10 (17.2)	0.80 (0.31-2.05)	0.64		
Clinical stage	II	3	1 (33.3)	1.00			
	III	8	4 (50.0)	2.34 (0.26-21.22)	0.45		
	IV	76	13 (17.1)	0.79 (0.10-6.18)	0.82		
Chemotherapy	No	39	8 (20.5)	1.00			
	Yes	48	10 (20.8)	1.13 (0.44-2.88)	0.80	1.02 (0.40-2.61)	0.97
Radiotherapy	No	76	17 (22.4)	1.00			
	Yes	11	1 (9.1)	0.43 (0.06-3.24)	0.41		
Total bilirubin level (continuous, mg/dL)		87	18 (20.7)	1.01 (0.99-1.03)	0.26		
Length of stricture (continuous, mm)		87	18 (20.7)	1.03 (1.00-1.07)	0.10		
Total procedure time (continuous, min)		87	18 (20.7)	1.00 (0.98-1.03)	0.75		
EST	No	27	5 (18.5)	1.00			
	Yes	60	13 (21.7)	1.19 (0.42-3.35)	0.75		
CSEMS type (1)	Partially	38	9 (23.7)	1.00			
	Fully	49	9 (18.4)	0.85 (0.34-2.15)	0.73		
CSEMS type (2)	Laser-cut	6	1 (16.7)	1.00			
	Braided	81	17 (21.0)	1.27 (0.17-9.73)	0.82		
Length of CSEMS	≤ 6 cm	48	8 (16.7)	1.00			
	7 cm	10	3 (30.0)	2.33 (0.61-8.89)	0.22		
	8 cm	29	7 (24.1)	1.63 (0.64-4.14)	0.45		
Diameter of CSEMS	< 10 mm	6	1 (16.7)	1.00			
	≥ 10 mm	81	17 (21.0)	1.37 (0.18-10.47)	0.76		
Angle of CSEMS (continuous, *per* 10°)		87	18 (20.7)	0.67 (0.51-0.87)	< 0.01	0.71 (0.54-0.92)	0.01

Data are presented as medians for continuous variables and as numbers for categorical variables. For categorical data, comparisons between groups were performed using the chi-squared test (or Fisher's exact test, when necessary, because of small sample sizes), whereas continuous data were compared using Mann-Whitney *U* test. ASA-PS: American Society of Anesthesiologist Physical Status classification; EST: Endoscopic sphincterotomy; CSEMS: Covered self-expandable metallic stents; IQR: Interquartile range; HR: Hazard ratio; CI: Confidence interval.

are elevated. Hence, we suggest the replacement of CSEMS even in asymptomatic patients if the liver enzyme levels are elevated and the CSEMS angle is < 130° while managing such patients. We believe that this information has great significance in the management of patients undergoing CSEMS placement in clinical practice. Additionally, by deploying a new CSEMS with a high AF as needed, it is possible to expect long-term maintenance without stent dysfunction.

Table 5 Details of patients who had recurrent biliary obstruction following covered self-expandable metallic stenting (< 130° group *vs* ≥ 130° group)

	< 130° group	≥ 130° group	*P* value
	n = 16	*n* = 71	
RBO, *n* (%)	7 (43.5)	11 (15.5)	0.07
	n = 7	*n* = 11	
Cause of RBO, *n* (%)	7 (100.0)	11 (100.0)	
Sludge formation or food impaction	3 (42.9)	8 (72.7)	
Tumor overgrowth	3 (42.9)	2 (18.2)	
Migration	1 (14.2)	1 (9.1)	
Tumor ingrowth	0 (0.0)	0 (0.0)	
Elevated liver enzymes, *n* (%)	6 (85.7)	5 (45.5)	

RBO: Recurrent biliary obstruction; CSEMS: Covered self-expandable metallic stents.

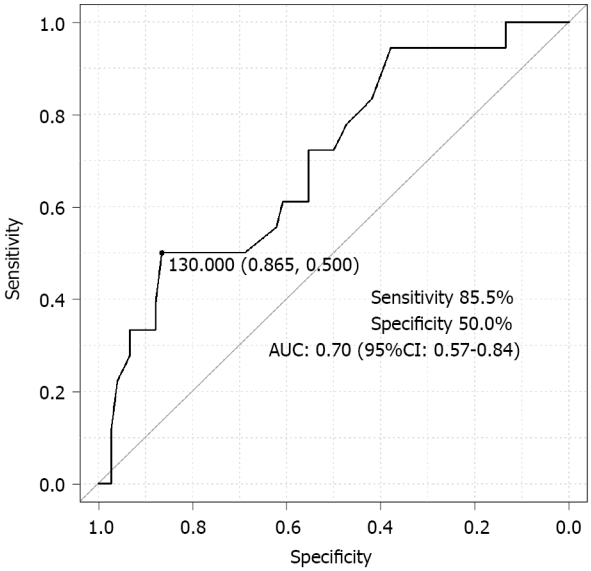

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Figure 4 Evaluation of accuracy by receiver operating characteristic curve analysis. AUC: Area under the curve; ROC: Receiver operating characteristic; CI: Confidence interval.

Our study has the following limitations. First, this was a retrospective cohort study conducted at a single center. We acknowledge that patient assignment to different interventions was subjected to selection bias. There were differences in the length, diameter, and type of CSEMS used in our study, which can influence RBO. However, in multivariate analyses, these factors did not significantly influence RBO. In our results, the angle after placement was a risk factor for RBO regardless of the selected CSEMS characteristics. A larger prospective multicenter study should be conducted to evaluate the obtuse angle of the CSEMS after placement in unresectable distal MBO. Second, we evaluated the angle of the CSEMS after placement using two-dimensional data. Although three-dimensional data, *i.e.*, CT or magnetic resonance imaging, are desirable, we could not evaluate the angle after placement on a unified modality or quantify it using three-dimensional data. However, no previous reports have assessed RBO based on the angle of the CSEMS. Therefore, we consider our method using two-dimensional data to be simple and highly versatile. Third, the actual results of the censored cases were unknown because follow-up data were collected only from medical records.

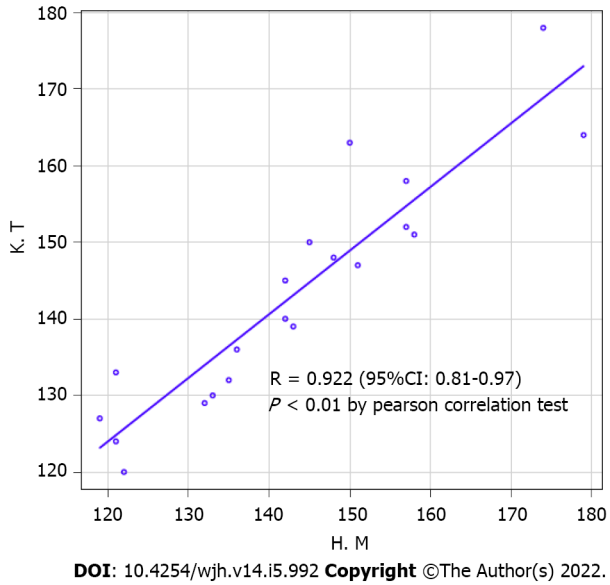

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Figure 5 Evaluation of consistency for the angle of covered self-expandable metallic stents after placement. CSEMS: Covered self-expandable metallic stents; CI: Confidence interval.

CONCLUSION

The angle of the CSEMS after placement is a risk factor for RBO, and the TRBO of the CSEMS with a low AF is shorter than that of other CSEMS. These novel results provide pertinent information for future stent management.

ARTICLE HIGHLIGHTS

Research background

Covered self-expandable metallic stents (CSEMS) cause recurrent biliary obstruction (RBO), which prevents the continuation of treatment and causes the quality of life in patients with unresectable distal malignant biliary obstruction (MBO) to be poor. To date, sludge formation and food impaction have remained to be major causes of RBO. Recently, CSEMS with a low axial force (AF) to improve compatibility with the bile duct have been used frequently, with increasing concerns that they are likely to cause early RBO as a result of sludge formation and food impaction.

Research motivation

We hypothesized that the time to RBO (TRBO) of CSEMS with a low AF was short. We considered that proving this hypothesis has great significance in the management of patients with CSEMS placement in the clinical practice.

Research objectives

We aimed to evaluate whether the angle of CSEMS after placement is a risk factor for RBO in patients with unresectable distal MBO.

Research methods

Finally, we included 87 patients in this study. We divided the patients into two cohorts, RBO group and non-RBO group, and evaluated the risk factors for RBO including the angle of CSEMS after the placement. Using the SYNAPSE PACS system, we measured the obtuse angle of CSEMS after placement on an abdominal radiograph.

Research results

We found that the angle of CSEMS after placement was an independent risk factor for RBO. Further, we demonstrated that the cut-off value for the angle of CSEMS after placement was 130°, and that time to RBO in the < 130° group was significantly shorter than that in the ≥ 130° group. In our study, among patients who caused RBO of CSEMS in the < 130° angle group, 86% had elevated liver enzymes in the latest laboratory data before the occurrence of RBO, compared with previous laboratory data, and all

patients were asymptomatic.

Research conclusions

The findings suggest that the angle of CSEMS after placement for unresectable distal MBO is a risk factor for RBO, and TRBO of CSEMS with a low AF is shorter than that of other CSEMS. Hence, while managing such patients, we suggest the replacement of CSEMS even in asymptomatic patients if the liver enzymes are elevated and the CSEMS angle is < 130°. Additionally, by deploying a new CSEMS with a high AF as needed, it could be possible to expect long-term maintenance without stent dysfunction.

Research perspectives

These results are novel and provide pertinent information for future stent management. However, further prospective studies with larger cohorts are needed to validate our findings.

FOOTNOTES

Author contributions: Tanoue K and Maruyama H contributed to study conception and design; Tanoue K and Maruyama H analyzed and interpreted the data; Tanoue K, Maruyama H, Ishikawa-Kakiya Y, Kinoshita Y, Hayashi K, Yamamura M, Ominami M, Nadatani Y, Fukunaga S, Otani K, Hosomi S, Tanaka F, Kamata N, Nagami Y, Taira K, and Watanabe T wrote, reviewed, and/or revised the manuscript; Fujiwara Y supervised the study; all authors reviewed and approved the final manuscript.

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ORIGINAL ARTICLE

Observational Study

Dietary phytochemical consumption is inversely associated with liver alkaline phosphatase in Middle Eastern adults

Zahra Darabi, Richard James Webb, Hassan Mozaffari-Khosravi, Masoud Mirzaei, Ian Glynn Davies, Sayyed Saeid Khayyatzadeh, Mohsen Mazidi

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Abstract

BACKGROUND

The hepatoprotective effects of phytochemicals are controversial. A dietary phytochemical index (DPI) has been suggested as an alternative method for quantifying the phytochemical content of foods.

AIM

To assess the DPI in relation to liver function tests among a representative sample of Iranian adults.

METHODS
A total of 5111 participants aged 35-70 years old were included in this cross-sectional study by a multistage cluster random sampling method. Dietary intakes were collected by a validated and reliable food frequency questionnaire with 121 items. DPI was calculated by the percent of daily energy intake taken from phytochemical-rich foods. Fasting serum concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) were determined. Linear regression was used to investigate the association between DPI and levels of liver enzymes using crude and adjusted models.

RESULTS
There was an inverse association between DPI score and serum ALP in the crude model (β = -0.05; $P < 0.001$). This association remained significant after adjustment for body mass index, age, smoking, energy intake, history of diabetes, and education (β = -0.03; $P = 0.01$). No significant associations were found between DPI score and serum levels of AST, ALT, and GGT. The individuals with the highest DPI scores consumed significantly higher amounts of fruits, vegetables, legumes, nuts, and cereals, yet were shown to have significantly higher serum total cholesterol and low-density lipoprotein cholesterol, as well as several other metabolic abnormalities.

CONCLUSION
Higher adherence to phytochemical-rich foods was associated with lower levels of ALP, but no change in other liver enzymes. Those with higher DPI scores also consumed food items associated with a healthier overall dietary pattern; however, they also presented several unexpected metabolic derangements. Additional randomised trials are needed to better determine the effects of phytochemical-rich foods on liver function.

Key Words: Diet; Phytochemical; Liver; Dietary phytochemical index; Dietary pattern; Phytochemical index; Iran; Middle East; Nutrition

Core Tip: It has been debated whether phytochemicals are hepatoprotective. Furthermore, to the authors knowledge, this has not previously been researched in an Iranian population. In our study we used a dietary phytochemical index to categorise participants in terms of their intake of dietary phytochemicals. We found that participants with higher intakes of phytochemicals had a healthier overall dietary pattern and lower levels of alanine aminotransferase, which may be suggestive of improved liver function. Despite this, several metabolic disturbances were also revealed in these participants.

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INTRODUCTION

The hepatoprotective effects of phytochemicals have been the subject of recent debate. This has stemmed from increasing evidence concerning the ability of these plant-derived bioactive components to beneficially modulate metabolic processes. Favourable changes have been previously demonstrated in a variety of disease states, including Type 2 diabetes, obesity and cardiovascular and renal disease[1]. Given the links of these metabolic aberrations with liver pathology it is reasonable to suggest that phytochemicals might infer a degree of hepatoprotection[1].

More specifically, studies investigating potential links between dietary phytochemicals and liver function have shown that compounds such as polyphenols, flavonoids, carotenoid and terpenoids, contained in food items such as grapes, tea, olives, nuts and legumes, may favourably mediate liver function when consumed, especially in the context of a low-calorie diet[2,3]. Several proposed mechanisms responsible for these effects may include the ability of phytochemicals to act as natural ligands for peroxisome proliferator-activated receptors, as well as favourably impacting upon mitochondrial beta-oxidation[4]. Furthermore, the ability of phytochemicals to reduce oxidative stress

and decrease transaminase activity are also thought to be influencing factors[1,5].

The Iranian population has high rates of hepatic abnormalities, such as non-alcoholic fatty liver disease (NAFLD), which affects approximately 4.1% of the population[6]. It is thought that this is perpetuated by concurrent high rates of metabolic syndrome; a cluster of risk factors known to be strongly associated with the development of NAFLD[7]. In addition to this, recent findings have also revealed that NAFLD is more likely to be present in Iranians with a higher socioeconomic class and is exacerbated by a Western dietary pattern, whereas a healthier traditional Iranian dietary pattern, presumably richer in dietary phytochemical-containing foods, is likely to be more protective[8]. Although there is evidence concerning the beneficial impact of dietary phytochemicals upon cardiometabolic risk, there are no studies to date which focus specifically on liver function in Iranian adults[9].

We aimed to determine, for the first time, the association between dietary phytochemical index (DPI) and liver function in Iranian adults. We used data derived from the Prospective Epidemiological Research Studies in Iran (PERSIAN) Cohort Study to create a 'dietary phytochemical index' (DPI), based on the work of McCarty, which takes into account the health promoting properties of phytochemicals [10]. This enabled us to test the hypothesis that higher DPI scores would equate to improved liver function.

MATERIALS AND METHODS

Study population

The present cross-sectional study is reported based on the Strengthening the Reporting of Observational studies in Epidemiology (STROBE) guideline[11]. The study was conducted in the context of the baseline data of Shahedieh Cohort Study, which is a part of the PERSIAN multicenter cohort study which was conducted using a representative sample of the Iranian adult population aged 35-70 years old[12]. The Shahedieh cohort study recruited about 10000 adults older than 35 living in two municipal areas of Yazd city (Zarch and Shahedieh), Yazd province, Iran. The study protocol of the PERSIAN cohort is provided in detail elsewhere[12,13]. In brief, healthy participants were selected by a multistage cluster random sampling method after they provided written informed consent. The eligible participants were invited to give blood samples and provide data on general characteristics, demographic, dietary intake, smoking, and other lifestyle related data. Anthropometric and blood pressure measurements were also conducted for all attendants. All data were collected by trained interviewers[12,13]. Data on 10,113 adults were provided. Participants with a history of current pregnancy, ovary removal, cardiac ischemia, myocardial infarction, stroke, renal failure, hepatitis B and C, and different types of cancer such as skin cancer, breast cancer, stomach cancer, colorectal cancer, and bladder cancer along with the history of hematopoietic cancers were excluded from the current study because of the possibility of an adjustment in diet (n = 1189). Data for participants with a history of alcohol consumption, fatty liver, and diabetes (n = 2241) were also omitted. Furthermore, we excluded those who left > 70 items unanswered on the food frequency questionnaire (FFQ) and those who under and over reported their dietary intake (daily energy intake less than 800 kcal/d or more than 7000 kcal/d) (n = 1433). The missing data consisted of n = 139 participants who were also excluded from the study. After the mentioned exclusions, 5111 participants remained for the present analysis. The study was also approved by the ethics committee of Shahid Sadoughi University of Medical Sciences (approval code: IR.SSU.SPH.REC.1397.161).

Laboratory assessment

Blood samples (25 mL) were taken when participants were in the fasted state (8 to 12 h before blood sampling). The blood was aliquoted into serum, buffy coat, and whole blood samples. Serum gamma-glutamyltransferase (GGT), alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were determined from serum samples by an auto-analyser (Analyzer BT1500) using Pars Azmun standard kits.

Dietary intake assessment

The study participants were interviewed by trained nutritionists to complete a semi-quantitative FFQ with 121 items, which asked about dietary intake over the past year[12]. To complete the information, two questions were asked from the participants about each food item: (1) The frequency of food consumption (number of times per month, week, or day the food was consumed) in the previous year, and (2) the amount of the food that was usually consumed every time (portion size based on the standard serving sizes commonly consumed by Iranians). All reported intakes were converted to g/day by using household portion sizes of consumed foods[14]. The USDA food database was used to calculate nutrient intakes[15].

Dietary phytochemical index calculation

The DPI score was computed based on the method presented by McCarty as follows: DPI = [daily energy derived from phytochemical-rich foods (kcal)/total daily energy intake (kcal)] × 100[10]. Fruits, vegetables, legumes, whole grains, nuts, soy products, seeds and olive oil were considered phytochemical-rich foods. The phytochemical content of potatoes is low and so potatoes were not included[16].

Anthropometric measurement

Anthropometric parameters (weight, height) were measured by a trained investigator. Weight was measured while the participants were wearing minimum clothing and without shoes using a digital scale (SECA, model 755, Germany). Participants' height was measured using a stadiometer with a precision of 0.5 cm. Body mass index (BMI) was calculated by dividing weight (kg) by height (meters) squared.

Assessment of other variables

Data on additional variables including marital status, smoking (never smoker/current smoker/ex-smoker), and multivitamin-mineral supplements use (yes/no) were obtained using questionnaires. People were asked about their usual physical activity in the last year and if they had seasonal jobs. The information gathered in the questionnaire was converted to the metabolic equivalent of task hours per week [metabolic equivalent of task (MET)-h/wk][17].

Statistical methods

Continuous and categorical variables were compared across quartiles of DPI scores using analysis of variance (ANOVA) and chi-square tests, respectively. Linear regression was applied in crude and different multi-variable adjusted models to examine the linear association between DPI scores and serum liver enzyme levels. Age, sex (male/female) and the energy intake (kcal/day) were adjusted in the first model; the second model also included BMI (kg/m^2). Additional adjustments were performed for physical activity, smoking and multivitamin supplement use in third model. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS, version 23.0 for Windows, 2006, SPSS, Inc, Chicago, IL, United States). *P* values less than 0.05 were considered statistically significant.

RESULTS

General characteristics of the study participants across quartile scores of DPI are shown in Table 1. There were no significant differences in BMI (P = 0.647), multivitamin mineral supplement use (P = 0.211) and gender (P = 0.071). However, individuals in the first quartile of DPI scores were significantly younger than those in the fourth quartile of DPI scores (46.4 ± 9.1 and 47.5 ± 9.5 y respectively (P < 0.001)) and had significantly lower systolic blood pressure (107.4 ± 16.2 and 109.1 ± 16.4 mm/Hg respectively (P < 0.001)). Those in the first quartile of DPI score also had significantly lower MET than those in the fourth quartile of DPI score (41.3 ± 6.8 and 42.0 ± 6.5 h/week respectively (P < 0.01)). Table 2 shows that there were also no significant differences in the levels of serum ALT (P = 0.225), AST (P = 0.562) and GGT (P = 0.338) between the quartiles of DPI scores. However, the participants in the first quartile of DPI scores had higher ALP levels compared to participants in the fourth quartile (185.7 ± 59.3 and 176.8 ± 52.7 U/L respectively (P < 0.01)). There were also significant differences in serum total cholesterol and low-density lipoprotein cholesterol (LDL-C) concentrations between those with the lowest DPI scores compared to those with the highest (189.9 ± 59.8 and 195.4 ± 39.0 mg/dL and 106.7 ± 55.1 and 111.4 ± 31.2 mg/dL respectively) (both P < 0.01). Fasting blood sugar was also lower in those with the lowest DPI scores compared to those with higher scores (96.0 ± 21.9 and 97.6 ± 32.7 mg/dL (P < 0.01)). Dietary intakes of study participants by quartile score of DPI is shown in Supplementary Table 1. Participants with greater DPI scores had higher intakes of vegetable, fruits, legumes, nuts, olive oils and olives, dairy, and dietary cholesterol (all P < 0.001). In addition, intakes of energy, carbohydrate, protein, fat, monounsaturated fatty acids (MUFA), saturated fatty acids (SFAs), and polyunsaturated fatty acids (PUFAs) were significantly lower among the subjects within the fourth quartile of DPI score compared to the first quartile (all P < 0.001). Vitamin B12, vitamin A and vitamin C intake were significantly higher among individuals in the fourth quartile of DPI score in comparison to the first quartile (all P < 0.001), but folate, magnesium, calcium, iron, vitamins B6, Niacin, Riboflavin and Thiamin intake were significantly higher among individuals in the first quartile of DPI score in comparison to the fourth quartile (all P < 0.001). The relationships between score of DPI and levels of liver enzymes are presented in Table 3. There was an inverse association between score of DPI and serum ALP levels in our unadjusted model (β = -0.05; 95%CI (-0.43 to -0.15) P < 0.001). Additionally, this inverse correlation remained significant after adjustment for confounding factors in Model I (β = -0.04 95%CI (-0.39 to -0.07); P < 0.01), Model II (β = -0.04 95%CI (-0.39 to -0.08); P < 0.01), Model III (β = -0.03 95%CI (-0.34 to -0.03); P < 0.019) and Model IV (β = -0.03 95%CI (-0.35 to -0.04) P = 0.014. No statistically significant

Table 1 General characteristics of study participants by quartiles score of dietary phytochemical index

	Q4 (N = 1278)	Q3 (N = 1278)	Q2 (N = 1278)	Q1 (N = 1277)	*P* value[1]
Age (Yr)	47.5 ± 9.5	45.9 ± 8.8	45.5 ± 8.8	46.4 ± 9.1	< 0.001
BMI (kg/m^2)	28.2 ± 4.9	28.3 ± 7.7	28.2 ± 10.9	27.9 ± 5.0	0.647
Waist circumference (cm)	94.1 ± 11.8	94.3 ± 11.5	94.2 ± 11.4	94.7 ± 12.1	0.668
WHR	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.8	0.9 ± 0.3	0.021
Systolic blood pressure (mm/Hg)	109.1 ± 16.4	106.8 ± 15.8	106.7 ± 15.1	107.4 ± 16.2	< 0.001
Diastolic blood pressure (mm/Hg)	67.3 ± 11.0	66.8 ± 11.0	66.8 ± 10.3	67.2 ± 10.6	0.451
Metabolic equivalent of task (h/wk)	42.0 ± 6.5	41.0 ± 6.6	41.6 ± 7.2	41.3 ± 6.8	< 0.01
WSI	0.2 ± 0.7	0.2 ± 0.7	0.2 ± 0.7	0.1 ± 0.7	< 0.001
Gender men (%)	578 (45.2)	543 (42.5)	609 (47.7)	548 (45.7)	0.071
Smoking					< 0.01
Never smoker (%)	1086 (85.0)	1088 (85.1)	1045(81.8)	1052 (82.4)	
Current smoker (%)	106 (8.3)	128 (10.0)	161 (12.6)	140 (11.0)	
Ex_smoker (%)	86 (6.7)	62 (4.9)	72 (5.6)	85 (6.7)	
Education					< 0.001
Uneducated and elementary (%)	536 (41.9)	506 (39.6)	501 (39.2)	612 (48.0)	
Middle and high school	487 (38.2)	535 (38.2)	541 (42.3)	472 (37.0)	
University or college degree	224 (17.5)	212 (16.6)	195 (15.3)	168 (13.2)	
Postgraduate	31 (2.4)	25 (2.0)	40 (3.1)	24 (1.9)	
Multivitamin mineral use					0.211
Never (%)	1244 (97.4)	1242 (97.2)	1231 (96.5)	1245 (97.6)	
Daily (%)	1 (0.1)	0	2 (0.2)	1 (0.1)	
Weekly (%)	4 (0.3)	1 (0.1)	1 (0.1)	0	
Monthly (%)	1 (0.1)	4 (0.3)	2 (0.2)	0	
Yearly (%)	27 (2.1)	31(2.4)	39 (3.1)	30 (2.4)	
Metabolic syndrome	213 (16.7)	225 (17.6)	216 (16.9)	233 (18.2)	0.713

[1]Obtained from one way Anova or Chi-squared tests for continuous and categorical variables, respectively.
Values are means ± SDs or *n* (%).
DPI: Dietary phytochemical index; BMI: Body mass index; WHR: Waist-to-hip ratio; WSI: Wealth score index.

association was found between levels of ALT, AST, GGT and score of DPI in the crude mode, which remained non-significant after adjustment for potential confounders.

DISCUSSION

In this study we aimed to determine if increased phytochemical consumption, consequently resulting in an increased 'dietary phytochemical index' (DPI) score, would be predictive of improved liver function in Iranian adults. Our principal finding was that DPI score was inversely related to ALP and that this relationship persisted, even after adjusting for multiple variables. No such relationship existed with the other liver enzymes, which were similar across the DPI quartile scores. Furthermore, perhaps unsurprisingly, there were also several differences in the overall dietary intake of the participants. Principally, those with higher DPI scores consumed more foods and nutrients suggestive of largely 'healthy' dietary patterns, as opposed to those with lower scores, who consumed greater quantities of foods and nutrients often associated with 'unhealthy' dietary patterns.

The homogeneity in levels of ALT, AST and GGT between those with varied DPI scores is a finding which is not in agreement with the literature. Previous studies have suggested that phytochemical consumption is associated with improvements in these particular liver enzymes[1]; however, this was

Table 2 Biochemical parameters of study participants by quartiles score of dietary phytochemical index

	Q4 (N = 1278)	Q3 (N = 1278)	Q2 (N = 1278)	Q1 (N = 1277)	*P* value[1,2]
ALT (U/L)	22.6 ± 15.4	22.3 ± 17.1	23.2 ± 17.3	21.9 ± 16.7	0.225
AST (U/L)	19.0 ± 7.4	19.0 ± 10.32	19.4 ± 8.5	19.1 ± 7.5	0.562
GGT (U/L)	27.9 ± 22.8	26.5 ± 23.5	27.2 ± 23.0	26.2 ± 27.7	0.338
ALP (U/L)	176.8 ± 52.7	180.5 ± 50.3	181.0 ± 49.7	185.7 ± 59.3	< 0.01
Cholesterol (mg/dL)	195.4 ± 39.0	191.9 ± 40.6	189.5 ± 38.8	189.9 ± 59.8	< 0.01
Triglyceride (mg/dL)	162.4 ± 96.4	155.0 ± 100.8	157.5 ± 99.8	152.5 ± 96.2	0.075
HDL-C (mg/dL)	53.6 ± 12.3	54.0 ± 12.1	52.9 ± 12.0	53.8 ± 12.4	0.116
LDL-C (mg/dL)	111.4 ± 31.2	107.9 ± 31.2	106.4 ± 30.9	106.7 ± 55.1	< 0.01
FBS (mg/dL)	97.6 ± 32.7	95.5 ± 20.0	94.4 ± 15.9	96.0 ± 21.8	< 0.01
BUN (mg/dL)	27.3 ± 7.6	27.0 ± 7.3	26.9 ± 7.1	27.4 ± 7.0	0.241
Creatinine (mg/dL)	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	0.135

[1]Values are means ± SDs.
[2]Obtained from one way Anova.
DPI: Dietary phytochemical index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transferase; ALP: Alkaline phosphatase; HDL: High-density lipoprotein; LDL: Low density lipoprotein; FBS: Fast blood sugar; BUN: Blood urea nitrogen.

not the case in the present study for reasons which are not entirely clear. This is also unexpected given the higher proportion of foods such as fruits, vegetables and nuts which were consumed by those with the highest DPI scores. Food items such as these have previously been associated with a more favourable overall dietary pattern; a feature associated with a decreased risk of NAFLD[18-21]. Furthermore, a reduction in calorie intake accompanied by a concomitant decrease in carbohydrate consumption, both factors which occurred in those with higher DPI scores in the present study, has also been previously associated with improvements in transaminase levels[20].

Despite the unexpected similarities in most liver enzymes, it is important to note that the concentration of ALP was significantly lower in those with higher DPI scores, even after adjusting for energy intake. This aligns with previous literature which also demonstrated that the consumption of phytochemicals is associated with improvements in ALP[22-27]. However, these studies have typically been carried out in animal models with the aim of alleviating hepatic damage resulting from aging and/or pharmacological agents *via* the delivery of phytochemical rich extracts as opposed to the dietary consumption of these compounds per se. Furthermore, as there is a dearth of human studies comparing the impact of dietary phytochemicals upon markers of liver damage it is difficult to make direct comparisons with the improvements in ALP shown by us. Despite this, it is noteworthy that this finding persisted after adjusting for a range of other variables, including metabolic syndrome. This is important, especially given the high rates of the disorder in Iran, its suspected role in NAFLD and because ALP is routinely utilised as a marker for liver disease[7,28]. More specifically, elevated ALP of hepatic origin is used as a marker of primary biliary cirrhosis which can indicate cholestatic liver disease[29]. This is possibly perpetuated by a decline in the tissue-specific environment, such as a deterioration of the HCO_3 umbrella; however, the exact mechanisms have yet to be fully elucidated[29]. As such, the driving factors underpinning the inverse association between DPI score and ALP found in the present study remain unknown.

While there are potentially hepatoprotective benefits of consuming a diet rich in dietary phytochemicals, it is noteworthy that those with higher DPI scores also had increased levels of serum cholesterol and LDL-C, as well as higher levels of fasting blood sugar and systolic blood pressure, even when these individuals were comprised of fewer current smokers and had similar rates of metabolic syndrome compared to those with lower DPI scores. These abnormalities associated with phytochemical consumption are not in agreement with previous research, with a recent review of the literature highlighting how these compounds are generally considered to infer positive effects upon a range of clinical markers including body weight, waist circumference and blood pressure and glucose[1]. Therefore, perhaps some of the observed cardiometabolic abnormalities may be related to other less favourable dietary factors. For example, those with higher DPI scores consumed a higher proportion of total dietary fats than those with lower scores and a smaller quantity of MUFA and PUFA per gram. Consumption of MUFA and PUFA are well known to favourably modulate both the concentration and size of LDL particles[30,31]. However, in the case of the current study it could be hypothesised that perhaps the reduced intake per gram of these fatty acids, along with the percentage increase in SFA intake may override any benefits normally attributed to MUFA and PUFA, leading to a net increase in

Table 3 Linear regression analysis of the correlations between score of dietary phytochemical index and levels of liver enzymes.

	P value	B (95%CI)
ALT		
Crude	0.474	0.02 (-0.002 to 0.06)
Model I	0.063	0.02 (-0.002 to 0.09)
Model II	0.109	0.02 (-0.009 to 0.08)
Model III	0.1	0.02 (-0.008 to 0.08)
Model IV	0.097	0.02 (-0.007 to 0.08)
AST		
Crude	0.751	-0.004 (-0.02 to -0.01)
Model I	0.902	-0.002 (-0.02 to 0.02)
Model II	0.773	-0.005 (-0.02 to 0.02)
Model III	0.9	-0.002 (-0.02 to 0.02)
Model IV	0.917	-0.002 (-0.02 to 0.02)
GGT		
Crude	0.154	0.02 (-0.01 to 0.11)
Model I	0.328	0.01 (-0.03 to 0.10)
Model II	0.433	0.01 (-0.04 to 0.09)
Model III	0.366	0.01 (-0.03 to 0.10)
Model IV	0.365	0.01 (-0.03 to 0.10)
ALP		
Crude	< 0.001	-0.05 (-0.43 to -0.15)
Model I	< 0.01	-0.04 (-0.39 to -0.07)
Model II	< 0.01	-0.04 (-0.39 to -0.08)
Model III	0.019	-0.03 (-0.34 to -0.03)
Model IV	0.014	-0.03 (-0.35 to -0.04)

Model I: Adjusted for energy intake, age and gender; Model II: Additionally adjusted for body mass index; Model III: Additionally adjusted for physical activity, supplement or multivitamin use, smoking, education and wealth score index; Model IV: Additionally adjusted for metabolic syndrome. DPI: Dietary phytochemical index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transferase; ALP: Alkaline phosphatase.

LDL-C.

Limitations and strengths

This study has several strengths. These being that the study is the first of its kind to investigate the associations between DPI score and liver function in an Iranian adult population, using a large sample size. Despite this our study has several limitations. These being that the study design is cross-sectional in nature, meaning that the findings cannot detect changes across time, which is important since the impact of diet is often only detected through longitudinal measurements. Similarly, as the participants in the study resided in a single province it is unlikely that our findings are extrapolatable to the Iranian population. Furthermore, the DPI scoring technique only creates an approximate estimation of the consumption of dietary phytochemicals and excludes certain items, which have no calorific value, such as tea, which is consumed in high quantities in Iran[10,32]. Also, the scoring technique fails to consider that the ratio of phytochemicals to calories varies greatly in plant foods, as do the health promoting properties of individual phytochemicals[10]. In addition to this, the logistic regression analysis, although offering insights into the relationship between dietary phytochemical intake and liver function, can succumb to high intercorrelations as well as residual confounding, potentially leading to the production of spurious relationships. It is also important to remember that certain medications may also have impacted upon our findings and although a medication history was taken when the PERSIAN cohort study was conducted this data was not included in our analysis. Finally, as our study is

ecological in nature, the exact biological mechanisms underpinning our findings are subject to speculation and further research to elucidate these aspects is therefore warranted.

CONCLUSION

To summarise, our findings reveal that an increased consumption of dietary phytochemicals is associated with beneficial reductions in serum ALP in an Iranian adult population. Furthermore, we also demonstrate that a higher DPI score is also accompanied with an overall increase in consumption of food items and nutrients associated with healthier dietary patterns. Despite these positive aspects, our findings also showed that those with higher DPI scores presented several metabolic disturbances compared to counterparts with lower scores. This suggests that there may be hepatoprotective effects associated with increased consumption of phytochemicals, but further research is required to determine the validity of these findings as well as any factors that may be driving unexpected metabolic abnormalities.

ARTICLE HIGHLIGHTS

Research background

Dietary phytochemicals are plant-derived bioactive compounds. It has been previously suggested that these compounds may be hepatoprotective; however, the existing literature concerning this is equivocal.

Research motivation

In addition to the debatable hepatoprotective nature of dietary phytochemicals, there has been little research investigating this specifically in an Iranian population.

Research objectives

To determine the if the intake of dietary phytochemicals is hepatoprotective.

Research methods

Participants recruited to the PERSIAN cohort study were asked to complete a validated food frequency questionnaire. We applied a dietary phytochemical index to this data in order to categorise participants based upon their phytochemical intake. We then used linear regression to investigate the association between the dietary phytochemical index and levels of liver enzymes using both crude and adjusted models.

Research results

We found significant and inverse associations between dietary phytochemical intake and alkaline phosphatase. This is possibly indicative of improved liver function. We also found that participants with higher intakes of dietary phytochemicals also had an overall healthier dietary pattern yet increased levels of serum cholesterol, low-density lipoprotein cholesterol, blood pressure and blood glucose.

Research conclusions

Although there may be hepatoprotective effects associated with increased dietary phytochemical intake in addition to a healthier overall dietary pattern, these may be accompanied by a a number of metabolic abnormalities.

Research perspectives

Future research should seek to determine the validity of our findings and to elucidate any factors which may be responsible for any metabolic abnormalities associated with an increased intake of dietary phytochemicals.

FOOTNOTES

Author contributions: Darabi Z, Mozaffari-Khosravi H, Mirzaei M, Khayyatzadeh SS, and Mazidi M conceived and designed the study, acquired and analysed the data; Darabi Z and Webb RJ interpreted the data and wrote the first draft of the manuscript; All authors critically revised the manuscript.

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ORIGINAL ARTICLE

Prospective Study

Prospective validation to prevent symptomatic portal vein thrombosis after liver resection

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Abstract

BACKGROUND

Portal vein thrombosis (PVT) after liver resection is rare but can lead to life-threatening liver failure. This prospective study evaluated patients using contrast-enhanced computed tomography (E-CT) on the first day after liver resection for early PVT detection and management.

AIM

To evaluate patients by E-CT on the first day after liver resection for early PVT detection and immediate management.

METHODS

Patients who underwent liver resection for primary liver cancer from January 2015 were enrolled. E-CT was performed on the first day after surgery in patients undergoing anatomical resection, multiple resections, or with postoperative bile leakage in the high-risk group for PVT. When PVT was detected, anticoagulant therapy including heparin, warfarin, and edoxaban was administered. E-CT was performed monthly until PVT resolved.

RESULTS

The overall incidence of PVT was 1.57% (8/508). E-CT was performed on the first day after surgery in 235 consecutive high-risk patients (165 anatomical resections, 74 multiple resections, and 28 bile leakages), with a PVT incidence of 3.4% (8/235). Symptomatic PVT was not observed in the excluded cohort. Multivariate analyses revealed that sectionectomy was the only independent predictor of PVT [odds ratio (OR) = 12.20; 95% confidence interval (CI): 2.22-115.97; $P = 0.003$]. PVT was found in the umbilical portion of 75.0% (6/8) of patients, and sectionectomy on the left side showed the highest risk of PVT (OR = 14.10; 95%CI: 3.17-62.71; $P < 0.0001$).

CONCLUSION
Sectionectomy on the left side should be chosen with caution as it showed the highest risk of PVT. E-CT followed by anticoagulant therapy was effective in managing early-phase PVT for 2 mo without adverse events.

Key Words: Portal vein thrombosis; Liver resection; Anatomical resection; Anticoagulant therapy; Hepatocellular carcinoma; Umbilical potion

Core Tip: This prospective study evaluated patients by contrast-enhanced computed tomography (E-CT) on the first day after liver resection for early portal vein thrombosis (PVT) detection and immediate management. Sectionectomy on the left side should be treated with caution as it showed the highest risk of PVT. E-CT on the first day and immediate anticoagulant therapy were effective in managing early-phase PVT for 2 mo without adverse events.

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INTRODUCTION

The rate of portal vein thrombosis (PVT) after liver resection occurs in 2.1%-11.1% of cases[1-5] and was unchanged in recent series[6-8] .While no symptoms are detected in the early phase, severe PVT can lead to life-threatening liver failure[9,10]. Immediate anticoagulant therapy after surgery is recommended in some cases[11]. However, patients who undergo liver resection may have coagulation disorders such as hepatocellular carcinoma, which makes it difficult to administer routine anticoagulant therapy. Therefore, early detection and immediate intervention for asymptomatic PVT are essential. The risk factors for PVT after liver resection include hepatic clumping and total operation time[1,2,4,7,8], resection scale and location[1,3], splenectomy[3], and portal vein reconstruction[4,5].

Studies have reported the occurrence of postoperative PVT in high-risk[1-3,7,8] or symptomatic cases in the first 5-7 d after liver resection[4,6]. However, there is a lack of information regarding the timing of PVT occurrence, approaches for its early detection, and the efficacy of early interventions. No study has focused on early detection and intervention before symptomatic PVT. Therefore, we conducted a prospective study and assessed patients considered to be at high risk for PVT after liver resection using screening with contrast-enhanced computed tomography (E-CT). E-CT was performed on the first day after liver resection, and immediate anticoagulant therapy was started when PVT was detected. We assessed the frequency, predictive factors, and efficacy of early intervention for PVT.

MATERIALS AND METHODS

Patients

This study was conducted in patients who underwent liver resection for primary liver cancer from January 2015 onward. E-CT was performed on the first day after surgery for patients at high risk of PVT, including those undergoing: Anatomical resection, multiple resections, or postoperative bile leakage. In previous studies, the frequency of PVT was hypothesized to be 5%. Consequently, a total of 400 resections was expected to detect 20 PVT events[1]. Our institution performs an average of 150 liver resections for primary liver cancer annually; thus, the study was estimated to take 3 years to complete. This study excluded patients with metastatic liver cancer, bile duct or portal vein reconstruction, laparoscopic surgery, contrast medium allergy, renal dysfunction, or thrombus requiring anticoagulant therapy. The liver, lungs, and legs of patients with abnormal preoperative serum D-dimer levels were screened by ultrasound and E-CT.

Surgical procedure and postoperative management

Liver resection was performed under general anesthesia with hypoventilation during liver transection. Total hepatic inflow occlusion was performed using Pringle's maneuver, in which the protocol called for a 15-min blood flow clamp and a 5-min release[12,13]. The liver parenchyma was transected using the

crush-clamping method, and the vessels were ligated using an energy device or silk thread[14-16]. At the end of the operation, routine intraoperative ultrasonography was performed to detect any thrombus in the liver. A drain was placed on the resected stump of the liver and removed 3 d after the operation [17]. Elastic bandages and intermittent air compression on the legs were routinely used to prevent thrombosis.

Evaluation of PVT and anticoagulant therapy

PVT was defined as a defect image that was confirmed in the portal phase of dynamic CT. The images were checked by a surgeon (Yoshida N) and 2 independent radiologists. When PVT was detected, anticoagulant therapy was performed according to the following protocol: Unfractionated heparin (10000-12000 units/d), warfarin (2 mg/d), and direct oral anticoagulant edoxaban tosylate hydrate (LIXIANA®, 30 mg/d). The target range of anticoagulant therapy was an activated partial thromboplastin time of 50-60 s and a prothrombin time-international normalized ratio of 1.5-2.0. In small cases of PVT, short half-life heparin was used just after the detection of PVT by E-CT, whereas in cases of large PVT, intravenous heparin combined with oral anticoagulation (warfarin or direct oral anticoagulant) was used as an initial treatment. Heparin was then changed to an oral anticoagulant 10 d after the operation. E-CT was performed in all treated cases for 1 wk after starting anticoagulant therapy to confirm the severity or new appearance of PVT. Anticoagulant therapy was continued when the PVT had disappeared by monthly E-CT after discharge. Patients who had no PVT on E-CT the first day after surgery did not undergo E-CT until discharge. All patients received E-CT at 2 or 3 mo after discharge as a routine follow-up post liver resection.

Statistical analyses

Statistical analyses were performed using JMP 10.0.2 (SAS Institute Inc., Cary, NC, United States). Data are expressed as the mean ± standard error of the mean. Categorical variables were compared using χ^2 tests, whereas continuous variables were compared using nonparametric Wilcoxon or parametric *t*-tests. Statistical significance was set at $P \leq 0.05$. Risk factors evaluated by univariate logistic regression ($P < 0.05$) were included in the multivariate analysis to determine the independent risk factors. This study protocol was approved by the ethics committee of Nihon University School of Medicine (Protocol No. RK-170214-3; Tokyo, Japan) and was registered in the UMIN Clinical Trials Registry under entry number UMIN000047362.

RESULTS

PVT analyses using patient flow

This study was started in January 2015 and discontinued in December 2018 after 4 years because the occurrence of PVT was lower than our hypothesis. The incidence of PVT was 8 (1.57%) among the 508 participants over the study period. E-CT was performed on the first day after liver resection in 235 patients (165 for anatomical resections, 74 for multiple resections, and 28 for bile leakage). The incidence of PVT in the high-risk group was 3.4% (8/235) (Figure 1). No cases of symptomatic PVT occurred in patients who met the exclusion criteria.

Risk factors associated with PVT after liver resection

In univariate analyses, the occurrence of PVT was significantly higher in women (50.0% *vs* 79.7%; $P = 0.04$) and in patients who had undergone sectionectomy (75.0% *vs* 24.2%; $P = 0.001$). In multivariate analyses, sectionectomy [odds ratio (OR) = 12.2, 95% confidence interval (CI): 2.22-115.97; $P = 0.003$] was the only independent predictor of PVT occurrence (Table 1). There were 2 postoperative deaths, neither of which was related to PVT. One patient with pneumonia underwent a pathological autopsy and did not show thrombus anywhere. One patient with liver failure and no PVT was observed on E-CT during the study.

PVT frequency and distribution

Six of the eight patients with PVT underwent anatomical resection. Most instances of PVT were found on the left side of the liver, with 62.5% (5/8) around the umbilical portion (UP) and one in the apex of the UP (12.5%). Only one instance was found at the resection stump of segment 8 and one in the anterior branch (Figure 2). All PVTs at the UP were found in the second-to third-order branches, with preserved peripheral blood flow from the thrombus at the time of detection. Among the procedures, 3 patients among 26 who underwent left lateral sectionectomy developed PVT (11.5%). PVT also developed in 2 of 3 patients undergoing left medial sectionectomy (66.7%) and in 1 of the 25 patients who underwent right posterior sectionectomy (4%). Among the procedures, sectionectomy on the left side was associated with the highest risk of PVT (OR = 14.10; 95%CI: 3.17-62.71; $P < 0.0001$) (Table 2).

Table 1 Risk factors associated with portal vein thrombosis after liver resection

Variables	Univariate analyses			Multivariate analyses		
	PVT (+), *n* = 8	PVT (-), *n* = 227	*P* value	OR	95%CI	*P* value
Back ground characteristics						
Age	69.9 ± 3.4	67.8 ± 0.6	0.54			
Male sex	4 (50.0%)	181 (79.7%)	0.04	0.25	0.05-1.29	0.10
Hepatocellular carcinoma (+)	7 (87.5%)	190 (73.7%)	0.77			
Multiple tumor	2 (25.0%)	72 (31.7%)	0.69			
Heavy drinker	2 (25.0%)	55 (24.2%)	0.96			
Portal vein embolization	0	12 (5.3%)	0.50			
Body mass index	24.8 ± 1.2	23.7 ± 0.2	0.39			
Preoperative anticoagulant therapy	1 (12.5%)	10 (4.4%)	0.29	7.95	0.32-106.81	0.17
Diabetes mellitus	2 (25.0%)	92 (40.5%)	0.38			
HBsAg (+)	1 (12.5%)	39 (17.2%)	0.73			
HCVAb (+)	2 (25.0%)	52 (22.9%)	0.89			
Platelet count as × 10^4/μL	18.6 ± 7.8	21.4 ± 1.5	0.72			
Albumin in g/dL	4.2 ± 0.2	4.2 ± 0.03	0.79			
Total bilirubin in mg/dL	0.7 ± 0.2	0.7 ± 0.03	0.92			
Prothrombin time, %	93.8 ± 3.8	93.4 ± 0.7	0.94			
D-dimer in ng/mL	1.7 ± 0.8	1.7 ± 0.2	0.97			
ICGR15, %	15.0 ± 2.2	11.7 ± 0.4	0.14	0.91	0.82-1.02	0.09
Liver damage B	0	10 (6.3%)	0.53			
Procedure						
Anatomical resection	6 (75.0%)	159 (70.0%)	0.76			
Segmentectomy	0	48 (21.2%)	0.14			
Sectiontectomy	6 (75.0%)	55 (24.2%)	0.001	12.20	2.22-115.97	0.003
Hemihepatectomy	0	56 (24.7%)	0.11			
Surgical outcomes						
Operatin time in min	324 ± 40	374 ± 8	0.21			
Hepatic clamping time in min	76 ± 15	89 ± 3	0.38	1.01	0.99-1.03	0.56
Blood loss in g	517 ± 186	455 ± 35	0.74			
RBC transfusion	1 (12.5%)	13 (5.7%)	0.43			
Surgical results						
Complication CD ≥ 3a	3 (37.5%)	50 (22.0%)	0.30			
Bile leakage	2 (25.0%)	26 (11.5%)	0.25	3.12	0.35-21.58	0.28
Mortality	0	2 (0.9%)	0.79			
Histological cirrhosis	1 (12.5%)	32 (14.1%)	0.90			

CD: Clavian-Dindo classification; CI: Confidence interval; HBsAg: Hepatitis B surface antigen; HCVAb: Hepatitis C virus antibody; OR: Odds ratio; PVT: Portal vein thrombosis; RBC: Red blood cell.

Treatment and outcomes

No adverse events were observed with anticoagulant therapy, and all patients were successfully treated (Table 3 and Figure 3). One patient (case 5) stopped treatment because the thrombus did not extend to the central side. The median period of PVT treatment was 55 d (range: 37-140 d).

Table 2 Comparisons of left and right sides for each procedure

		PVT (+), *n* = 8	PVT (-), *n* = 227	*P* value	OR	95%CI
Partial resection		2 (25.0%)	68 (30.0%)	0.76	0.78	0.15-3.96
Segmentectomy	Left	-	8 (3.5%)	0.59		
	Right	-	40 (17.6%)	0.19		
Sectiontectomy	Left	5 (62.5%)	24 (10.6%)	< 0.0001	14.10	3.17-62.71
	Right	1 (12.5%)	31 (13.7%)	0.93	0.90	0.11-7.60
Hemihepatectomy	Left	-	37 (16.3%)	0.21		
	Right	-	19 (8.4%)	0.39		

CI: Confidence interval; OR: Odds ratio; PVT: Portal vein thrombosis.

Table 3 Portal vein thrombosis cases, treatments, and outcomes

Case	No. of patients	Age	Sex	Diagnosis	Extent of resection	PVT location	Anticoagulation	Outcome	PVT treatment period in d
1	65	71	Male	HCC	Left medial sectionectomy	UP	Heparin, warfarin	Resolved	53
2	135	83	Male	ICC	Left lateral sectionectomy	Lt branch, UP	Heparin, warfarin	Resolved	37
3	151	50	Female	HCC	Left lateral sectionectomy, partial resection (S1)	UP	Heparin, warfarin	Resolved	63
4	214	75	Female	HCC	Left lateral sectionectomy	UP	Warfarin	Resolved	59
5	385	78	Female	HCC	Partial resection (S8)	P8 stump	Edoxaban	Stable	46
6	391	70	Male	HCC	Right posterior sectionectomy	Anterior branch	Heparin, warfarin	Resolved	44
7	417	75	Female	HCC	Partial resection (S4, S7)	UP	Heparin, edoxaban	Resolved	140
8	475	57	Male	HCC	Left medial sectionectomy	UP	Heparin, edoxaban	Resolved	58

HCC: Hepatocellular carcinoma; ICC: Intrahepatic cholangiocarcinoma; PVT: Portal vein thrombosis; UP: Umbilical potion.

DISCUSSION

The incidence of PVT was lower (1.56%) in this study than in previous reports[3,4,6]. In the high-risk group, E-CT on the first day after surgery had an incidence of the initial phase of asymptomatic PVT of 3.4% (8/235), which was successfully treated with anticoagulant therapy. The results of this study demonstrated that PVT formed as early as the first day after liver resection. To date, most studies have found PVT at 5-7 d after liver resection[1-3,7,8]. In this phase, peripheral blood flow is obstructed and impaired by a large thrombus. Anticoagulant therapy takes a long time to resolve the thrombus, and some patients experience severe liver failure[3,6]. Early anticoagulant therapy is an ideal option for patients at high-risk for PVT; however, no study has reported the initiation of PVT. Consequently, in this study, we aimed to detect early-stage PVT in a high-risk group of patients using E-CT. This enabled the prompt initiation of anticoagulant therapy and thus preserved peripheral blood flow from PVT.

The risk factors for PVT after liver resection include operating procedure or scale[1,3], operation or hepatic clamping time[1,2,4,7,8], portal vein reconstruction, and complications[3,5]. Sectionectomy is the only independent predictor of PVT occurrence. Sectionectomy requires ligation of the second branch root of the liver, which is more complex than hemihepatectomy. With regard to the sites of PVT, six of the eight PVTs identified in this study were found in the UP and 5 patients had undergone sectionectomy on the left side. A previous report suggested a higher PVT incidence in the UP than in other sites, and that left lateral sectionectomy was a risk factor for PVT[6]. Thrombi occur due to three factors (*i.e.*, stasis of blood flow, hypercoagulable state, and endothelial injury), known as Virchow's triad[18]. Blood stasis occurs due to the shape of the UP, which flows dorsal to ventral and caudal to cranial, especially following liver transection. The initial site of PVT is unknown, but this study detected small PVTs in the UP. Turbulent flow caused by reduced liver parenchyma after liver resection and a change

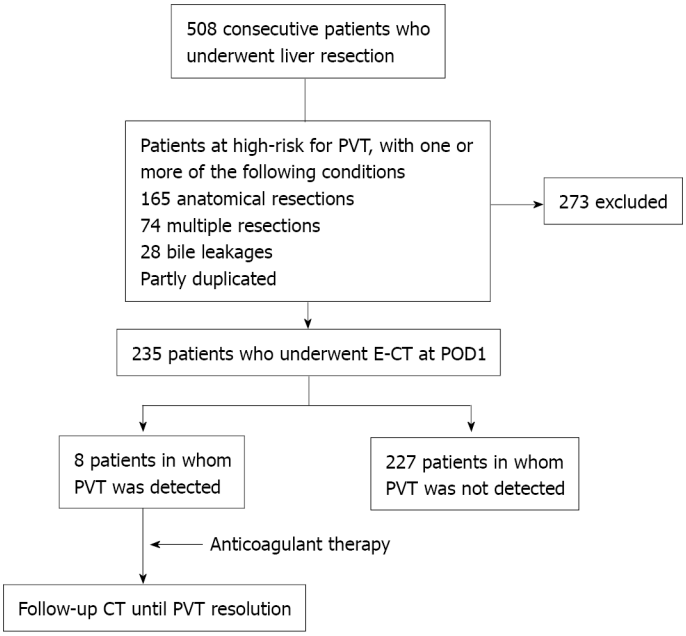

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Figure 1 Patient flow of the analysis of portal vein thrombosis. Contrast-enhanced computed tomography was performed on the first day in 235 patients, who were categorized as high risk by three factors: Anatomical resection (165 patients), multiple resections (74 patients), and bile leakage (28 patients). Eight patients were detected with portal vein thrombosis in the high-risk group. E-CT: Contrast-enhanced computed tomography; POD: Post operative day; PVT: Portal vein thrombosis.

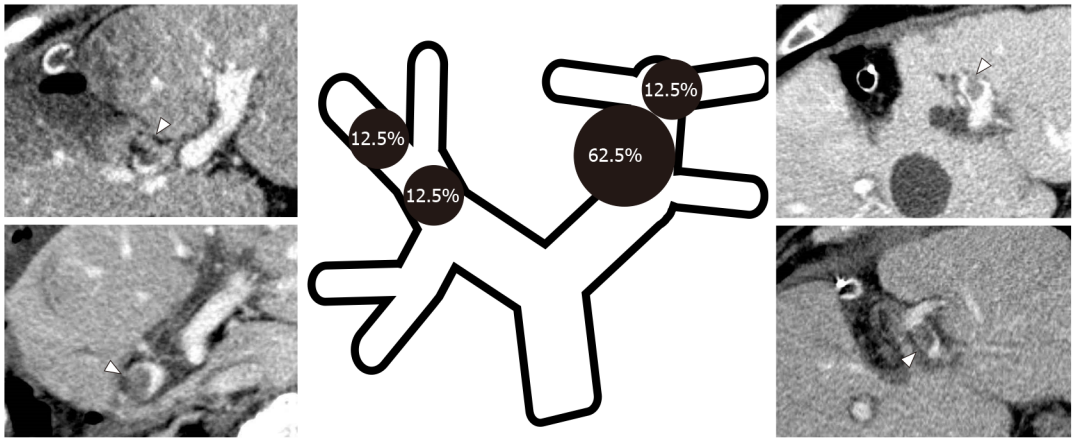

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Figure 2 Portal vein thrombosis distribution and frequency. The sites of portal vein thrombosis included those around the umbilical portion (UP) (62.5%, 5/8 patients) and in the apex of the UP (12.5%, 1 patient), as well as the P8 resection stump (12.5%, 1 patient) and the anterior portal vein branch (12.5%, 1 patient).

in the shape of the UP in cases of sectionectomy on the left side may increase the risk of PVT.

Although this was a prospective study, it had several limitations. First, the incidence of PVT was lower than expected. The statistical power to analyze the risk factors for PVT was not optimal therefore, this protocol is ongoing to analyze larger population in the future. Second, E-CT and anticoagulant therapy were contraindicated. Third, cost-effectiveness is low for E-CT; therefore, routine ultrasonography may be more practical for the high-risk group in our study. Fourth, E-CT alone was insufficient to detect a small PVT on day 1. Therefore, it is necessary to combine coagulation makers and images.

CONCLUSION

In conclusion, sectionectomy on the left side should be cautiously managed, as this patient group showed the highest risk of PVT. Immediate anticoagulant therapy from the first day after surgery is recommended for patients at high risk for PVT.

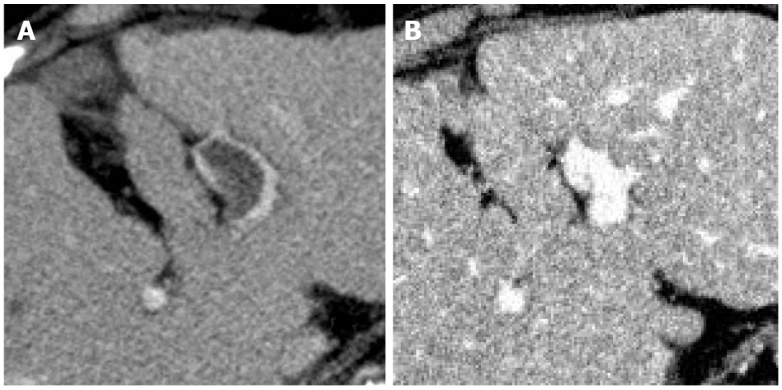

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Figure 3 Before and after anticoagulation therapy. A: In case 7, portal vein thrombosis (PVT) was found in the umbilical portion; B: PVT disappeared 140 d after starting anticoagulant therapy.

ARTICLE HIGHLIGHTS

Research background

Portal vein thrombus (PVT) is one of the potentially lethal complication after liver resection; however, its etiology and the way for immediate treatment is unsettled.

Research motivation

Based on our experience, we tried to resolve hepatic failure due to huge PVT.

Research objectives

The study was conducted in patients who underwent open liver resection for cancer in our institution.

Research methods

Retrospective but retrospectively collected cohort.

Research results

In a total of 235 patients, 8 had major PVT. We successfully treated the patients with anticoagulant therapy without adverse events. No hepatic failure observed through this study.

Research conclusions

Performing enhanced computed tomography (CT) on post-operative day 1 is an effective option to find a thrombi at the portal vein close to the surgical site.

Research perspectives

The early detection of PVT by enhanced CT is a promising way to avoid hepatic failure after liver resection.

FOOTNOTES

Author contributions: Yoshida N collected the patient's data; Yamazaki S designed this study; Masamichi M contributed to the proofreading of manuscript; Okamura Y and Takayama T supervised the writing of the manuscript.

Institutional review board statement: The ethics committee of Nihon University School of Medicine approved this clinical study.

Clinical trial registration statement: This study was registered in the UMIN Clinical Trials Registry under entry number UMIN000047362.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: Authors have no conflict-of-interest of this study.

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SYSTEMATIC REVIEWS

Prognostic non-invasive biomarkers for all-cause mortality in non-alcoholic fatty liver disease: A systematic review and meta-analysis

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Abstract

BACKGROUND

Non-alcoholic fatty liver disease (NAFLD) represents a growing public health concern, with patients having higher risk of morbidity and mortality. It has a considerably high prevalence in the general population, estimated 20%-40% in Europe, and is asymptomatic until late in the disease course. It is therefore important to identify and validate tools that predict hard outcomes such as mortality for use in clinical practice in risk-stratifying NAFLD patients.

AIM

To evaluate available evidence on the use of non-invasive test(s) as prognostic factors for mortality in NAFLD.

METHODS

We performed electronic searches of Medline and EMBASE (Ovid) until 7th

January 2021 of studies in NAFLD populations. Prognostic markers included serum biomarkers, non-invasive scoring systems, and non-invasive imaging. The population included all spectrums of disease severity, including NAFLD and non-alcoholic steatohepatitis (NASH). Outcomes included all-cause, and cardiovascular mortality. All non-invasive tests were synthesised in a narrative systematic review. Finally, we conducted a meta-analysis of non-invasive scoring systems for predicting all-cause and cardiovascular mortality, calculating pooled hazard ratios and 95% confidence (STATA 16.1).

RESULTS

Database searches identified 2850 studies – 24 were included. 16 studies reported non-invasive scoring systems, 10 studies reported individual biomarkers, and 1 study reported imaging modalities. 4 studies on non-invasive scoring systems (6324 participants) had data available for inclusion in the meta-analysis. The non-invasive scoring system that performed best at predicting all-cause mortality was NAFLD fibrosis score (NFS) [pHR 3.07 (1.62-5.83)], followed by fibrosis-4 index [pHR 3.06 (1.54-6.07)], BARD [pHR 2.87 (1.27-6.46)], and AST to platelet ratio index [pHR 1.90 (1.32-2.73)]. NFS was also prognostic of cardiovascular-related mortality [pHR 3.09 (1.78-5.34)].

CONCLUSION

This study reaffirms that non-invasive scoring systems, especially NFS, are reliable prognostic markers of all-cause mortality and cardiovascular mortality in NAFLD patients. These findings can inform clinical practice in risk stratifying NAFLD patients.

Key Words: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Biomarkers; Non-invasive; Prognosis; Mortality

Core Tip: Non-alcoholic fatty liver disease (NAFLD) represents a growing public health concern, with an estimated prevalence in European general populations of 20%-40% and epidemiological projections of significant future increase in prevalence. NAFLD patients are at increased risk of morbidity and mortality, so it's important to validate non-invasive prognostic markers for predicting mortality in NAFLD. This systematic review highlighted several non-invasive prognostic markers including biomarkers and imaging modalities. This meta-analysis showed that NAFLD fibrosis score is a useful prognostic marker for all-cause and cardiovascular mortality, which can be implemented in clinical practice to risk stratify and target high risk NAFLD patients.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) represents a growing public health concern, with an estimated prevalence in European general populations of 20%-40% in various studies[1], and epidemiological projections of significant future increase in prevalence[2] owing to its bidirectional association with other growing metabolic diseases such as type 2 diabetes mellitus (T2DM) and dyslipidaemia[3]. Epidemiological studies have shown that NAFLD patients have higher risk of morbidity, and mortality from all causes, and specifically from cardiovascular and liver-related causes[4-6]. Hence, it is important to identify and validate tools that predict hard outcomes such as mortality for use in clinical practice.

NAFLD is characterised by excessive hepatic fat accumulation, defined by the presence of steatosis in > 5% of hepatocytes. This can be ascertained histologically, by liver biopsy, or radiologically, including by magnetic resonance imaging (MRI) or proton magnetic resonance spectroscopy (^{1}H-MRS). NAFLD encompasses a wide spectrum of liver disease ranging from non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH). NASH includes liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC), which carries a significantly worse clinical prognosis[7]. Liver biopsy is the gold standard for histological assessment of NAFLD. Fibrosis, quantified by liver biopsy, has been validated as being an

important prognostic measure of disease-related outcomes and mortality[8,9]. However, liver biopsy has well-established limitations including its risk and availability, making it a less than ideal tool for widespread and repeated use in clinical practice and clinical research.

International guidelines now recommend the use of ultrasound, non-invasive biomarkers and scoring systems as reliable and validated tools to diagnose NAFLD[7]. There are no guidelines advocating use of non-invasive tests for prognostic purposes in NAFLD. Increasing the evidence available on the prognostic value of non-invasive tests for mortality in NAFLD would facilitate their inclusion in international guidelines, which would facilitate risk stratification of NAFLD patients and more intensive targeting of higher risk groups.

Several non-invasive scores for liver fibrosis that combine serum tests, clinical and demographic data have been developed and validated to stratify levels of liver fibrosis. These include the NAFLD fibrosis score (NFS), fibrosis-4 index (FIB-4), and Enhanced Liver Fibrosis score (ELF). The association between biopsy-proven liver fibrosis and more advanced disease, with poor outcomes of morbidity and mortality has been well-described. In particular, a recent systematic-review and meta-analysis of studies on patients with NAFLD or NASH found that fibrosis stage measured by liver biopsy had an unadjusted increased risk of all-cause mortality, liver-related mortality, liver transplantation, and liver-related events[9]. Given that increasing degree of biopsy-proven liver fibrosis is associated with mortality, it is plausible that non-invasive measures of liver fibrosis might find the same association. Indeed, studies have shown that non-invasive scoring systems can predict important clinical outcomes such as mortality. One systematic review and meta-analysis of 5 studies of NFS and all-cause mortality has successfully validated the association[10]. A second systematic review and meta-analysis has evaluated the association between NFS, and a further two scoring systems, FIB-4 and AST to platelet ratio index (APRI), and mortality. This also confirmed the association between NFS and mortality, in a meta-analysis of 5 studies, but did not find an association between APRI or FIB-4 and mortality[11]. Finally, a recent systematic review and meta-analysis of non-invasive scoring systems (NFS, APRI, FIB-4, and BARD) and histological scoring systems associated with important clinical outcomes, including liver disease decompensation and mortality, once again validated the prognostic ability of NFS in relation to mortality but rejected that of the remaining scoring systems[12]. These studies have not evaluated the ability of scoring systems to predict cause-specific mortality, namely cardiovascular or liver-related mortality; two of the commonest causes of death in NAFLD patients.

The pathophysiology of NAFLD is multifactorial and multisystem. In addition to genetic predisposition, there is close relation to endocrine and metabolic dysfunctions[13-16]. This suggests several factors are at play in disease progression and poor outcomes, and several biomarkers not necessarily specific to liver function may be useful in outcome prediction. Overall, in clinical practice, different non-invasive markers can be combined to achieve a series of clinical uses. This includes, in primary care, identifying those individuals who have risk factors for metabolic syndrome and insulin resistance and are therefore at higher risk of having NAFLD. EASL guidelines presently recommend that individuals with insulin resistance or features of metabolic syndrome should undergo diagnostic procedures for NAFLD. The individual markers quoted are waist circumference, arterial pressure, serum triacylglycerols, fasting glucose and HDL cholesterol. Guidelines also recommend individuals with obesity, which can be defined as BMI > 30, as well as persistently raised liver enzymes should be screened for NAFLD. It is plausible such markers could also have a prognostic implication in patients with NAFLD. Some studies have successfully addressed this issue, linking the presence of T2DM and insulin resistance, as well as renal impairment, with poor outcomes, including mortality, in NAFLD patients[17,18].

Certain genetic polymorphisms have also been implicated in susceptibility to NAFLD and, indeed, more severe disease. The clinical application of this is currently limited, though guidelines do mention genotyping may be considered in select cases and in clinical studies. An example of a potential future clinical application of genotyping in NAFLD is a clinical study in 152 children that developed a risk score based on 4 genetic polymorphisms that predicts presence of NASH[19].

EASL guidelines advocate the use of ultrasonography as a first line diagnostic test for hepatic steatosis, seeing as, despite its limited ability to detect low grade steatosis, it is reliable in identifying moderate and severe steatosis. It is preferred in clinical practice to MRI due to lower cost and better availability. Non-invasive imaging modalities including ultrasound, elastography and MRI have been shown in individual studies to play an important prognostic role for clinically significant outcomes such as mortality[20,21]. To date, no systematic review has evaluated the prognostic use of these non-invasive modalities in NAFLD.

The main aim of this systematic review was to evaluate available evidence on the use of any non-invasive test, including serum biomarkers, non-invasive scoring systems, and imaging modalities, in predicting all-cause mortality, and disease-specific mortality, in NAFLD. We aimed to validate one or a combination of measures that can be used as prognostic factors for mortality in NAFLD.

MATERIALS AND METHODS

The systematic review was conducted following iPreferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) process[22] and was registered with the International Prospective Register of Systematic Reviews (PROSPERO) and protocol for this systematic review was published on the PROSPERO website.

Registration number: CRD42020201207.

Search strategy

We searched MEDLINE *via* OvidSP (January 1946 to 7th January 2021) and EMBASE *via* OvidSP (January 1947 to 7th January 2021). A citation search of key included studies using Google Scholar and examining references was also conducted. We restricted our search to human only and English language studies.

A detailed search strategy using both indexing languages (MeSH, EmTree) and free text key words was developed in consultation with a university librarian. We manually searched the reference lists of all included papers for other relevant primary articles. Appendix 1 displays the final search strategy used for one of the database searches. A senior librarian from university of Nottingham was consulted to finalise the search strategy. A combination of the terms below was used for the final search strategy (PICOTS tool).

Population or Condition of interest: "Non-alcoholic fatty liver or non-alcoholic steatohepatitis", "NASH or NAFLD or NAFL", "fatty liver", "Liver fibrosis", "cirrhosis", "Liver Disease".

Prognostic Factors: "Fibroscan", "Transient Elastography", "magnetic resonance elastography", "Elasticity Imaging Techniques", "Liver Biomarkers", "The Enhanced Liver Function test (ELF)", "Hepascore", "BARD score", "NFS", "Fibrometer NAFLD", "FibroTest", "FIB-4", "APRI", "FLI", "HSI", "SteatoTest", "LAP", "ION", "NAFLD-LFS", "Liver Function test$", "Liver function", "Liver enzymes", "Liver test", "blood test", "blood marker" "serum marker", "non-invasive" "ALT", "AST", "GGT", "AST/ALT ratio", "platelets", "triglycerides", "HbA1c", "plasma glucose", "fasting plasma glucose", "insulin", "cholesterol".

Outcomes: "mortality", "death", "all-cause mortality", "cardiovascular", "liver-related", "extrahepatic", "malignancy", "cancer", "diabetes".

For this review NAFLD was defined as "excessive hepatic fat accumulation in the liver, as characterised by the presence of steatosis in more than 5% of hepatocytes. The term NAFLD encompasses all spectrum of liver disease including non-alcoholic fatty liver (NAFL), non-alcoholic steatohepatitis (NASH), various stages of liver fibrosis, and cirrhosis".

Inclusion and exclusion criteria

For the purposes of our study, we included all adult patients with confirmed NAFLD according to agreed international diagnostic criteria (including imaging or biopsy based) of any spectrum, including NASH[7]. We included any non-invasive biomarker used in predicting disease specific or all-cause mortality. These included but were not limited to serum biomarkers, imaging, and combined scoring systems. The study outcome was defined as disease specific and all-cause mortality. We included studies conducted in any setting *i.e.*, primary, or secondary care. We included any observational study (retrospective, prospective, cohort, and case-control studies), and interventional study.

We excluded studies that did not diagnose NAFLD in their study population according to international guidelines; or did not exclude other causes of chronic liver disease. We excluded studies using invasive markers such as liver biopsy and studies reporting non-quantifiable markers such as presence of a comorbidity and studies reporting outcomes other than mortality, or combined outcomes that included mortality. We also excluded cross-sectional studies and systematic reviews and meta-analyses (although these were used to aid manual reference searching). Finally, we excluded studies only available as abstracts if the full paper could not be obtained.

Study selection

Initial title and abstract screening was done using Rayyan. QCRI and Microsoft Excel (2016). Article screening was done by two independent reviewers (NC and MS, and conflicts resolved by AS). Following shortlisting, three independent reviewers identified studies for inclusion by full text review (MS, AS and NC). Where the two reviewers disagreed about eligibility of studies for inclusion, disagreement was resolved through discussion. A third reviewer (GPA) was available to resolve any further disagreements but was not required. Reasons for exclusion of ineligible studies were recorded and the selection process recoded in a PRISMA flow diagram and "Characteristics of Excluded Studies" table.

Data extraction

A modified data extraction form was created using Cochrane CHARMS checklist as a guide. For each study included in the systematic review, two reviewers (NC and DZ) extracted the data using a standardised template. Extracted data was checked by a third reviewer (AK) prior to meta-analysis. Data collected included study characteristics (study design, length of follow-up, method of NAFLD

diagnosis, number of participants, comorbidities, NAFLD severity), index test features (type of biomarker, cut off thresholds, how they were determined and context of use), study outcomes, mortality data (including relative risk, hazard ratio, and/or any reported outcome measure), statistical analysis and adjustment methods.

Risk of bias assessment

Two reviewers independently assessed the quality of individual studies using the Quality in Prognostic Studies (QUIPS) tool, and determined risk of bias rating (high, moderate, low) for each study based on information presented in the published study.

Data synthesis and statistical analysis

We aimed to create a systematic narrative review, as we anticipated there would be considerable diversity in the design, index tests, and outcomes used in individual studies. We sought to include detailed tables and figures to display characteristics and findings (including numerical outcomes) of included studies, as well as bias ratings.

We assessed feasibility and appropriateness of conducting meta-analysis for the primary outcome, the prognostic effect of individual non-invasive tests for mortality in NAFLD. We considered conducting a meta-analysis of adjusted and unadjusted prognostic effect estimate of individual non-invasive tests by pooling any accepted measure of all-cause or disease-specific mortality of included studies. Meta-analysis was performed where 2 or more studies reported the same outcome measure for mortality for a given non-invasive test, having used equivalent cut-off values and statistical methods and in a similar study population. We excluded studies that had overlapping study population due to data duplication.

We performed a meta-analysis of multivariable adjusted hazard ratios and 95% confidence intervals of individual studies, calculated the pooled hazards ratio and 95% confidence interval with p value for overall effect.

The between-studies heterogeneity was measured by the Q test and Higgins's inconsistency index (I^2). In line with the Cochrane Handbook[23], we interpreted heterogeneity values of 0%-40% as low heterogeneity, 30%-60% as representing moderate heterogeneity, 50%-90% as representing substantial heterogeneity and 75%-100% as representing considerable heterogeneity. The p value for Cochrane's Q statistic was also calculated to evaluate the statistical significance of the heterogeneity, considering a $P < 0.05$ as statistically significant.

Where the heterogeneity was statistically significant the results of the random effects analysis (DerSimonian-Laird method) are reported. Where heterogeneity was not a concern the results of the fixed effects analysis (inverse variance method) are reported.

Data was analysed using STATA (version 16.1).

RESULTS

The study selection process is summarized in a PRISMA flow diagram (Figure 1). Initial searches identified a total of 2850 records, which were narrowed down to 1725 records after exclusion of duplicates. One hundred and forty-five records were selected for full-text review. References and reasons for exclusion of full-text articles are detailed in Appendix 1. After full-text review, 24 articles were judged to meet our inclusion and exclusion criteria.

Systematic review

Individual serum and imaging prognostic markers: Overall, 10 studies reported individual biomarkers (2 studies on bilirubin, 2 studies on HbA1C, 2 studies on albumin, 1 study on Apolipoprotein A1, Haptoglobin, GGT, platelets, serum ferritin, prothrombin time, TSH, serum vitamin E and Vitamin E:Cholesterol, serum Vitamin D, and PNPLA3 genotype). One study reported an imaging-based prognostic marker. The individual characteristics, outcomes, and conclusions of these studies are summarized in Supplementary Table 1.

In summary, out of the 10 studies reviewed, 9 found a statistically significant association between a serum marker and risk of all-cause mortality in NAFLD patients. Only 3 reported a statistically significant association between a serum marker and cardiovascular mortality. Ferritin was found in 1 study to be a prognostic marker of all-cause mortality in NAFLD. Bilirubin has in 2 studies been found to be a marker for all-cause mortality. One study found HbA1C to be predictive of all-cause and cardiovascular mortality in NAFLD and non-NALFD participants; and one further study found HbA1C was prognostic of all-cause and liver-related mortality in NAFLD participants. Vitamin D level has been investigated in 1 study in relation to its prognostic ability for mortality and was found to only be prognostic of Alzheimer's disease mortality but not of all-cause, or other cause-specific mortality. Low TSH has been found in 1 study to be prognostic of all-cause and cardiovascular mortality in NAFLD but not in non-NAFLD population. Low platelet count, low albumin, and high GGT was prognostic for all-cause and liver-related mortality in 1 study. One study found that low ApoA1 and high haptoglobin

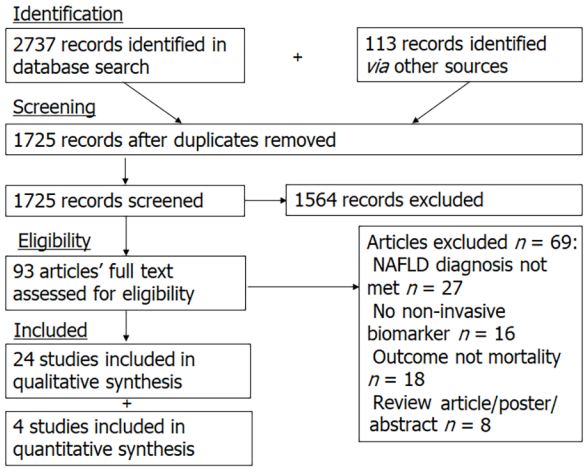

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Figure 1 PRISMA flow diagram for study search.

were prognostic of all-cause and cardiovascular mortality. One study found that both serum vitamin E and lipid-corrected vitamin E were negatively associated with all-cause mortality only in non-diabetic NAFLD participants, but not in pre-diabetic or diabetic NAFLD participants. One study found that the homozygous PNPLA3 I148M (rs738409) GG genotype showed an increase in all-cause mortality in the general population and NAFLD population.

Only one study of non-invasive imaging modalities met our inclusion criteria. This was a study of 2245 NAFLD participants in China and France, which found that LSM > 12 kPa (≤ 12 as reference) was prognostic for all-cause mortality, with a HR 2.85 (1.65 – 4.92).

Non-invasive scoring systems: Overall, 16 studies reported scoring systems (11 studies on NFS, 5 studies on FIB-4, 7 studies on APRI, 2 studies on BARD, 1 study on FibroTest, SteatoTest-2, NashTest-2, renal impairment, ASCVD, Hepascore). The studies reporting prognostic value for mortality of non-invasive scoring systems are summarised in Supplementary Table 2.

All 11 studies that included NFS found that it performed well in predicting all-cause mortality. Two studies also investigated cardiovascular mortality, 1 of which found NFS had a prognostic value, and a further study found that NFS is a prognostic marker for cerebro-cardiovascular mortality. One study investigated NFS and liver-related mortality and found that it is not a prognostic marker.

All 5 studies that included FIB-4 found that it was a prognostic marker for all-cause mortality. Two studies investigated the prognostic value of FIB-4 and liver-related mortality and one had positive results. One study found that FIB-4 was prognostic of cardiovascular mortality and one further study found that FIB-4 was prognostic of cerebro-cardiovascular mortality.

APRI was investigated for its prognostic value for all-cause mortality in 3 studies, with positive results in 2 studies. APRI was also investigated in 1 further study for liver-related mortality and 1 study for cardiovascular mortality, both of which had negative results. BARD was found to be a prognostic marker for all-cause mortality in 2 studies. FibroTest was found to be a prognostic marker for all-cause and cardiovascular mortality, but not liver-related mortality, in 1 study. NashTest-2 and SteatoTest-2 were found not to be prognostic markers for all-cause, liver-related, or cardiovascular mortality. Renal impairment (measured by eGFR or albumin-creatinine ratio) was found to be a prognostic marker for both all-cause and cardiovascular mortality in 1 study. ASCVD score was also found to be a prognostic marker for all-cause and cardiovascular mortality in 1 study. Hepascore was found to be a prognostic marker for all-cause mortality in 1 study.

Meta-analysis

Non-invasive scoring systems were the only prognostic markers reported in two or more studies, to enable pooling of results *via* meta-analysis. A total of 4 studies were included in data analysis. The study characteristics for the 4 studies are summarized in Table 1.

The 4 included studies recruited a total of 6324 NAFLD participants. Two studies included participants with NAFLD diagnosed by liver biopsy, 1 study included participants with NAFLD diagnosed by imaging presence of hepatic steatosis, and 1 study included participants with NAFLD diagnosed by USFLI score ≥ 30.

All were retrospective cohort studies. Study participants with NAFLD had a weighted mean age of 48.2 years, 36.9% had hypertension, 14.3% had diabetes, and were overweight with a mean BMI of 30.4. The 4 studies included 2 with cohorts from the United States, 1 from Sweden, and 1 multinational cohort (United States, United Kingdom, Australia, Italy, Thailand, Iceland).

Table 1 Characteristics of studies included in the meta-analysis

Study characteristics	Le *et al*[18]	Kim *et al*[29]	Hagstrom *et al*[30]	Angulo *et al*[31]
Year	2019	2013	2019	2013
Country (setting)	United States (community care)	United States (community care)	Sweden (secondary care)	United States, United Kingdom, Australia, Thailand, Italy, Iceland (secondary care)
Study design	Retrospective cohort study	Retrospective cohort study	Retrospective cohort study	Retrospective cohort study
NAFLD diagnostic method	USFLI[1]	Ultrasound[2]	Liver biopsy[3]	Liver biopsy[3]
Number of NAFLD participants	4680	4079	646	320
Average length of follow-up (yr)	Not specified, however follow-up period was 1999-2016	14.5 (median)	19.9 (mean)	8.7 (median)
Proportion of males (%)	56.3	50.9	62	43
Mean age (yr)	52.6	46.2	50	52
Caucasians (%)	74.8	75.8	Not specified	92
Mean BMI (kg/m^2)	34.3	29.05	28	33
Cardiovascular disease (%)	13.3	7.1	Not specified	Not specified
Hypertension (%)	52.3	32.4	30	47.5
Diabetes (%)	24.4	9.5	14	36.2
Smoking (%)	45	55.2	Not specified	Not specified
Deaths (*n*)	683	778	214	22
Low NFS[4] (%)	32.4	67.5	76.2	39.1
Intermediate NFS[4] (%)	51.7	28.2	5.1	37.5
High NFS[4] (%)	17.3	4.2	2.3	23.4

[1]USFLI ≥ 30 and exclusion of liver disease of other aetiology.
[2]Ultrasound hepatic steatosis and exclusion of liver disease of other aetiology.
[3]Liver biopsy confirmed non-alcoholic fatty liver disease (NAFLD) and exclusion of liver disease of other aetiology.
[4]The same NAFLD fibrosis score (NFS) cut-offs have been used in all studies: High NFS (> 0.676), intermediate NFS (-1.455-0.676), low NFS (<-1.455).
NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index; NFS: NAFLD fibrosis score.

The scoring system used, outcomes and main conclusions for the 4 included studies are summarised in Supplementary Table 3. Risk of bias for included studies was assessed by using the QUIPS tool. Overall, studies included in the meta-analysis were low – moderate risk of bias (Supplemen-tary Table 4).

After considering the feasibility of pooling results for analysis, we were able to conduct a meta-analysis of several non-invasive scoring systems and all-cause mortality, and a meta-analysis of NFS and cardiovascular-related mortality.

Overall, our analysis found that non-invasive scoring systems can predict all-cause mortality in patients with NAFLD, with higher scores having a higher pooled hazard ratio for mortality than intermediate scores, when compared with low scores (Table 2). The non-invasive scoring system that performed best at predicting all-cause mortality when comparing "high" with "low" scores was NFS, pHR 3.07 (1.62-5.83), followed by FIB-4, pHR 3.06 (1.54-6.07), BARD, pHR 2.87 (1.27-6.46), and finally APRI, pHR 1.90 (1.32-2.73). These pHR were all statistically significant ($P < 0.05$). When comparing "intermediate" and "low" scores, NFS, FIB-4, and BARD, but not APRI were also statistically significant for prognosis of mortality, with intermediate scores showing a higher pHR for all-cause mortality, though the individual values were lower than the respective values for "high" score in each scoring system. The forest plots for NFS are shown in Figure 2 and for FIB-4 in Figure 3. The forest plots for the remaining analyses can be found in Supplementary Figures 1 and 2.

We also report that NFS was associated with cardiovascular-related mortality, with higher scores being prognostic of higher mortality risk (Figure 2). "High" NFS had a pHR of 3.09 (1.78-5.34), and "intermediate" NFS had a pHR of 2.12 (1.41-3.17).

Table 2 Meta-analysis of non-invasive scoring systems and all-cause mortality and cardiovascular-related mortality

Comparison categories[1]	No. studies	Studies included	Heterogeneity: I^2 (P value for Q)[2]	Pooled HR (95%CI)	P value for overall effect (pHR)
All-cause mortality					
NFS high *vs* low	4	Le *et al*[18], 2019; Kim *et al*[29], 2013; Hagstrom *et al*[30], 2019; Angulo *et al*[31], 2013	75.7%(0.006)	3.07 (1.62 – 5.83)	0.001
NFS Int. *vs* low	4	Le *et al*[18], 2019; Kim *et al*[29], 2013; Hagstrom *et al*[30], 2019; Angulo *et al*[31], 2013	81.5%(0.001)	1.91 (1.18 – 3.09)	0.008
FIB-4 high *vs* low	3	Kim *et al*[29], 2013; Hagstrom *et al*[30], 2019; Angulo *et al*[31], 2013	73.0%(0.025)	3.06 (1.54 – 6.07)	0.001
FIB-4 Int. *vs* low	3	Kim *et al*[29], 2013; Hagstrom *et al*[30], 2019; Angulo *et al*[31], 2013	0.0%(0.396)	1.60 (1.33 – 1.91)	< 0.001
APRI high *vs* low	3	Kim *et al*[29], 2013; Hagstrom *et al*[30], 2019; Angulo *et al*[31], 2013	0.0%(0.589)	1.90 (1.32 – 2.73)	0.001
APRI Int. *vs* low	3	Kim *et al*[29], 2013; Hagstrom *et al*[30], 2019; Angulo *et al*[31], 2013	0.0%(0.411)	0.98 (0.76 – 1.26)	0.887
BARD high *vs* low	2	Hagstrom *et al*[30], 2019; Angulo *et al*[31], 2013	45.1%(0.177)[3]	2.87 (1.27 – 6.46)	0.011
BARD Int. *vs* low	2	Hagstrom *et al*[30], 2019; Angulo *et al*[31], 2013	0.0%(0.862)	1.64 (1.21 – 2.23)	0.001
Cardiovascular mortality					
NFS high *vs* low	2	Le *et al*[18], 2019; Kim *et al*[29], 2013	0.0%(0.317)	3.09 (1.78 – 5.34)	< 0.001
NFS Int. *vs* low	2	Le *et al*[18], 2019; Kim *et al*[29], 2013	0.0%(0.759)	2.12 (1.41 – 3.17)	< 0.001

[1]For Non-alcoholic fatty liver disease fibrosis score, cut-off values were "low" < -1.455 (reference group), "intermediate" -1.455 – 0.676, and "high" > 0.676. For fibrosis-4 index, cut-off values were "low" < 1.30 (reference group), "intermediate" 1.30-2.67, and "high" > 2.67. For AST to platelet ratio index, "low" < 0.5 (reference group), "intermediate" 0.5-1.5, and "high" > 1.5. For BARD, "low" 0-1 (reference group), "intermediate" 2-3, and "high" 4.
[2]Where the heterogeneity is statistically significant the results of the random effects analysis (DerSimonian-Laird method) are shown. Where heterogeneity is not a concern the results of the fixed effects analysis (inverse variance method) are shown. The P value is for the Cochran's Q statistic.
[3]For the analysis of BARD high *vs* low the results of the random effects analysis are shown as the I^2 value suggests moderate heterogeneity is present, however note the low number (n = 2) of studies included.
NAFLD: Non-alcoholic fatty liver disease; NFS: NAFLD fibrosis score; FIB-4: Fibrosis-4 index; APRI: AST to platelet ratio index; BARD score: BMI ≥ 28 kg/m^2 (1 point), AST/ALT ratio ≥ 0.8 (2 points), and diabetes mellitus (1 point).

We report heterogeneity using I^2 and Cochrane's Q statistic and found that several of the analyses reported moderate to considerable heterogeneity. However, the significance of this is uncertain due to the small number of studies included in the analyses. Due to the small number of studies involved in each analysis, we were unable to investigate sources of heterogeneity *via* statistical methods such as sensitivity analyses, sub-group analyses and/or meta-regression. For the same reason we did not assess publication bias *via* the usual methods such as funnel plot as this was uninformative with such a low number of included studies.

DISCUSSION

This systematic review identified a substantial number of individual observational studies reporting several non-invasive markers of prognostic value for mortality in NAFLD, including individual blood markers, imaging modalities, and non-invasive scoring systems. Only non-invasive scoring systems were examined in a sufficient number of studies to enable meta-analysis of the results. Our analysis reaffirms previous evidence; with higher scores in non-invasive scoring systems, there is a stepwise prognostic value for all-cause mortality. NFS appears to be the most reliable among the non-invasive scores, with highest pHR and greatest number of included studies and patients. Another non-invasive marker with very similar performance in predicting all-cause mortality was FIB-4. The pHR, confidence intervals, and heterogeneity levels of FIB-4 and NFS with all-cause mortality were indeed very similar. This can likely be attributed to all 4 of the individual components of the FIB-4 score (age, AST, platelets, and ALT) being part of the NFS (which in addition contains BMI, impaired fasting glucose or diabetes,

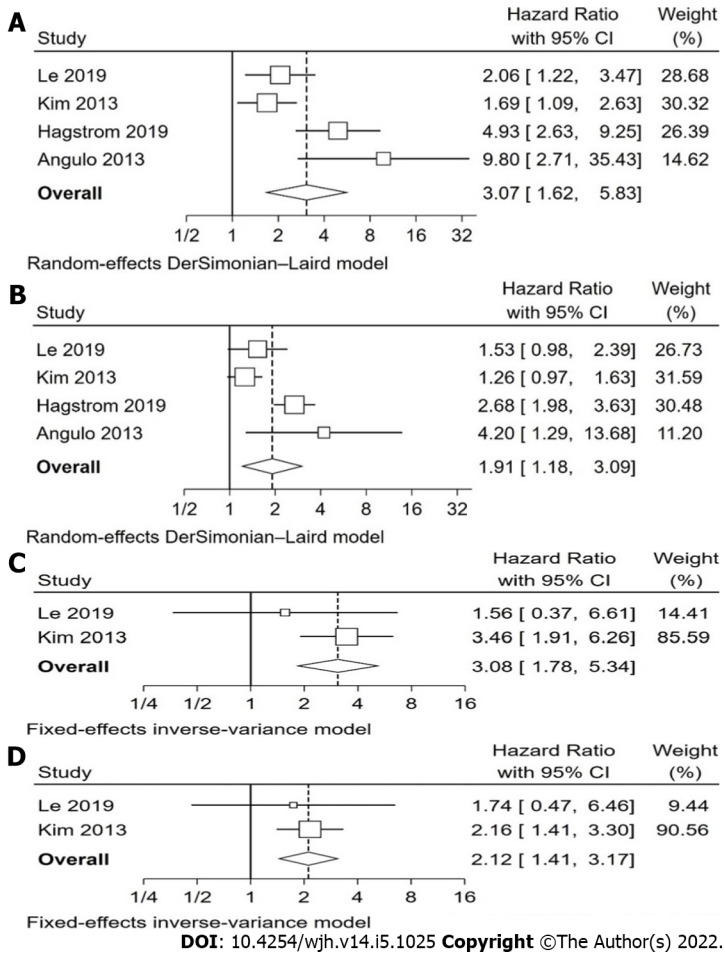

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Figure 2 The forest plots for non-alcoholic fatty liver disease fibrosis score. A: Non-alcoholic fatty liver disease (NAFLD) fibrosis score (NFS) high *vs* low and all-cause mortality; B: NFS intermediate *vs* low and all-cause mortality; C: NFS high *vs* low and cardiovascular mortality; D: NFS Intermediate *vs* Low and cardiovascular mortality.

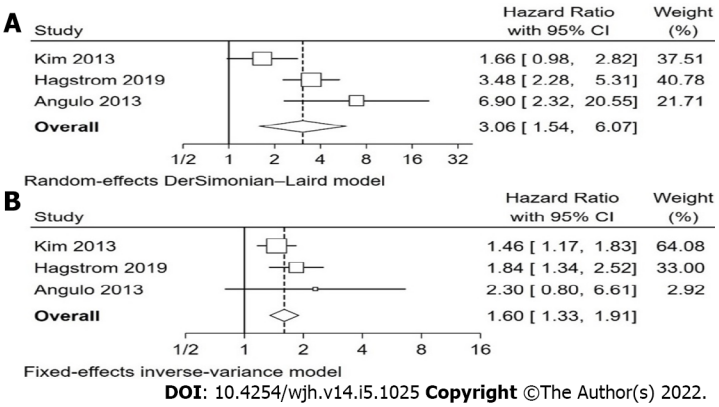

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Figure 3 The forest plots for fibrosis-4 index. A: Fibrosis-4 index (FIB-4) high *vs* low and all-cause mortality; B: FIB-4 intermediate *vs* low and all-cause mortality.

and albumin). It is encouraging to find a scoring system with fewer components seems to have a similar performance, as it may be easier to implement in clinical practice. However, our study found only 3 studies, with a total of 5045 NAFLD patients, that evaluated the prognostic performance of FIB-4. This is significantly less than the 9725 NAFLD patients included in the analysis of NFS and all-cause mortality. Further epidemiological studies are warranted to enable a head-to-head comparison of NFS and FIB-4 performance to help develop clinical guidelines on the best non-invasive scoring system to use in clinical practice.

Further, for the first time in the literature, our study reports that NFS has a prognostic value for cardiovascular-related mortality in patients with NAFLD. Although only 2 studies were included in this meta-analysis, they included a large number of participants and events, with 8759 NAFLD patients, and 1461 deaths. The main cause of death in NAFLD patients is cardiovascular disease[24], our findings highlight it is possible to predict those NAFLD patients at higher risk of cardiovascular death such that more intensive clinical care can be provided to modify cardiovascular risk.

An important limitation of this meta-analysis is that few studies were included, leading to high heterogeneity. From the baseline study characteristics (Table 1), one can infer there were differences in study design and population which may explain this heterogeneity, namely differences in the setting and country of study, the method of NAFLD diagnosis, age, sex, and prevalence of different comorbidities. The high levels of heterogeneity ultimately limits the generalisability of the results of our meta-analysis. Our definition of NAFLD included all spectrums of disease, and, in the inclusion criteria for the population included in our study, we sought to evaluate both NAFL and NASH, however very few studies included subgroups comprising NASH. Indeed the only studies who did were studies where NAFLD was diagnosed *via* liver biopsy (Supplementary Table 1). This is likely to be due to the fact that, currently, international and national clinical guidelines recommend for NASH to be diagnosed histologically by liver biopsy, so studies where NAFLD was diagnosed by imaging and non-invasive scores would not be able to include NASH as a subgroup. In addition there aren't robust, validated non-invasive markers to identify NASH independent of fibrosis. So, it is unsurprising that in our systematic review we weren't able to identify any relationship between NASH and mortality.

Cohort studies have consistently shown association of fibrosis stage in NAFLD with overall and disease specific mortality[9,25,26]. The algorithms that we have identified include parameters such as age, BMI and type 2 diabetes which are well recognised risk factors for cardiovascular and all-cause mortality. Therefore, it is understandable that particular biomarkers are also associated with all-cause mortality.

Previous systematic reviews and meta-analyses report non-invasive scoring systems are prognostic of all-cause mortality in NAFLD[11,12]. These authors also report NFS is the best non-invasive tool for prognosis and risk-stratification of all-cause mortality in NAFLD patients. However, their analysis included studies using different NFS cut-off values, which may make the results less precise, and seem to have missed out the largest observational study of NFS and mortality to date[18]. Despite including less studies, our meta-analysis stringently scrutinised individual studies, ensuring the study population, non-invasive biomarker cut-offs, and multivariable adjustment were equivalent and therefore comparable. For instance, the study by Golabi *et al*[21], which was included in the Liu *et al*[12] analysis, reports HRs for all-cause mortality in "low" and "high" NFS groups, however they do not state what their reference group was. Further, their study population was extracted from NHANES III (1988-1994) data, which is the same population used in another of the studies included in their analysis. This would introduce bias due to data duplication.

The main limitations of our study were derived from the design and reporting of primary included studies. Several individual studies reported non-invasive markers having a prognostic use for mortality in NAFLD, however these were not replicated in different studies to enable a meta-analysis. It is well-recognised NAFLD is associated with extra-hepatic disease, and commonest causes of death include diabetes-related and extra-hepatic cancers, as well as cardiovascular disease. It is unsurprising that studies found markers including HbA1C[27], renal impairment[18] and ferritin[28] demonstrated good prognostic value for mortality in NAFLD when adjusting for other variables. Further studies aiming to better characterise prognostic markers for disease-specific mortality in NAFLD are warranted, to enable a more targeted approach for risk stratification and reduction in mortality of NAFLD patients. Future studies should also consider the prognostic role of imaging-based tests. One prospective observational study of 2245 participants found liver stiffness measurement using transient elastography had very good performance in identifying patients at predicting overall survival and liver events[20]. Transient elastography is a non-invasive, increasingly widespread test that may in future prove to be a useful complement to non-invasive biomarkers and liver biopsy in risk-stratifying NAFLD patients.

Biopsy-proven liver fibrosis has been well-described as being prognostic for mortality in NAFLD, with higher stages of fibrosis being prognostic of higher rates of mortality[9]. Our study adds to the available literature supporting NFS as a simple, non-invasive marker for biopsy-proven fibrosis that has a growing body of evidence suggesting it as a useful surrogate marker to predict important clinical outcomes. Further studies assessing whether a reduction in NFS value then translates to a reduction in mortality are crucial in establishing the use of NFS in clinical practice to improve outcomes in NAFLD patients.

CONCLUSION

In conclusion, our study reaffirms non-invasive scoring systems, especially NFS, is a reliable prognostic marker of all-cause mortality in NAFLD patients. We further report NFS can be used specifically to predict cardiovascular-related mortality, and our systematic review has highlighted several other non-

invasive prognostic markers for mortality in NAFLD. These findings can be applied to clinical practice to stratify patients needing further investigation such as liver biopsy, closer follow-up such as referral to specialist liver services, and more intense treatment including addressing metabolic risk factors. With the increasing prevalence of NAFLD in the global population and general strain on healthcare systems, the ability to stratify NAFLD patients according to the risk of adverse outcomes can have a crucial role on clinical practice and help guide future research in NAFLD.

ARTICLE HIGHLIGHTS

Research background

Non-alcoholic fatty liver disease (NAFLD) represents a growing public health concern, highly prevalent in the general population, and with wide range of disease severity and prognosis.

Research motivation

Some NAFLD patients are at increased risk of morbidity and mortality, so it's important to validate non-invasive prognostic markers for predicting mortality in these patients, to guide risk stratification and more intense clinical focus on high risk patients.

Research objectives

The aim of this systematic review and meta-analysis was to evaluate available evidence on the use of non-invasive test(s) as prognostic factors for mortality in NAFLD.

Research methods

The authors performed electronic searches of Medline and EMBASE (Ovid) until 7th January 2021 of studies in NAFLD populations. We conducted a meta-analysis of non-invasive scoring systems for predicting all-cause and cardiovascular mortality, calculating pooled hazard ratios and 95% confidence (STATA 16.1).

Research results

The authors identified multiple individual non-invasive biomarkers and imaging modality that had a prognostic value in NAFLD patients. Non-invasive scoring systems were the only marker to have been studied in a sufficient number of studies to permit meta-analysis. The non-invasive scoring system that performed best at predicting all-cause mortality was NAFLD fibrosis score (NFS) [pHR 3.07 (1.62-5.83)], followed by fibrosis-4 index (FIB-4) [pHR 3.06 (1.54-6.07)], BARD [pHR 2.87 (1.27-6.46)], and AST to platelet ratio index [pHR 1.90 (1.32-2.73)]. NFS was also prognostic of cardiovascular-related mortality [pHR 3.09 (1.78-5.34)].

Research conclusions

This study reaffirms that non-invasive scoring systems, especially NFS, are reliable prognostic markers of all-cause mortality and cardiovascular mortality in NAFLD patients. Further, we have identified multiple individual biomarkers and imaging modalities that have prognostic value.

Research perspectives

NFS and FIB-4 may be of value in clinical practice in risk-stratification of NAFLD patients with highest risk of mortality. Several other individual serum and imaging markers identified by this systematic review could be studied further to evaluate and validate their prognostic ability.

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FOOTNOTES

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CASE REPORT

Biliary obstruction following transjugular intrahepatic portosystemic shunt placement in a patient after liver transplantation: A case report

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Abstract

BACKGROUND

Transjugular intrahepatic portosystemic shunt (TIPS) is a method used to decrease portal hypertension. Biliary stricture is the rarest of the complications associated with this procedure with only 12 cases previously reported in the literature. None of these cases have documented the resolution of biliary stenosis induced by a stent graft. The only curative solutions reported are liver transplantation or bypassing the stenosis with an artificial biliary tract using advanced endoscopic techniques.

CASE SUMMARY

This is the first reported case of biliary obstruction secondary to TIPS placement in a transplanted liver. In our patient, a portosystemic shunt was created to treat severe veno-occlusive liver graft disease manifesting itself primarily by fluid retention. A cholestatic liver lesion and cholangitis with abscesses developed due to a stent graft-induced stricture in the dorsal segment of the right hepatic duct and the stricture diminished following percutaneous drainage. Endoscopic drainage was performed after unsuccessful removal of the percutaneous catheter

resulting in a bilio-cutaneous fistula. Although the liver graft now functions well, the stricture remains refractory even after 44 mo of treatment.

CONCLUSION
Biliary strictures caused by TIPS in both transplanted and native livers seem refractory to endoscopic treatment.

Key Words: Biliary stricture; Transjugular intrahepatic portosystemic shunt; Liver trans-plantation; Sinusoidal obstruction syndrome; Literature review; Case report

Core Tip: Biliary stricture is the most unusual complication of transjugular intrahepatic portosystemic shunt (TIPS) implantation yet to be reported in liver transplant recipients. In our patient, TIPS was successfully used to treat the rare liver graft disease, sinusoidal obstruction syndrome. Cholangitis with abscess formation caused by a TIPS-induced stricture of the right hepatic duct was successfully treated by percutaneous and subsequent endoscopic drainage and resulted in preserved liver graft function. However, the stenosis persisted even after long-term endoscopic treatment. The outcome of our case is comparable to those reported in a native liver where no case was resolved using only biliary drainage.

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INTRODUCTION

Transjugular intrahepatic portosystemic shunt (TIPS) is a procedure in which an artificial portosystemic shunt is applied to decrease portal hypertension. The most common indications are refractory ascites and uncontrolled variceal bleeding which often serves as a bridge to liver transplantation. Common complications of TIPS include encephalopathy, cardiac failure, occlusion and infection. Serious biliary complications are rare and occur in less than 1% of cases[1].

TIPS insertion can create a tract between the shunt and non-vascular structures, typically resulting in a biliary-TIPS fistula. This fistula can cause haemobilia and jaundice and is associated with acute and chronic TIPS occlusion[2]. Such complications have been significantly reduced with the use of covered stent grafts. Biliary stenosis secondary to TIPS placement is extremely rare with only 12 cases documented (7 case reports and 1 case series) (Table 1). Interestingly, a single-center retrospective study found TIPS-related segmental intrahepatic cholestasis in 4 of 135 intervened patients (2.9%)[3]. However, this observation has yet to be confirmed by further studies.

This report documents the very first case of a TIPS-related bile duct stricture in a liver graft.

CASE PRESENTATION

Chief complaints

Dyspnoea in a patient early after liver transplantation.

History of present illness

A 40-year-old Caucasian male underwent orthotopic liver transplantation for alcoholic liver cirrhosis (see Figure 1 for timeline). He received a full graft from an ABO-compatible 74-year-old cadaveric donor. Cold ischemia time was 3 h 21 min. The transplantation was performed using piggyback technique with side-to-side caval anastomosis (Belghiti Technique) and standard end-to-end hilar anastomoses. The duration of the procedure was 2 h 52 min. The postoperative course was uneventful. The immunosuppressive regimen consisted of tacrolimus, mycophenolate mofetil and corticosteroids. A month later, the patient presented with symptomatic ascites, large right-sided pleural effusion and normal liver function. Upper endoscopy revealed esophageal varices and the consequent catheterization of the hepatic veins showed a portosystemic gradient of 28 mmHg. Liver biopsy revealed microvascular damage corresponding to sinusoidal obstruction syndrome (SOS) (Figure 2). The patient was

Table 1 Characteristics of previously published cases of transjugular intrahepatic portosystemic shunt-induced biliary strictures

Ref.	Indication for TIPS placement	Interval between TIPS placement and biliary complications	Clinical presentation	Imaging finding	Treatment	Outcome	Long-term follow-up
Peynircioglu *et al*[12]	OH, in HCV cirrhosis	3 mo	Jaundice, acute cholangitis	RHD stenosis by TIPS	PTC, impassable stricture, external biliary drainage	Decrease in bilirubin; sepsis due to infectious colitis, colon perforation, death	-
Duller *et al* [13]	OH in cirrhosis (aetiology not reported)	4 mo	Jaundice	RHD stenosis by TIPS, fistula, biloma	TIPS replacement with a polytetrafluoroethylene-covered Wallstent, biloma drainage	Regression of biloma; SSC	OLTx
Paterno *et al* [14]	OH, in HCV cirrhosis	Immediate	Jaundice	Malposition of TIPS in CBD, obstruction of LHD and RHD at confluence	OLTx with HJA	Uneventful recovery	
Karlas *et al* [15]	RA in ALC	18 mo (TIPS placement); 10 mo (TIPS extension)	Jaundice	Branch of RHD compression by TIPS extension	None, non-compliant patient	-	OLTx KI for continuous alcohol abuse
Korrapati *et al*[1]	RA in BCS	Immediate	Cholestatic liver lesion	LHD stenosis by TIPS	ERCP with biliary stent placement	Regression of cholestasis, persistent stenosis with stent replacement at 2 mo	Not reported
Meng *et al* [16]	OH, in cirrhosis (aetiology not reported)	5 d	Jaundice	RHD stenosis by TIPS	PTC, impassable stricture, external biliary drainage	Normalisation of bilirubin	External drainage in situ for 2 yr, one episode of mild cholangitis
	OH, in cirrhosis due to schisto-somiasis	10 d	Jaundice	RHD stenosis by TIPS	PTC, impassable stricture, external biliary drainage	Normalisation of bilirubin	Enrolled on WL for OLTx
Bucher *et al* [3]	RA + HRS in ALC	72 mo	Asymptomatic	Compression of segmental bile duct (SVII) by TIPS	None	-	Resolution of imaging finding after 2 yr; death due to metastatic HCC
	RA in ALC	83 mo	Asymptomatic	Compression of segmental bile duct (SVII) by TIPS, cystic congestion of the intrahepatic bile ducts (SVII)	None	-	Stable on F-U
	RA + HRS in ALC	17 mo	Jaundice	Compression of segmental bile duct (SV) by TIPS	Failed ERCP (stricture not achieved); PTC KI for ascites; ATB prophylaxis	Lost to F-U (continuous alcohol abuse)	-
	RA in BCS	0.4 mo	Cholestatic liver lesion	Stenosis of segmental bile duct (SI) by TIPS; liver abscess (SI)	Percutaneous drainage of abscess, failed ERCP (impassable stricture)	Normalisation of liver enzymes	"Unremarkable"
Zhang *et al* [17]	Recurrent colonic variceal bleeding due to CTPV	3 d	Jaundice	CBD stenosis by TIPS	Percutaneous and endoscopic drainage	Refractory stenosis, recurrent cholangitis; magnetassisted endoscopic biliaryduodenal anastomosis after 33 mo due to TIPS	

ALC: Alcoholic liver cirrhosis; ATB: Antibiotics; BCS: Budd-Chiari syndrome; CBD: Common bile duct; CTPV: Cavernous transformation of portal vein; F-

U: Follow-up; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; KI: Contraindicated; HJA: Hepaticojejunostomy; LHD: Left hepatic duct; OH: Oesophageal haemorrhage; OLTx: Orthotopic liver transplantation; RA: Refractory ascites; RHD: Right hepatic duct; WL: Waiting list.

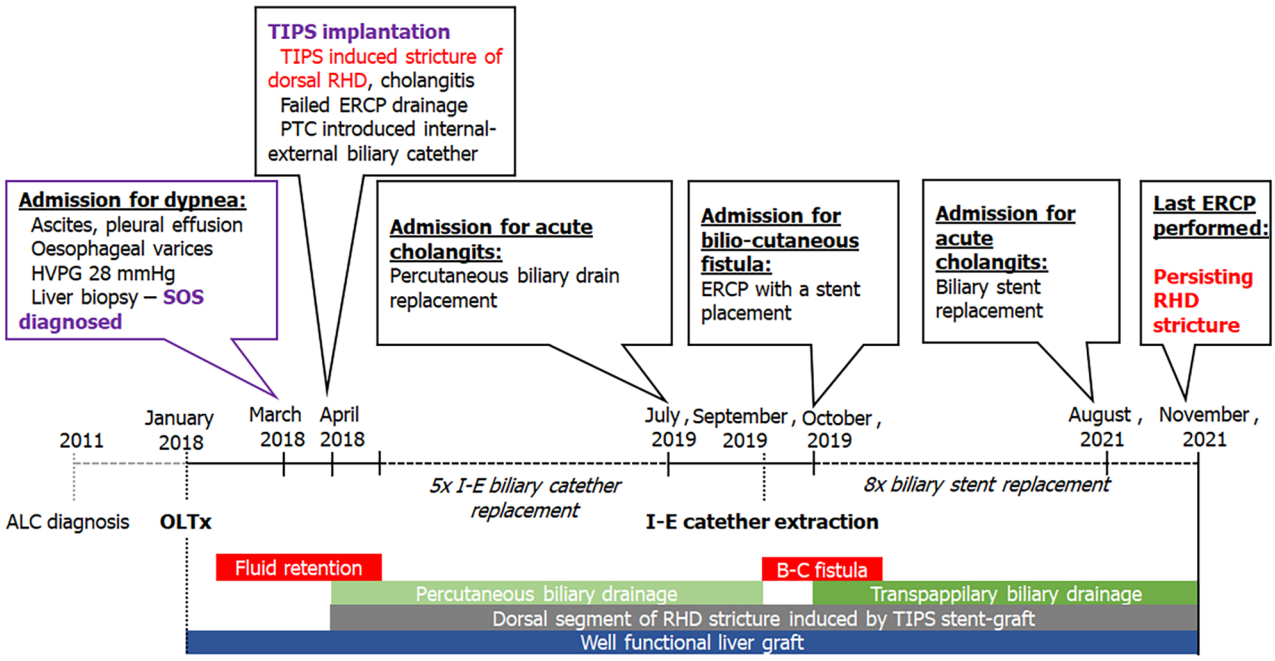

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Figure 1 Timeline of the reported case. ALC: Alcoholic liver cirrhosis; B-C: Bilio-cutaneous; I-E: Internal-external; OLTx: Orthotopic liver transplantation; RHD: Right hepatic duct; SOS: Sinusoidal obstruction syndrome.

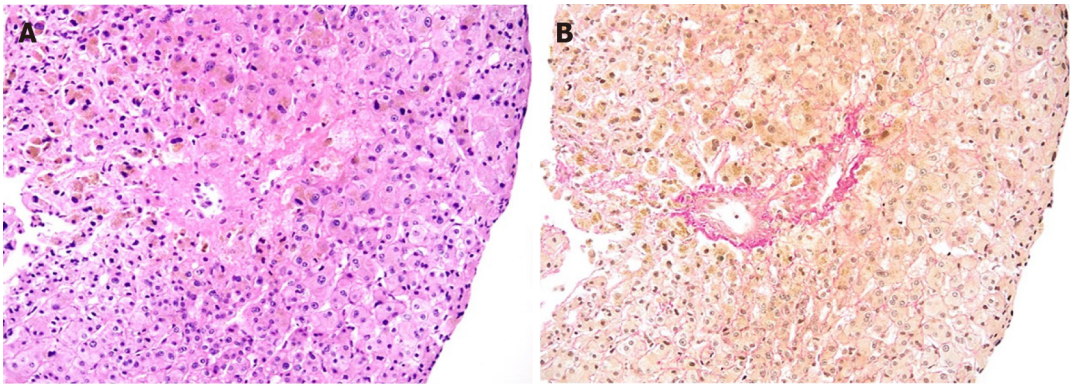

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Figure 2 Histological findings in liver biopsy. Centro lobular vein with wall edema and narrowing of the lumen by connective tissue, focal obstructive fibrosis of the surrounding sinuses; hematoxylin-eosin (A) and Elastica van Gieson (B) staining, original magnification x 100.

subsequently indicated for placement of a TIPS stent graft. This procedure was successfully performed and reduced the portosystemic gradient to 11 mmHg (Figure 3). Immediately after placement of the shunt, a rise in cholestatic enzyme levels was observed.

History of past illness

In the pre-transplant period, the patient was diagnosed with alcoholic liver cirrhosis but displayed no other significant comorbidities. He suffered from several severe episodes of hepatic encephalopathy (including coma) and was also treated for hepatorenal syndrome. At the time of transplantation, he had a MELD score of 27 and his Child-Pugh class was C (14 points).

Personal and family history

The patient had a history of alcohol addiction but had been abstaining from alcohol for 2 years before

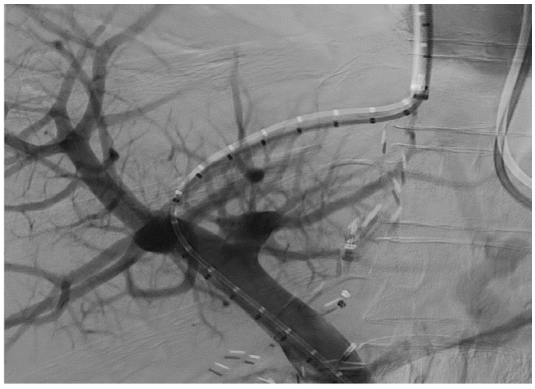

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Figure 3 Transjugular intrahepatic portosystemic shunt implantation.

liver transplantation. He had smoked 20 cigarettes a day for 20 years prior to quitting 18 mo prior to the present illness. His family history was unremarkable.

Physical examination

Upon initial examination, the patient was sarcopenic, pale, anicteric and dyspneic at rest with an oxygen saturation of 88%. He had tachycardia at 102 pulses per minute but blood pressure was within normal values. Breath sounds were inaudible on the right side and percussion in the right chest was dull. The patient exhibited large ascites and a palpable liver but no lower-limb oedema. No remarkable abdominal wall collateral vessels were noted on physical examination. After TIPS placement, we observed a significant regression in physical signs of both ascites and pleural effusion.

Laboratory examinations

Upon admission, bilirubin and liver enzymes were within normal limits (total bilirubin 0.86 mg/dL, AST 18.67 U/L, ALT 26.51 U/L, ALP 96.58 U/L and GGT 62.99 U/L). Total serum protein was 46.5 g/L and the albumin level was 23.7 g/L. After shunt placement, we observed an elevated ALP (292.74 U/L) and GGT (435.51 U/L), but levels of bilirubin, AST and ALT remained at normal values.

Imaging examinations

The initial abdominal ultrasound showed marked hepatomegaly (20.5 cm in the craniocaudal length) and massive ascites. After TIPS placement, liver ultrasound revealed a patent shunt with appropriate blood flow. Subsequent magnetic resonance analysis revealed tight stenosis of the right dorsal hepatic duct caused by the compression of the previously placed stent graft with prestenotic tract dilation and multiple small abscesses (Figure 4).

FINAL DIAGNOSIS

The patient was diagnosed with cholangitis and abscess formation due to biliary stenosis secondary to TIPS.

TREATMENT

Intravenous antibiotics were administered. Endoscopic cholangiography confirmed our previous magnetic resonance finding. However, an attempt at biliary stent placement proved unsuccessful (Figure 5A). Following failed endoscopic drainage, an internal-external catheter was placed percutaneously (Figure 5B).

OUTCOME AND FOLLOW-UP

Percutaneous intervention led to a prompt reduction in cholestasis and the disappearance of hepatic abscesses. As a result, the patient was discharged. During follow-up, ascites and pleural effusion resolved completely. After 17 mo involving five replacements of the percutaneous drain, we observed a significant regression in biliary stenosis allowing for drainage extraction (Figure 5C). Shortly

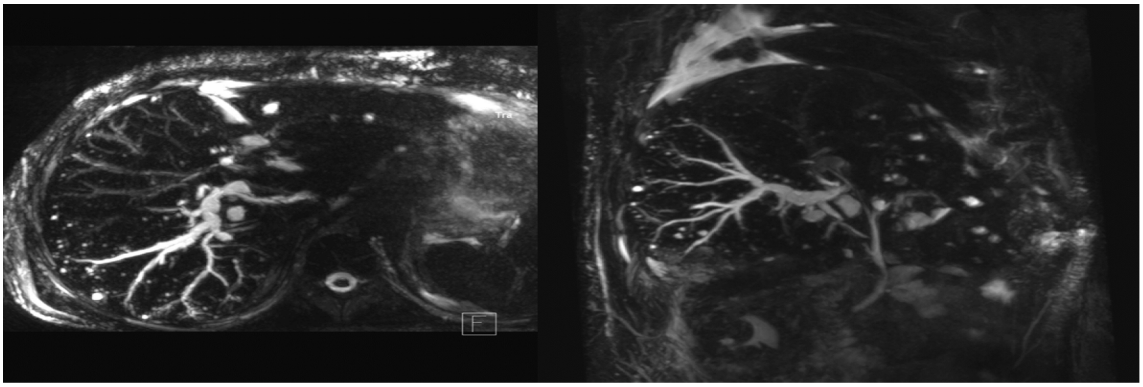

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Figure 4 Magnetic resonance imaging scan after transjugular intrahepatic portosystemic shunt placement. Dilation of dorsal branch of right hepatic duct with multiple small abscesses of the right lobe.

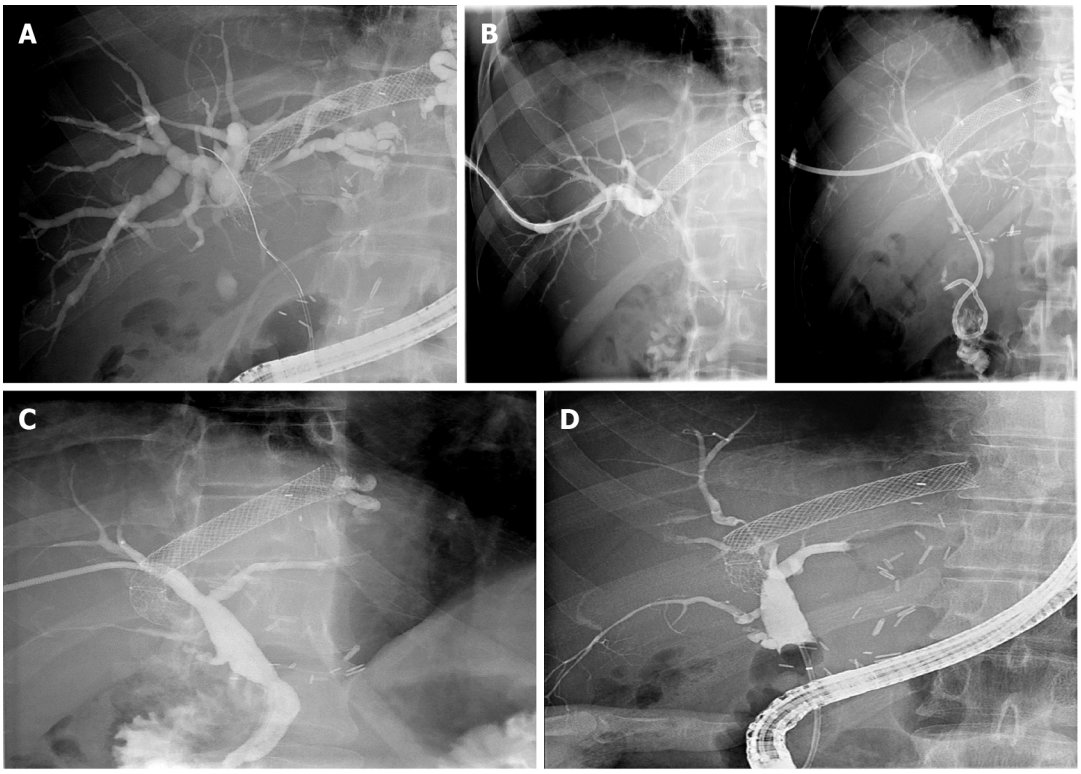

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Figure 5 Images of cholangiogram. A: Endoscopic cholangiogram. Tight stenosis in the dorsal segment of the right hepatic duct caused by the transjugular intrahepatic portosystemic shunt stent graft; stent not placed; B: Percutaneous cholangiogram. Stricture passed with a wire; external-internal catheter placed in duodenum; C: Percutaneous cholangiogram. Apparent regression of the visualized stricture with drain removed; D: Eight endoscopic cholangiograms. Persistent stricture of the right hepatic duct.

afterwards, however, the patient presented with a biliary leak due to opening of the cutaneous channel. Endoscopic cholangiography revealed persistent biliary stenosis. Endoscopic stent placement resulted in the resolution of the biliary fistula. Over the next 26 mo, the patient underwent eight ERCPs with repeated balloon dilations and stent replacements. Stricture location and anatomy do not allow for metal stent implantation and the stenosis has thus far proved refractory to treatment (Figure 5D). To date, the patient has suffered from two episodes of acute cholangitis and the liver graft has remained functional.

DISCUSSION

Biliary complications are common in liver transplant recipients occurring in approximately 30% of patients[4]. Among the most common are anastomotic (AnS) and non-anastomotic strictures (nAnS), with incidence of 6%–12% and 0.5%–10%, respectively[5]. Anastomotic strictures account for up to 80% of all stenotic complications and respond excellently to endoscopic treatment, especially those that occur early (< 3 mo after OLTx)[4]. AnS require the placement of repetitive multiple plastic stents or self-expandable metal stents to achieve sufficient radial tension. Recurrence of the stricture after treatment is uncommon, representing less than 20% of cases[6]. In contrast to AnS, endoscopic treatment of nAnS is difficult and often unsuccessful, resulting in compromised graft survival[7]. We know of no other reported case of non-anastomotic stricture in a transplanted liver due to TIPS placement.

Hepatic sinusoidal obstruction syndrome (SOS; previously known as veno-occlusive disease) is a rare endothelial disorder that occurs particularly after exposure to drugs or toxins. Obstruction of the terminal hepatic venules and sinusoids is typical following hematopoietic cell transplantation and as a consequence of myeloablative preparation (in up to 15% of patients)[8]. SOS is also infrequently observed in liver transplant recipients (with an approximate prevalence of 2%)[9], presumably as an immune-mediated condition related to rejection episodes or azathioprine therapy. SOS is also associated with inferior graft and patient survival[9,10]. The typical clinical manifestation of the disease is a triad of hepatomegaly, fluid retention and jaundice; treatment usually relies on fortification of immunosuppression. A few other pharmacological agents can be used for SOS therapy, but evidence of their efficacy in solid organ transplant recipients is limited. It has been documented that in the case of severe disease refractory to medical treatment, TIPS placement prior to re-transplantation is a justifiable therapy in patients after liver transplant; however, very few cases have been reported in the literature [11].

In our case, veno-occlusive disease manifesting in hepatomegaly and portal hypertension was successfully treated by TIPS intervention. Cholestatic injury developed as a consequence of biliary stenosis induced by the TIPS stent graft. The tight stenosis initially only allowed for percutaneous drainage, which was then converted to internal biliary stents after an unsuccessful attempt at removal. To date, even after a total follow-up time of 44 mo, endoscopic treatment has failed to resolve the stenosis. The patient remains dependent on repeated stent exchange due to refractory and persistent right hepatic duct stenosis.

Our observations are consistent with the outcomes of previously reported cases. Biliary stenosis due to TIPS has been reported in twelve patients thus far. In ten of these (83%) cases, patients were symptomatic and required treatment. Seven patients were indicated for biliary drainage: ERCP was attempted in three patients and percutaneous therapy in four patients. ERCP as a first-line treatment was technically successful only in one of the three patients, failing in the remaining two. One patient was contraindicated for percutaneous drainage due to ascites, but ERCP subsequently failed and the patient was lost to follow-up. In the other patient, laboratory findings normalized solely due to percutaneous drainage of a hepatic abscess proximal to the stricture. Surprisingly, however, the long-term outcome of this patient was designated "unremarkable" in spite of the stenosis having not been resolved. A primary percutaneous approach was successful in all of the remaining four cases. However, in three of these cases, only external drainage due to an impassable stricture was possible, with the fourth patient later converted to internal drainage. Altogether, successful stenting of TIPS-induced stenosis has been reported in only two published cases, both having either unknown or unfavorable long-term outcomes. While in the first patient only a very short 2-mo follow-up was reported, in the second patient the stricture remained refractory even after almost 3 years of percutaneous and subsequent endoscopic drainage.

The above observations may be attributable to characteristic differences between TIPS-induced stenosis and other etiologies. In TIPS-induced stenosis, ducts narrow due to external compression of the metal stent graft. In these instances, radial force cannot be completely reverted by balloon dilation or implantation of plastic stents or drains, as with, for example, fibrotic stenosis. Resolution of symptomatic TIPS-induced biliary strictures was only achieved in four of the ten patients: three by liver transplantation and one by magnet-assisted endoscopic biliary-duodenal anastomosis. Of the remaining six patients, one died from colon perforation, two were lost to follow-up due to continuous abuse of alcohol, one patient remained on internal biliary drainage and another on external biliary drainage. In the last patient, the stricture became unexpectedly asymptomatic after percutaneous drainage of a liver abscess induced by stenosis.

CONCLUSION

We present here a peculiar case of a liver transplant recipient with a unique non-anastomotic biliary stricture caused by a TIPS stent graft implanted as a treatment for a very rare post-transplant complication, hepatic sinusoidal obstruction syndrome. To our knowledge, this extraordinary case is the first to document biliary complication due to TIPS placement in a transplanted liver. In our patient,

cholestatic liver injury was reverted by successful biliary drainage, resulting in preserved graft function. To date, even after a long follow-up period, the stenosis remains refractory to endoscopic treatment, with the patient dependent on repeated stent replacements. These results correspond with outcomes from previously published rare cases of TIPS-induced biliary strictures in native livers, in which complete resolution was only achieved as a result of liver transplantation or advanced endoscopic techniques.

FOOTNOTES

Author contributions: Hucl T, Peregrin J and Macinga P created the conception and design of this research; Macinga P and Hucl T wrote the paper, Gogova D and Jarosova J reviewed the literature and contributed to manuscript drafting; Raupach J performed transjugular intrahepatic portosystemic shunt placement; Janousek L performed liver transplantation and provided surgical consultations; Honsova E performed histological examination; Novotny J performed percutaneous transhepatic biliary drainage and interpreted the imaging findings; Hucl T, Spicak J, Taimr P and Peregrin J were responsible for the revision of the manuscript for important intellectual content; All authors revised the manuscript and issued final approval for the version to be submitted.

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LETTER TO THE EDITOR

Reply to "Six-minute walking test performance is associated with survival in cirrhotic patients" to the editor

Carla Malaguti, Carlos Alberto Mourão-Junior, Júlio Maria Chebli

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Abstract

Use of the six-minute walk test has been proposed as a prognostic marker in liver cirrhosis. In the Letter to the Editor presented here, the authors highlight some important points, which were raised after the article was published in the November issue of the *World Journal of Hepatology*.

Key Words: Six-minute walking test; Liver cirrhosis; Hospital admission and mortality

Core Tip: We advocate the use of the six-minute walk test, a practical and simple way to assess risk and provide a better understanding of how exercise limitation can directly affect the survival of cirrhotic patients; however, we emphasize the importance of interpreting it by using appropriate reference equations for a given population.

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TO THE EDITOR

The six-minute walk test (6MWT) is an easy-to-perform, inexpensive, and highly reproducible test to assess exercise capacity[1-3]. It also provides the most comprehensive prognostic information on many chronic health conditions[1]. Recently, our research group showed the prognostic clinical value of 6MWT in regard to predicting the risk of clinical decompensation in patients with compensated cirrhosis, adding clinical prognostic value in the evaluation[4].

In the November issue of the *World Journal of Hepatology* (*WJH*), Pimentel *et al*[5] reported the predictive capacity for mortality in patients with liver cirrhosis using the distance covered in the 6MWT over a 1-year period. The interesting results of that study seem to be in line with findings from other populations, in which the distance covered in the 6MWT predicts mortality and decompensation[6,7]. It must be noted that the study of Pimentel *et al*[5] was conducted in Brazil, and the reference equation used to determine predicted values of distance covered in the 6MWT was proposed by Enright *et al*[8] and based on a North American (United States) population.

The 6MWT is better interpreted if reference values are obtained using equations developed using a sample from the same country. Different authors have proposed reference values to predict the expected "normal" distance to be covered by a given patient[8-13]. Moreover, many equations with similar predictors are available in Brazil[10-13], despite different coefficients of determination. Negreiros *et al*[14] compared six reference equations developed in Brazil and observed that the equation proposed by Britto *et al*[13] estimated the most accurate results of distance covered by healthy Brazilian men. This is particularly relevant because Machado *et al*[15] demonstrated a low agreement between the reference equations of Enright *et al*[8] and Britto *et al*[13] (Kappa = 0.39).

Furthermore, studies carried out in different countries with healthy adults and older adults showed that the amount and intensity of physical activity vary considerably, depending on several factors, such as ethnicity, education level, and socioeconomic level[16,17]. In the study by Pitta *et al*[18], Brazilian patients with chronic obstructive pulmonary disease had a higher level of physical activity in daily life than Austrian patients, despite the high prevalence of comorbidities in Brazilian patients (*e.g.*, hypertension, diabetes, and osteoporosis). These results suggest that socioeconomic status and ethnic predictors for physical inactivity play a different role in inactive populations; for instance, South American patients are more active than patients in Central Europe due to worse socioeconomic conditions; they also have a higher degree of ethnic miscegenation. These factors certainly impact exercise capacity during the 6MWT. And as a result of this impact, using a reference equation in a given population is crucial.

We congratulate Pimentel and colleagues for their important contribution regarding the predictive capacity of distance covered in the 6MWT for mortality of patients with liver cirrhosis. However, considering the statements reported herein, it seems reasonable to recommend using available reference equations based on a national study.

FOOTNOTES

Author contributions: Malaguti C, Mourão-Junior CA, and Chebli JM were responsible for the conception, interpretation of the data, making critical revisions and final approval of the version of the article to be published; All authors of this manuscript contributed to its development.

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LETTER TO THE EDITOR

Role of biliary complications in chronic graft rejection after living donor liver transplantation

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Abstract

Postoperative biliary complications remain a substantial challenge after living donor liver transplantation, especially due to its heterogeneous clinical presentation.

Key Words: Chronic graft rejection; Biliary complications; Living donor liver transplantation; Graft survival; Cholangiopathy

Core Tip: In clinical practice, post-transplant cholangiopathy is a multifactorial process, including not only biliary complications like biliary leakage, biliary infection and biliary stricture, idiopathic post-transplant chronic hepatitis, fibrosing cholestatic hepatitis, and viral infections like cytomegalovirus but also chronic graft rejection. The post-transplant cholangiopathy substantially influences graft, as well as patient outcome and survival. Therefore, it is of outmost importance to distinguish the underlying etiology while simultaneously appreciating the heterogeneous nature of post-transplant cholangiopathy. A better understanding of clinical and histopathological features can result in an improved therapy strategy.

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TO THE EDITOR

With great interest, we read the article by Guirguis *et al*[1] entitled "Biliary complications in recipients of living donor liver transplantation: A single-center study". The study presents the findings on 169 recipients of right-lobe living-donor liver transplantation, which were followed for at least 1 year, or until graft or patient loss occurred. Based on their data on biliary leakage, biliary infection, and biliary stricture, as well as the presence of chronic graft rejection (CGR) and failure, the authors conclude that biliary infection/complications are an independent risk factor for CGR and graft failure in their study population.

While we congratulate the team on its attempt to unravel the impact of biliary complications on graft survival and mortality, we believe that some conclusions drawn in the article must be critically addressed.

First, the authors reference a study on a pediatric study population and deduce that biliary infection is a risk factor for CGR. However, in the mentioned study by Tannuri *et al*[2], the authors merely conclude that the occurrence of ductopenia is linked to a poor prognosis in pediatric patients with CGR. Furthermore, the authors outline main pathological changes that indicate CGR, such as vanishing bile duct syndrome and obliterative arteriopathy. Hence, Tannuri *et al* interpret ductopenia as the result of CGR, not its cause.

On that note, we want to turn the attention to well-defined classifications of CGR, especially in liver grafts for children and adults, respectively. In the updated *International Banff Schema for Liver Allograft Rejection*, Demetris *et al*[3] describe the features of CGR in accordance to histopathological findings of explanted liver tissue. Hereby, leading indicators of advanced CGR are outlined. These include, amongst others, loss of bile ducts (BD) of more than half of portal tracts, as well as the discovery of a foam cell obliterative arteriopathy in rejected tissue. Meanwhile, the loss of BD in less than 50% of portal tracts, BD degeneration, perisinusoidal fibrosis, and inflammation are considered preliminary findings for CGR after liver transplantation.

Second, in their retrospective multivariate analysis, Horster *et al*[4] reported on their 12-year experience with 352 liver transplant recipients. They identified HCV serostatus and high peri-transplantation viral serum loads as independent risk factors for postoperative anastomotic strictures. While non-anastomotic strictures, the presence of bile leaks, and subsequent treatment interventions worsened graft outcome in all patients, no increase of CGR was detected. Notably, biliary complications and HCV serum positivity exerted additive effects, although individually they did not alter the risk for graft loss. However, HCV-positive patients with BCs displayed significantly worse graft outcomes. The authors did not conclude that biliary infections would lead to CGR.

Although we understand that distinguishing between CGR and other causes of post-transplant cholangiopathy (PTC) might be histologically challenging and clinically difficult, it is of great significance to characterize the underlying etiology in order to provide our patients with the best available treatment and procedures. Thus, we appreciate CGR as one possible cause of the post-transplant cholangiopathy.

Leading to increased patient morbidity and mortality after liver transplantation, these entities require highly experienced physicians for a clear distinction and prompt intervention. The term PTC encompasses a wide range of histological donor bile duct aberrations, including biliary stricture, cast formation to full thickness and, even, bile duct necrosis with intrahepatic biloma development. As per definition, the presence of thrombosis, severe stenosis of the hepatic artery, or underlying chronic autoimmune disease (*i.e.*, primary sclerosing cholangitis) is excluded from the definition of PTC[5]. We conclude that other causes of PTC can be mistaken for suspected chronic graft failure with assumed biliary etiology. Thus, additional measures should be taken to prevent misdiagnosis in this highly susceptible patient collectives.

In essence, we are delighted to see the efforts at Ain Shams University, Egypt to better understand clinical observations on biliary complications after right lobe living donor liver transplantation, in order to sustainably achieve better patient outcomes. Nevertheless, the cases of biliary-based CGR should be validated by carefully distinguishing this uncommon condition from multifocal biliary pathologies of other etiologies. Adequately powered, prospective study designs with larger study populations could effectively contribute to a better understanding and improved therapy options.

FOOTNOTES

Author contributions: Obed A designed the research; Obed A, Füzesi L, and Jarrad A performed research and revised the letter; Bashir A and Obed A analyzed the data; Obed A and Bashir A wrote the letter.

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