

World Journal of *Hepatology*

World J Hepatol 2021 June 27; 13(6): 620-716



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The primary aim of *World Journal of Hepatology* (*WJH*, *World J Hepatol*) is to provide scholars and readers from various fields of hepatology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

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INDEXING/ABSTRACTING

The *WJH* is now abstracted and indexed in PubMed, PubMed Central, Emerging Sources Citation Index (Web of Science), Scopus, China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database. The *WJH*'s CiteScore for 2019 is 5.8 and Scopus CiteScore rank 2019: Hepatology is 22/61.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: Li-Li Wang; Production Department Director: Xiang Li; Editorial Office Director: Xiang Li.

NAME OF JOURNAL

World Journal of Hepatology

ISSN

ISSN 1948-5182 (online)

LAUNCH DATE

October 31, 2009

FREQUENCY

Monthly

EDITORS-IN-CHIEF

Nikolaos Pylsopoulos, Ke-Qin Hu, Koo Jeong Kang

EDITORIAL BOARD MEMBERS

<https://www.wjgnet.com/1948-5182/editorialboard.htm>

PUBLICATION DATE

June 27, 2021

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INSTRUCTIONS TO AUTHORS

<https://www.wjgnet.com/bpg/gerinfo/204>

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<https://www.wjgnet.com/bpg/GerInfo/287>

GUIDELINES FOR NON-NATIVE SPEAKERS OF ENGLISH

<https://www.wjgnet.com/bpg/gerinfo/240>

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<https://www.wjgnet.com/bpg/GerInfo/288>

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<https://www.wjgnet.com/bpg/gerinfo/208>

ARTICLE PROCESSING CHARGE

<https://www.wjgnet.com/bpg/gerinfo/242>

STEPS FOR SUBMITTING MANUSCRIPTS

<https://www.wjgnet.com/bpg/GerInfo/239>

ONLINE SUBMISSION

<https://www.f6publishing.com>

Glutathione-S-transferases genes-promising predictors of hepatic dysfunction

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Conflict-of-interest statement: The authors declare no conflict of interest.

Open-Access: This article is an open-access article that was

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Abstract

One of the most commonly known genes involved in chronic diffuse liver diseases pathogenesis are genes that encodes the synthesis of glutathione-S-transferase (GST), known as the second phase enzyme detoxification system that protects against endogenous oxidative stress and exogenous toxins, through catalisation of glutathione sulfuric groups conjugation and decontamination of lipid and deoxyribonucleic acid oxidation products. The group of GST enzymes consists of cytosolic, mitochondrial and microsomal fractions. Recently, eight classes of soluble cytoplasmic isoforms of GST enzymes are widely known: α -, ζ -, θ -, κ -, μ -, π -, σ -, and ω -. The GSTs gene family in the Human Gene Nomenclature Committee, online database recorded over 20 functional genes. The level of GSTs expression is considered to be a crucial factor in determining the sensitivity of cells to a broad spectrum of toxins. Nevertheless, human GSTs genes have multiple and frequent polymorphisms that include the complete absence of the *GSTM1* or the *GSTT1* gene. Current review supports the position that genetic polymorphism of GST genes is involved in the pathogenesis of various liver diseases, particularly non-alcoholic fatty liver disease, hepatitis and liver cirrhosis of different etiology and hepatocellular carcinoma. Certain GST allelic variants were proven to be associated with susceptibility to hepatological pathology, and

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Manuscript source: Invited manuscript

Specialty type: Gastroenterology and hepatology

Country/Territory of origin: Ukraine

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: March 17, 2021

Peer-review started: March 17, 2021

First decision: May 2, 2021

Revised: May 6, 2021

Accepted: June 3, 2021

Article in press: June 3, 2021

Published online: June 27, 2021

P-Reviewer: Jha RK, Pham TTT

S-Editor: Zhang L

L-Editor: A

P-Editor: Wang LL



correlations with the natural course of the diseases were subsequently postulated.

Key Words: Glutathione-S-transferase; Non-alcoholic fatty liver disease; Drug induced liver disease; Liver cirrhosis

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Core Tip: Current review provide data regarding impact of genetic polymorphism of glutathione-S-transferase (GST) genes in the pathogenesis of various liver diseases, particularly non-alcoholic fatty liver disease, hepatitis and liver cirrhosis of different etiology and hepatocellular carcinoma. Certain GST allelic variants were proven to be associated with susceptibility to hepatological pathology and correlations with the natural course of the diseases were subsequently postulated.

Citation: Prysyazhnyuk V, Sydorchuk L, Sydorchuk R, Prysiashniuk I, Bobkovych K, Buzdugan I, Dzuryak V, Prysyazhnyuk P. Glutathione-S-transferases genes-promising predictors of hepatic dysfunction. *World J Hepatol* 2021; 13(6): 620-633

URL: <https://www.wjgnet.com/1948-5182/full/v13/i6/620.htm>

DOI: <https://dx.doi.org/10.4254/wjh.v13.i6.620>

INTRODUCTION

Glutathione-S-transferases (GSTs) are group of phase II detoxification enzymes that catalyses the conjugation of glutathione (GSH) to a variety of endogenous and exogenous electrophilic compounds. It is without doubts that phase I enzyme reaction catalyses the incorporation of a functional group to a foreign compound, resulting in the formation of an intermediate metabolite. However, many of intermediates contain high potent chemical groups that can react with different cellular components including DNA, proteins and lipids[1,2]. This presence of intermediate metabolites can lead to multiple adverse health effects. Intermediate substances undergo phase II metabolism to form highly hydrophilic and less chemically active compounds, facilitating their excretion through bile or urine. Moreover, before being eliminated from the body, an extraneous compound can directly take part in phase II bypassing phase I detoxification. Phase II enzymes deactivate and detoxify foreign compounds unlike phase I enzymes which serves as activation metabolism, and therefore referred to as detoxification enzymes[3-5]. The aim of the current review was to overview up-to-date data and sum up results of own investigations regarding the distribution of GST genes polymorphisms, possible mechanisms of their involvement in the processes of desintoxication, drugs metabolism and cancerogenesis, and their role in the natural course of various liver diseases.

GSTs are presented by the cytosolic and membrane-bound microsomal super-family members. The groups of microsomal GSTs are structurally distinct from the cytosolic enzymes as they are rather homo- and heterotrimerise than dimerise in order to form a solitary active site. Microsomal GSTs are known to be the primary players in the endogenous metabolism of certain important substances like prostaglandins and leukotrienes. In contradistinction to microsomal GSTs, cytosolic GSTs are highly polymorphic and can easily be divided into eight sub-classes: α , μ , ω , π , θ , ζ , σ , and ω -. The π and μ classes of GSTs play a regulatory role in the mitogen-activated protein kinase pathway participating in cellular survival and death signaling *via* protein-protein interactions with c-Jun N-terminal kinase 1 (JNK1) and apoptosis signal-regulating kinase (ASK1). JNK and ASK1 are in turn activated in response to cellular stress[6-8].

GSTs are broadly distributed in the living world, from single cell organisms like bacteria to various plants, animals, and humans. Plant GSTs include the ϕ , τ , θ , ξ and λ classes; the θ and ξ have analogues in animals, too. Moreover, the ξ and θ classes are numerous in non-vertebrate animals. Advocating that the ancestral progenitor for mammalian GSTs, probably arose from the θ class GSTs based on significant homology between the θ class GST and a dichloromethane dehalogenase enzyme from the prokaryote methylobacteriaceae, belonging to the genus of rhizobiales which is

known to be able to undergo genetic transformation and become competent for DNA uptake close to the end of the exponential growth phase[9-12].

The review of the GSTs gene family in the Human Gene Nomenclature Committee (HGNC), online database, shows 23 (as for beginning of 2021) functional genes contained within the group[13], which is a minor upgrade from the last decade, when there were only 21 of such genes reported. However, the number of subfamilies varies from 16 to 26 in different sources, and some genes of the group were determined as encoding membrane-bound enzymes having GST-like activity, but these genes are not related to the GSTs gene family evolutionarily. These genes include *GST-κ1* [glutathione S-transferase kappa 1 (*GSTK1*), *GST13*, HGNC: 16906, Chromosome 7q34], and microsomal glutathione S-transferase 1 (*MGST1*, Chromosome12p12.3) microsomal glutathione S-transferase 1-like 1 (prostaglandin E synthase-PTGES, *MGST-IV*, *PIG12*, *MGST1-L1*, *TP53I12*, HGNC: 9599, Chromosome 9q34.11), microsomal glutathione S-transferase 2 (*MGST2*, *MGST-II*, HGNC: 7063, Chromosome 4q31.1), and microsomal glutathione S-transferase 3 (*MGST3*, *GST-III*, HGNC: 7064, Chromosome 1q24.1). The known human GSTs gene family consists of six subfamilies-α (GSTA-alpha), μ (GSTM-miu), ω (GSTO-omega), π (GSTP-pi), θ (GSTT-theta) and ξ (GSTZ-zeta)[14].

Probably, naming of GSTs genes can cause confusion, because both GSTW and GSTO names are similarly used for GST omega (ω) subfamily marking, and GSTT or GSTQ are concurrently used for GST theta (τ) subfamily listing in different sources. The reason for this lack of certainty originates from the HGNC's rules. Moreover, quite similar nomenclature problems were reported with the mouse GST genes[14,15].

Nonetheless, while only human GSTs are of valid clinical significance, other GSTs genes are of notable interest as this may explain both the connections and developments of human GSTs. The soluble GSTs can be subdivided into the cytosolic and mitochondrial forms, only GSTκ is exclusively mitochondrial, while *GSTA1*, 4, *GSTM1* and *GSTP1* encode both cytosolic and mitochondrial forms. The rest of the GSTs genes encode cytosolic proteins only. Note worthily, a vast number of GSTs were first identified in non-mammalian organisms, and were later recognised in humans and mammals[16-18], however most of the mammalian GSTs have been extensively studied and classified according to commonly assented criteria.

NON-HUMAN GSTS

Reports concerning plant GST enzyme revealed its involvement in catalysing the detoxification of the herbicide atrazine by conjugation to the endogenous γ-L-glutamyl-L-cysteinyl-glycine in sorghum and maize plants, which initiated a research that focuses on the detoxification of various herbicides and other toxic xenobiotic compounds in plants[19]. GSTs exhibit catalysis of the conjugation between various xenobiotics with electrophilic centres and the nucleophilic GSH, tagging the xenobiotic for vacuolar sequestration. The resulting γ-L-glutamyl-L-cysteinyl-β-alanine conjugates were much less toxic and more water-soluble than the original xenobiotics. It was shown that multiple plant GSTs participate in antioxidative protection due to their glutathione peroxidase activity[20].

The floral GSTs are mostly cytosolic and can represent up to 2% of soluble proteins. They have the ability to manifest auxin-inducibility and have ligandin function as well to participate in auxin transport. GSTs play a significant role during the normal metabolism of plant secondary products like anthocyanins[21]. The understanding of GSTs' role in endogenous floral processes and metabolic substrates had been still far from complete in contrast to the vast knowledge collected about their detoxification function[20,22].

Likewise, in human genome, floral GSTs enzymes are encoded by large gene families. The genome of the model plant *Arabidopsis thaliana* harbors 54 GST genes, which are grouped into seven distinct classes in plants. The well-studied large GSTF and GSTU classes are specific to plants, whilst the smaller GSTZ and GSTT classes exist in animal and human tissues. Lesser data is obtainable about the three outlying minor classes including GSTL, dehydroascorbate reductases, and tetrachloro-hydroquinone dehalogenase[21,23].

HUMAN GSTS

Human GSTs genes have multiple and frequent polymorphisms, including the complete absence (up to 20%-50% in some groups and populations) of the *GSTM1* or the *GSTT1* gene. The prevalence of the null genotype of *GSTT1* and *GSTM1* genes are heterogeneous amongst different ethnic populations. The *GSTT1* deletion is found in 20% of Caucasians and 80% of Asians[24]. While *GSTM1* zero genotype is detected in 38%-67% of Caucasian individuals, 33%-63% in East Asians and 22% to 35% in Africans and African Americans[25]. The substitution of adenine for guanine in nucleotide position 313 in the *GSTP1* gene leads to a reduction in the GST enzymatic activity which plays a significant role in the development of various diseases[26].

Following deficit in evident GSTs activities may lead to impaired detoxication of environmental substances, like toxins, carcinogens or drugs that may consequently generate clinically worth problems in patients lacking these genes[14,27-29].

GSTA, GSTM, and GSTP are over expressed in rat model of hepatic neoplasms (preneoplastic nodules) and the increased levels of these isoenzymes are assumed to provide the multidrug-resistant phenotype observed in these lesions. The majority of human tumors and human tumor cell lines express significant amounts of GSTP. The mechanisms responsible for over expression of GSTs, implicate transcriptional activation, stabilization of either messenger ribonucleic acid or protein, and gene amplification. In humans, remarkable interindividual differences are present in the expression of GSTA, GSTM, and GSTT. However, the exact molecular basis for the variation in GSTA is not known; missing of certain GSTM and GSTT classes can be attributed to deletion of the *GSTM1* gene in 50% of the population and deletion of the *GSTT1* gene in 16% of the population. The biological consequences of failure to express hGSTM1 or hGSTT1 protein can include higher susceptibility to some types of malignancies including skin, colon, bladder, and possibly lung cancer[10,30].

The level of GSTs expression is considered to be a crucial factor in determining the sensitivity of cells to a broad spectrum of toxins. The most abundant mammalian GSTs are the GSTA, GSTM and GSTP, however the biological control of these families is complex as they exhibit species-, age-, sex-, tissue-, and tumor-specific patterns of expression. Moreover, GSTs as shown above are regulated up and down by a broad spectrum of xenobiotics and drugs, with a significant number of these substances occurring naturally as non-nutritional components in modern food. It is obvious that humans are exposed regularly to such compounds[10].

Majority of chemical compounds, acting as GSTs inducers or inhibitors, have effect on transcriptional activation of GSTs genes through either antioxidant-responsive element, xenobiotic-responsive element, GSTP enhancer I, or glucocorticoid-responsive element[31,32].

The probability of GSTs is regulated *in vivo* by reactive oxygen species which is based on evidence that is not only but some of the most potent. GSTs inducers are capable of generating free radicals by redox-cycling, but hydrogen peroxide has been shown to strongly induce GSTs in plant and mammalian cells. An induction of GST by reactive oxygen species would appear to represent an adaptive response as GSTs detoxify some of the toxic peroxide-, carbonyl-, and epoxide-containing metabolites produced within the cell during oxidative stress[33-35].

Several functional studies of individual GSTs showed that they can positively contribute to host resistance against various microorganisms, whereas some physiologic mechanisms undergo further studying. Notwithstanding, the elevated total GST enzyme activities and notable accumulation of multiple GST transcripts and proteins was often observed in numerous host-pathogen interactions[23,36]. GSH is the most important non-protein thiol compound in several organisms and plays an important role in signaling and host defense reactions in infection. GSTs' participation in antioxidative react together with the crucial cellular antioxidant GSH in order to eliminate lipid hydroperoxides that accumulate in infected tissues, is clearly their distinguishable function[37-39].

Substantiation of GSTs genes from some commensals and parasites that may have immunomodulatory effect towards the immune system is growing, based on the involvement of separate profiles of cytokine gene transcription and different patterns of cell growth. Both antioxidants and oxidative stress manifest prompt transcription effect on many of the GSTs genes, which leads to increased protection of the cell against insult caused by environmental chemicals and drugs[40-42].

Possible interactions between host and microorganisms may result in three different ways: resistance gene (R-gene) mediated resistance, basal resistance and virulence. The first one (R-gene mediated), hypersensitive-type resistance is based on a specific interaction of a bacterial effect or gene product with the R-gene of the host organism.

R-gene mediated type of resistance is commonly corresponded with the localised cell death in infected host. It is unspecific, in case of basal resistance recognition; opposite to the R-gene mediated cell death, as genetically alien organisms are recognised based on their common molecular patterns. Induction of basal resistance is not associated with perceptible symptoms, in contrast to the hypersensitive-type R-gene mediated cell death. Poor host defense results in virulence[32,43].

Several members of the cytosolic GSTA, GSTM, GSTP, GSTT, microsomal transferases MGST2 and MGST3, are up-regulated by a wide spectrum of foreign compounds including but not limited to fumaric acid, thiazolidinediones, dexamethasone, phenobarbital, β -naphthoflavone, oltipraz, sulforaphane, coumarin, *etc.*[42]. The mechanism explaining this gene expression induction includes the aryl hydrocarbon receptor, and rostrane receptor, the Pregnane X receptor, nuclear factor erythroid 2-related factor 2, CAATT/enhancer binding protein- β , and peroxisome proliferator-activated receptor- γ , which connects GSTs with other pathogenetic mechanisms, genes, and clinical conditions that include insulin resistance, diabetes mellitus type 2, arterial hypertension and abdominal obesity[44].

Due to the fact that GSTs play a determinative role in the detoxification of xenobiotics, their down- or up-regulation may obviously affect biological effects and metabolism of many biologically active compounds, industrials and environmental pollutants. Several studies have demonstrated the potency of some flavonoids to modify the expression of GSTs and their activities. Furthermore, real effect of flavonoid compounds on GSTs strongly hinge on concentration, remedy administration duration, chemical structure of particular flavonoid, as well as on GST origin and isoform. To add confusion, *in vitro* and *in vivo* studies results are often inconsistent, incongruous or conflicting. Notwithstanding, prudent use of a flavonoid enriched diets, which may potentially induce GSTs are commonly beneficial, however the uncontrolled intake of certain flavonoids like catechins and quercetin in high doses as a dietary supplement may threaten health in consequence of GST inhibition. Moreover, combined use of certain flavonoids with drugs (acetaminophen, cisplatin, cyclophosphamide, and simvastatin) or xenobiotics (acrylamide, isocyanates polycyclic aromatic hydrocarbons, and chlorpyrifos), which are GSTs substrates, might have significant pharmacological and toxicological consequences[45].

GSTs genes often, demonstrate high inductivity through various stimuli of both abiotic and biotic origin. For example, salicylic acid (SA) showed prompt inducible effect on multiple GSTs. Some of the GSTs genes (*GSTF2*, *GSTF8*, *GSTF10*, *GSTF11*) are recognised determining SA-binding receptor proteins, though the biological relevance of SA binding to these GSTs needs further study[36,46-48].

Similar behavior may be observed in other genes involving in hepato-pancreatic conditions like angiotensin-converting enzyme gene and peroxisome proliferator-activated receptors- γ gene[49]. We can presume, that there is little evidence of specific precise cellular hepatic alteration mechanisms resulted from GST enzymes dysfunction or corresponding genetics' dysregulations.

NONALCOHOLIC FATTY LIVER DISEASE

Due to the studies of possible difference in the distribution frequency of allelic variations in the *GSTP1* A313G polymorphism, it has been established that G allele is spread significantly and more frequent in patients with nonalcoholic fatty liver disease (NAFLD) than in healthy individuals ($\chi^2 = 5.69$, $P = 0.017$) in Ukrainian population (Table 1)[50]. This data is consonant with the results of Hashemi *et al*[51], who have demonstrated that G allele of *GSTP1* gene is a risk factor for NAFLD formation. It was investigated, that total bilirubin level in blood of NAFLD patients with GG genotype of A313G polymorphism of *GSTP1* gene was higher as compared to AA genotype and AG genotype carriers. Presence of G allele was also associated with increased alanine aminotransferase activity, which was noticed to be significantly higher in NAFLD patients AG, and GG genotypes carriers as compared to patients with AA genotype [52].

Pro-and anti-inflammatory cytokines and adipokines profile varies in NAFLD patients with different polymorphic variants of the *GSTP1* gene (A313G) in particular. Homozygous patients with G allele are characterised by higher level of interleukin-10 (IL-10) in the blood as compared to patients with the AA and AG genotypes, that may occur potentially in response to the increase in the tumor necrosis factor- α (TNF- α) concentration, which proved the increased activity of inflammation processes[53,54]. NAFLD patients were investigated with low adiponectin levels in the blood in

Table 1 Distribution of polymorphic variants of the A313G polymorphism of the *GSTP1* gene in patients with nonalcoholic fatty liver disease and healthy individuals

Genotypes of the gene <i>GSTP1</i>	Patients with NAFLD, <i>n</i> = 104		Healthy individuals, <i>n</i> = 45	
	Absolute number, <i>n</i>	%	Absolute number, <i>n</i>	%
AA	47	45, 2%	28	62, 2%
AG	42	40, 4%	16	35, 6%
GG	15	14, 4%	1	2, 2%
A-allele	136	65, 4%	72	80, 0%
G-allele	72	34, 6%	18	20, 0%

NAFLD: Nonalcoholic fatty liver disease.

comparison with healthy people[55]. Moreover, according to Li *et al*[56] low adiponectin level is associated with the progression of steatohepatitis. The adiponectin concentration was lower in patients with NAFLD and AG and GG genotypes than in those with the AA genotype, indicating a worse adipokine profile for the NAFLD natural course[50]. A reverse tendency has been determined for leptin, however its blood level was higher in NAFLD patients with AG and GG genotypes as compared to those with the AA genotype[50]. This elevation of the leptin content in the *GSTP1* G allele carriers was, probably, associated with a high TNF- α concentration stimulating leptin production[57]. The aforementioned can prove the development of the leptin-resistance syndrome more severe in this cohort of patients[58]. In general, these observations indicate the formation of adipokine imbalance in the examined patients with AA genotype, which is typical for patients with NAFLD[59] which causes elevated leptin concentration against decrease adiponectin level in the blood[60].

Deletion polymorphic variants of *GSTT1* and *GSTM1* genes prevalence amongst NAFLD patients was approximately the same as their distribution between healthy individuals in Ukrainian population. These data are partially different from those suggested by Hori *et al*[61] who reported higher frequency of *GSTM1* null genotype in NAFLD patients as compared to control in the Japanese. There were not any notable differences in the parameters of the synthetic, detoxification, excretory liver functions together with activity of cytolytic and cholestatic syndromes and lipid profile in NAFLD patients with deletion of *GSTT1* and *GSTM1* genes and patients with functional allele of these genes[62]. It agrees with Rafiee *et al*[63] who also did not define importance contrasts in cholesterol and triglycerides plasma levels in individuals with different polymorphic variants of the studied genes. Interestingly, earlier studies of Maciel *et al*[64] suggested that double deletion genotypes of *GSTM1* and *GSTT1* genes were associated with hypertriglyceridemia.

Elevated TNF- α level in the blood is typical for NAFLD patients as compared to healthy individuals[65]. Jamali *et al*[66] proposed an algorithm involving TNF- α for predicting NAFLD/non-alcoholic steatohepatitis. Importantly, that null-genotype of *GSTT1* gene goes with higher TNF- α concentration as compared with patients having allele variant of *GSTT1*, and thereby indicate the activation of proinflammatory segment of cytokine profile and inflammatory processes[62]. Note worthily, TNF- α is one of the key factors involved in the insulin resistance, inflammation and apoptosis in case of NAFLD[67], thus its elevated level could be a predictor of aggravated liver injury in NAFLD patients with null-genotype of *GSTT1* gene.

Certain peculiarities in adipokine profile were detected regarding *GSTM1* genotype. Leptin plasma level was significantly higher in patients with null-genotype of *GSTM1* gene as compared to NAFLD patients with functional allele. This elevation of leptin content in null-genotype *GSTM1* carriers was probably associated with a high TNF- α concentration that stimulates leptin production[57]. Deletion polymorphism of *GSTT1* and *GSTM1* genes in patients with NAFLD was associated with lower content of restored glutathione, catalase activity. And in the case of carrier of zero genotype of *GSTM1* gene; it was also with higher level of reaction products of thiobarbituric acid in blood as compared to patients with functional allele of the gene[68].

DRUG INDUCED LIVER INJURY AND HEPATITIS

Prevalence of G allele of *GSTP1* (A313G) gene did not differ notably in chronic hepatitis patients in comparison with healthy individuals in Ukrainian population, however, presence of G allele was associated with higher activity of cytolytic syndrome lower restored glutathione blood content in comparison with patients AA genotype carriers[69]. *GSTP1* Ile/Val genotype was significantly more frequent in the patients with chronic hepatitis B infection and in patients with cirrhosis than in healthy individuals in Turkey; *GSTP1* Val/Val genotype was even more frequent in these patients[70]. In addition, these authors denoted relation between *GSTP1* gene polymorphism and hepatitis stage. In fact, as Ile/Val and Val/Val genotype frequencies increased so did the stages of the disease and tendency grow towards cirrhosis[70].

In our previous study, it was found that deletion genotype of *GSTM1* and *GSTT1* in patients with chronic hepatitis were representative to those in healthy individuals. Qi *et al*[71], have discovered that the genes *GSTM3* and *GSTP1* promoter methylation, which causes dysfunction of intracellular antioxidant defense system, more frequently occurs in patients with acute and chronic liver failure in case of hepatitis B virus, compared to patients with compensated viral hepatitis. Determination of methylated promoters of *GSTP1* and *GSTM3* genes can serve as a prognostic factor in the development of acute and chronic liver failure in these patients. It was found that *GSTO2* mutant genotypes were increased with progression, and the degree of hepatitis B virus (HBV) infection and the patients had mutant *GSTO2* genotypes such as (A/G, and G/G) were more susceptible for more severe HBV disease progression. The authors of the aforementioned study concluded that people with A/G and G/G genotype for *GSTO2* are more prone to develop hepatic failure[72]. Certain investigations have driven to the relation of *GST* gene polymorphism and drug induced liver injury. It was discovered almost twenty years ago, that homozygous null mutation at the *GSTM1* gene might predispose to hepatotoxicity for drugs used for the treatment of tuberculosis[73]. This statement was supported in the following studies revealing *GSTT1* homozygous null polymorphism may be a risk factor of antituberculosis drug-induced hepatotoxicity in Caucasians[74]. Meanwhile, presence of at least one functional allele of *GSTM1* was significantly more frequent amongst the groups with higher grades of liver toxicity for antituberculosis drugs in Brazilians[75]. Contrarily, *GSTT1* and *GSTM1* were not related to increased antituberculosis drug induced liver injury in Indian citizens[76]. By now, certain researchers[77] have linked troglitazone intoxication in the development of chronic diffuse liver diseases with the double-zero genotype *GSTT1* and *GSTM1* genes, considering its consequence of insufficient activity of detoxification defense systems, low activity of conjugation of sulfuryl groups. It has been shown that the zero genotype of *GSTT1* gene increases the risk of drug-induced liver damage in particular, due to the use of isoniazid[78]. Finally, in meta-analysis, it was found that null *GSTM1* genotype was responsible for higher susceptibility to drug induced liver disease related to antituberculosis medications in East Asian population, but not the Indians or Caucasians[79]. There were no confirmed relationships between null genotype of *GSTT1* gene and this kind of drug induced liver disease[79]. On the other hand, Wu *et al*[80] investigated that patients with tuberculosis A allele carriers of *GSTP1* gene (A313G) have a higher risk of anti-tuberculosis drug-induced hepatotoxicity development.

LIVER CIRRHOSIS

With regards to the report of Burim *et al*[81] study of susceptibility to cirrhosis and pancreatitis in alcoholic, concerning the GST and cytochromes 450 genes polymorphism, revealed that *GSTP1* Val allele carriers were at higher risk of both diseases. Ghobadloo *et al*[82] discovered the association of cryptogenic cirrhosis with Val/Val *GSTP1* genotype which might be explained by low detoxification activity of protein that implicate this polymorphism as a risk factor for occurrence of the disease. Goncharova *et al*[83] showed that patients with liver cirrhosis AA genotype carriers have 2.5 times higher survival rate compared with the patients with the GG and AG genotypes of *GSTP1* gene.

Khan *et al*[84] showed an increase in risk to alcoholic cirrhosis in patients with *GSTM1* null genotype when compared with non-alcoholic or alcoholic controls. A much higher risk to alcoholic liver cirrhosis was observed in patients carrying combination of null genotypes of *GSTM1* and *GSTT1*[84]. The authors of the

mentioned study found interaction of GSTs with variant genotype of manganese superoxide dismutase, which detoxifies free radicals, or cytochrome P450 2E1 that generates free radicals, and resulted in several fold increase in risk to alcoholic liver cirrhosis. Thus, conclude the possible gene-gene interaction in modulating the risk of the alcoholic liver cirrhosis development[84]. However, in another study from Brazil, no differences were found in the prevalence of the *GSTM1* and *GSTT1* null genotypes between control non-alcoholics and alcoholics with liver cirrhosis, as well as alcoholics without disease and alcoholics with liver cirrhosis[81]. Several older studies also have got different conclusions regarding the impact of *GSTM1* null genotype on the appearance of liver cirrhosis in patients with alcohol abuse. Specifically, Harada *et al* [85] in Japanese and Savolainen *et al*[86] in Finland found an increased risk of liver cirrhosis associated with the *GSTM1* null genotype in chronic alcoholics. Whilst, Frenzer *et al*[87] in Caucasian population and Rodrigo *et al*[88] in Spanish adults have not reported any. Brind *et al*[89] have found higher prevalence of zero *GSTT1* genotype in patients with alcoholic liver disease compared to patients who do not consume alcohol. Meanwhile *GSTT1* null genotype was not found to vary importantly between liver cirrhosis related to hepatitis B infection and healthy individuals[90]. At the same time, patients with *GSTM1* null genotype are at risk of progression of liver disease as the frequency of *GSTM1* null genotype was found to be significantly higher in chronic hepatitis B, hepatitis B cirrhosis and cryptogenic cirrhosis as compared with controls [90]. Moreover, the link between *GSTM1*, but not *GSTT1* null genotype and cryptogenic cirrhosis was found in Iranian population[82]. Komuro *et al*[91] in their investigations of primary biliary cirrhosis concluded that genotypic difference of *GSTM1* and *GSTT1* did not relate to susceptibility of this disease, nevertheless serum titer of anti-mitochondrial antibody of *GSTM1* null and *GSTT1* null patients were significantly higher than those of *GSTM1* positive and/or *GSTT1* positive patients. Baclic *et al*[92] also postulated that polymorphism in *GSTM1* null genotype seems to be associated with an increased risk of chronic liver disease amongst Filipinos.

HEPATOCELLULAR CARCINOMA

The GST null genotype has been examined to have an association with various malignancies including cancers of the bladder[93], gastric[94], colon[95], and lung[96]. K. Wu *et al*[97] investigated that *GSTP1* 313 G/G polymorphism is a strong predisposing risk factor for bladder cancer. Meanwhile, data regarding the role of GST gene polymorphism on the hepatocellular carcinoma (HCC) is sporadic. Qu *et al*[98] have found single nucleotide polymorphism (SNPs) *GSTO2* rs7085725 and *GSTP1* rs4147581 were significantly associated with the overall survival of HCC patients and suggested to use them alone or in combination as potential prognostic markers for HCC patients. Particularly, according to the author's suggestion, SNP of *GSTP1* (rs4147581) could have a predictive biomarker in HCC patients aged ≤ 55 years[98]. *GSTM1* and *GSTT1* polymorphisms appear to be associated with a modest increase in the risk of HCC in Egyptian patients[99]. *GSTT1* null genotype was associated with more than 2-fold increased risk for HCC development in patients with hepatitis associated with hepatitis C virus (HCV) as compared to the control group. However, *GSTM1* null genotype was found to have a protective effect when hepatitis patients were considered in Indian population[100]. Meanwhile, in older study it was found that the *GSTT1*-null genotype alone did not affect risk of HCC development in HBV, but the *GSTM1*-null genotype was associated with a decreased risk for early-onset HCC[101]. The meta-analysis by Li *et al*[102], involving results of 46 related studies with more than 15 thousands of patients showed that both *GSTM1* null genotypes and *GSTT1* null genotypes increased the risk of HCC, while *GSTM1*-*GSTT1* dual-null genotypes increased the risk of HCC to a higher extend. Interestingly, during ethnicity consideration, this connection was significant only for Asians, and not for Caucasians and Africans. In older meta-analysis by Shen *et al*[103] *GSTM1* and *GSTT1* null genotype was found to be associated with higher risk of HCC with a similar ethnic pattern. *GSTP1* rs1138272 (341C>T) polymorphism was found to have a protective effect on liver cancer development in a high-risk HCV/HBV-positive population in Caucasian ethnicity[104]. *GSTP1* genetic polymorphisms (*i.e.*, Ile105Val, rs1695) were not associated with HCC risk in Asian population, European and African[105,106]. Higher *GSTP1* levels in tumor tissues indicated a better overall survival and disease-free survival for HCC patients[107]. The mentioned authors have found that *GSTP1* could decrease p-Akt in liver cancer cell lines and may inhibit alfa-fetoprotein expression. *GSTP1*'s inhibition on cancer progression may be accomplished by arresting the cell

cycle at the G1/S transition in HCC cells[108]. *GSTA1* TT genotype was more frequent in HCC than in non-HCC patients, suggesting that individuals carrying this genotype could be associated with 2-fold higher risk of developing HCCs[109]. *GSTM1* and *GSTT1* null genotypes are associated with an increased HCC risk in Chinese population with higher risk typical for double null genotype. Furthermore, in another meta-analysis, it was investigated that null genotype of *GSTT1* was associated with HCC susceptibility in Asians, and both *GSTT1* and *GSTM1* genes deletion were associated with higher susceptibility. *GSTP1* Ile105 Val gene polymorphism was not correlated with this disease, however, polymorphisms in *GSTM1* and *GSTT1* genes are not related to the incidence of HCC in a high-risk Spanish population[110]. Marahatta *et al*[111] provided the support for the difference in genotypic distribution for GSTO1* A140D between hepatocellular carcinoma and cholangiocarcinoma.

CONCLUSION

Current review supports the position that genetic polymorphism of *GST* genes is involved in the pathogenesis of various liver diseases, specifically in non-alcoholic fatty liver disease, hepatitis and liver cirrhosis of different etiology and hepatocellular carcinoma. Certain *GST* gene allelic variants were proven to be associated with susceptibility to hepatological pathology and correlations with the natural course of the diseases were postulated. Still the data obtained in different studies sometimes is controversial and even conflicting. Thus, more investigations involving larger numbers of patients are needed.

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Wilson's disease: Revisiting an old friend

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Author contributions: Ampuero J is the guarantor of the article; Lucena-Valera AL, Perez-Palacios D, Muñoz-Hernandez R, and Ampuero J drafted the manuscript; Romero-Gómez M, and Ampuero J performed a critical review of the manuscript; all authors approved the final version of the article, including the authorship list.

Supported by Consejería de Salud. Junta de Andalucía, No. PI_0039_2017; and Junta de Andalucía, No. 201799903406796.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

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Abstract

Wilson's disease (WD) is a rare condition caused by copper accumulation primarily in the liver and secondly in other organs, such as the central nervous system. It is a hereditary autosomal recessive disease caused by a deficiency in the ATP7B transporter. This protein facilitates the incorporation of copper into ceruloplasmin. More than 800 mutations associated with WD have been described. The onset of the disease frequently includes manifestations related to the liver (as chronic liver disease or acute liver failure) and neurological symptoms, although it can sometimes be asymptomatic. Despite it being more frequent in young people, WD has been described in all life stages. Due to its fatal prognosis, WD should be suspected in all patients with unexplained biochemical liver abnormalities or neurological or psychiatric symptoms. The diagnosis is established with a combination of clinical signs and tests, including the measurement of ceruloplasmin, urinary copper excretion, copper quantification in liver biopsy, or genetic assessment. The pharmacological therapies include chelating drugs, such as D-penicillamine or trientine, and zinc salts, which are able to change the natural history of the disease, increasing the survival of these patients. In some cases of end-stage liver disease or acute liver failure, liver transplantation must be an option to increase survival. In this narrative review, we offer an overview of WD, focusing on the importance of clinical suspicion, the correct diagnosis, and treatment.

Key Words: Wilson's disease; Copper; ATP7B; Ceruloplasmin; Chelator; Liver disease

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Manuscript source: Invited manuscript

Specialty type: Gastroenterology and hepatology

Country/Territory of origin: Spain

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: January 24, 2021

Peer-review started: January 24, 2021

First decision: March 8, 2021

Revised: March 21, 2021

Accepted: May 8, 2021

Article in press: May 8, 2021

Published online: June 27, 2021

P-Reviewer: El-Shabrawi MH, Janicko M

S-Editor: Fan JR

L-Editor: Webster JR

P-Editor: Wang LL



Core Tip: Wilson's disease (WD) is a rare metabolic disorder caused by the deposition of copper in organs, particularly in the liver and the brain. As the symptoms and clinical presentation can be highly variable, WD is not always suspected. A detailed but practical review is presented to assist clinicians in the diagnosis and management of WD.

Citation: Lucena-Valera A, Perez-Palacios D, Muñoz-Hernandez R, Romero-Gómez M, Ampuero J. Wilson's disease: Revisiting an old friend. *World J Hepatol* 2021; 13(6): 634-649

URL: <https://www.wjgnet.com/1948-5182/full/v13/i6/634.htm>

DOI: <https://dx.doi.org/10.4254/wjh.v13.i6.634>

INTRODUCTION

Wilson's disease (WD) is an autosomal-recessive monogenic disorder characterized by an excessive accumulation of copper, firstly described in 1912 by Kinnear Wilson. The World Health Organization estimates the global prevalence of WD to be between 1/10000 and 1/30000[1]. It is caused by mutations in the *ATP7B* gene, which encodes a transporter protein with ATPase activity. This transporter is involved in incorporating copper into apoceruloplasmin, which is finally eliminated in bile. When a mutation affects the *ATP7B* transporter, free copper is released into the bloodstream and is removed by urine instead of feces[2]. Therefore, *ATP7B* is essential for copper biliary excretion[3].

In this review, we aimed to revise the clinical aspects of WD, including diagnosis, clinical manifestations, and the therapeutic approach, and discuss the future treatment of the disease.

GENETICS

The *ATP7B* gene is located on chromosome 13q14.3 and comprises 20 introns and 21 exons, encoding a protein of 165 amino acids[4,5], whose function is the incorporation of copper into ceruloplasmin. Currently, more than 800 mutations have been discovered in the gene[6], of which 380 have confirmed involvement in the pathogenesis of the disease[7,8]. Although mutations have been reported in almost all exons[5], they mainly affect the central regions of the gene (both 8 and 14 exons are the most frequently affected). The most common mutations are H1069Q and R778L in European and Asian populations, respectively[2,4]. Approximately 90%-98% of WD subjects are heterozygous, showing different mutations in each of the alleles encoding the *ATP7B*. On the other hand, the phenotype and the penetrance of WD can be extremely variable. Even patients carrying two disease-causing mutations do not necessarily have a demonstrable alteration of copper metabolism[9]. Some of the proposed reasons are differences in copper intake, individual antioxidant capacity or susceptibility to liver fibrosis, and hormonal influences[10].

The potential role that epigenetics could have in the gene expression of the disease should be highlighted. Some experimental models have shown changes in DNA methylation through breast milk enriched with methyl groups that could be related to the clinical manifestation of WD[11].

Considering the probability of late-onset, the fact of having asymptomatic cases, and the phenotypic variability, it seems vital to evaluate the previous and next generation of the index case[12]. Both the European Association for the Study of the Liver (EASL) and the American Association for the Study of Liver Diseases (AASLD) recommend an appropriate study of the index case taking into account the family history of liver- and brain-related disease[7,13]. These guidelines propose to assess the patient's siblings since the risk of WD is 25% (by presenting two mutations in both alleles). Subsequently, other first-degree family members should be evaluated, although the risk decreases to 0.5%[12].

CLINICAL MANIFESTATIONS

There is a wide variety of symptoms involved in WD, which predominantly affect the liver and brain (Table 1). Although WD may be present at any age, it is more common between the ages of 5 and 35. However, it should be investigated in patients with liver failure due to an unknown cause and those with liver disease and neuropsychiatric symptomatology[13]. Asymptomatic patients are commonly diagnosed during the family screening process[7].

Liver symptoms

Liver symptoms of WD occur mainly during childhood and adolescence[10]. In these cases, liver involvement appears up to 10 years before neurological manifestations[7]. The clinical spectrum ranges from asymptomatic patients, with mild analytical alterations, to subjects with fulminant liver failure. In this scenario, there are forms of acute (from acute hepatitis to fulminant liver failure) and chronic presentation (from steatosis to compensated and decompensated cirrhosis)[14].

Asymptomatic forms usually have only hepatomegaly, discretely elevated transaminases, or are identified during the screening of an index case[15].

Acute presentation: WD should be suspected in a patient with acute hepatitis, in which viral hepatitis is ruled out. Symptoms are similar to acute viral hepatitis, with jaundice and abdominal pain[14]. This situation, including acute liver injury (manifested by coagulopathy) or acute liver failure (with hepatic encephalopathy), occurs predominantly in women[16]. Beyond these signs and symptoms, the elevation of hemoglobin, cholinesterase, and low alkaline phosphatase are characteristic of acute WD. Sometimes, hemolytic anemia with a negative Coombs test is presented, one of the diagnostic criteria of WD[7]. WD causes 2%-5% of acute liver failure events, showing a fatal prognosis in the absence of liver transplantation (LT) [14].

Chronic hepatitis and cirrhosis: Typically, it starts as a slight transaminase elevation that progresses slowly to fibrosis and, finally, cirrhosis. When it manifests itself as cirrhosis, there is an increased risk of mortality[8]. Sometimes, patients may show splenomegaly uniquely as a sign of portal hypertension. In particular, young patients over three years of age showing cirrhosis should be evaluated for WD[15]. On the other hand, WD can initially be confused with autoimmune hepatitis, as they occur at a similar age and are manifested by jaundice and increased transaminases and gammaglobulins[14]. Also, WD has been described as causing hepatic steatosis, which is identified in up to 15% of biopsies[17].

Neurological symptoms

Neurological involvement typically appears after liver manifestations. WD affects the central nervous system mainly through extrapyramidal system dysfunction and bulbar involvement. The most common symptom is dysarthria, particularly in the early stages of the disease[8]. The neurological presentation can also be manifested by tremors, parkinsonism, or involuntary movements, even by cerebellar dysfunction, chorea, or hyperreflexia[14]. Furthermore, dystonia affecting the face and jaw is characteristic, producing a typical sign (Wilson's face)[15]. Also, a postural tremor is common in WD patients[7].

Psychiatric symptoms

Psychiatric symptoms must be considered in WD. In fact, patients showing these symptoms often suffer a delayed diagnosis[18]. In fact, approximately one third of patients develop psychiatric symptoms as the initial manifestation[7]. Typical symptoms are depression and anxiety[14], although changes in behavior or personality or impulsivity can occur[19]. In addition, affective disorders are more common than psychotic spectrum disorders.

Ocular manifestations

Kayser-Fleischer's (KF) ring represents a frequent manifestation of WD, which affects the Descemet membrane of the cornea. The slit-lamp examination shows a brown-gold colored ring on the periphery of the cornea[20]. It is present in more than 90% of patients with WD showing neurological involvement but only in half of cases with liver disease. Notably, the KF ring does not affect vision, and its disappearance has been seen in patients undergoing effective treatment and LT[15]. Although it is one of the most typical features of WD, this ring has been described in cholestatic syndromes and other diseases[21].

Table 1 Clinical manifestations

Wilson's Disease Clinical Manifestations	
Liver	Hepatomegaly, jaundice, pain in right hypochondria, asthenia, elevation of transaminases, acute liver injury, acute liver failure, cirrhosis (compensated and decompensated), ACLF, steatosis
Neurological	Dystonia, tremor, dysarthria, dysphagia, Parkinson, chorea
Psychiatric	Behavioral changes, depression, anxiety, psychosis, school performance deficit, sexual disinhibition
Eye	Kayser-Fleischer Ring, Cataract
Hematologic	Hemolytic anemia, coagulopathy, thrombopenia
Renal	Acute renal failure, nephrolithiasis, urolithiasis, renal tubular acidosis
Musculoskeletal	Arthropathy, muscle weakness
Other	Heart disease, pancreatitis, hypoparathyroidism

ACLF: Acute-on-Chronic Liver Failure.

Other symptomatology

As copper can be accumulated in different organs and systems, WD has been associated with arthropathy[22], recurrent muscle weakness due to hypokalaemia[23], cardiomyopathy[24], symptomatic urolithiasis[25], pancreatitis[26], cases of hypoparathyroidism[27], and infertility[28,29].

DIAGNOSIS

There are no specific diagnostic tests for WD (Table 2). Instead, a combination of clinical signs and symptoms and some tests are required to achieve the final diagnosis.

Ceruloplasmin

Ceruloplasmin is the leading copper transporter protein, carrying 90% of serum circulating copper. It is synthesized in the liver and excreted into the circulation from hepatocytes, mostly as holoceruloplasmin (containing six copper atoms) and the remainder as apoceruloplasmin (not joined to copper)[30]. Ceruloplasmin levels may be determined enzymatically by its copper-dependent oxidase activity or by immunological assays. The immunological assay measures the total ceruloplasmin level but not the ceruloplasmin oxidase activity. Therefore, normal levels of ceruloplasmin do not rule out low oxidase activity and WD. For this reason, the use of enzymatic assays is more appropriate[31]. Blood ceruloplasmin levels are typically low (< 0.2 g/L) in patients with WD and neurological involvement. However, they may be higher in up to half of patients with WD[32]. On the other hand, ceruloplasmin levels are not decreased only in WD, but can be reduced in other conditions such as renal or enteric protein loss, malabsorption, end-stage liver disease, or aceruloplasminemia[33]. In addition, up to 20% of healthy heterozygous carriers have low non-pathological levels of ceruloplasmin. Ceruloplasmin is also an acute-phase reactant and may be elevated in inflammation or infections, resulting in false negatives in WD patients with both characteristics[7].

Serum copper

Serum copper decreases proportionally with ceruloplasmin levels. WD should be considered when normal or elevated serum copper levels along with decreased ceruloplasmin are identified, as this indicates an increase in the concentration of non-ceruloplasmin-bound copper[34]. However, in patients with deficient ceruloplasmin levels, low total serum copper levels can be found even though free copper (albumin-bound copper or non-ceruloplasmin bound copper) may be increased. For this reason, only the determination of free copper is important as total serum copper mostly reflects ceruloplasmin-bound copper. To calculate free copper, serum copper must be subtracted from the value of ceruloplasmin and multiplied by 3 (each ceruloplasmin molecule provides 3 mg of copper). Patients with WD have free copper levels between 10-20 mg/dL and symptomatic individuals have levels > 20 mg/dL[35]. Free copper levels may also be increased in cholestatic syndromes and copper intoxication[36] and

Table 2 Diagnosis tests for Wilson's disease

Test	Normal values	Wilson disease	False negative	False positive
Ceruloplasmin	0.2-0.4 g/L	< 0.2 g/L	Increased levels: Hepatic inflammation Estrogen Pregnancy Infection Children Overestimation by immunological assay	Low levels: Malabsorption Malnutrition Aceruloplasminemia Menkes' disease Terminal liver disease Nephropathy with renal protein loss Excess zinc ingestion Healthy heterozygotes WD
Non ceruloplasmin bound copper	< 0.3 µg/dL	> 10 µg/dL	Overestimation of ceruloplasmin by immunological assay	Increased levels: Cholestatic syndromes Acute liver failure Copper intoxication
Urinary copper excretion	< 0.6 µmol/24 h; < 40 µg/24 h	> 1.6 µmol/24 h; > 100 µg/24 h	Incomplete collection; Children	Increased levels: Cholestatic syndromes Autoimmune hepatitis Chronic active liver disease or hepatocellular necrosis Healthy heterozygotes WD
Liver biopsy	< 50 µg/g; < 0.8 µmol/g	> 250 µg/g; > 4 µmol/g	Uneven copper distribution	Increased levels: Cholestatic syndromes Idiopathic copper toxicosis disorders
Kayser Fleischer rings	Absence	Present: Neurological WD Absence: 50% hepatic WD Asymptomatic WD		Primary biliary cholangitis

WD: Wilson's disease.

strikingly elevated in acute WD liver failure due to the sudden release of copper from the liver.

Determining free copper is challenging due to the inadequacy of ceruloplasmin determination methods. It is preferable to use enzymatically determined ceruloplasmin levels when calculating free copper, but they do not detect apoceruloplasmin and overestimate ceruloplasmin. For this reason, the determination of ceruloplasmin non-bound copper is not commonly used as a diagnostic method[37]. In 2009, a new method called exchangeable copper (CuEXC) was proposed for the direct determination of labile copper. It can be performed routinely and allows a direct and accurate measurement of copper overload, representing an extrahepatic biomarker[38]. For instance, values greater than 2.08 mmol/L suggest a high risk of severe neurological disease[39]. Additionally, CuEXC facilitates calculation of the relative exchangeable copper. When its threshold is higher than 18.5%, this biomarker reaches a sensitivity and specificity close to 100% in WD diagnosis, without the presence of false negatives [40,41]. Therefore, it could differentiate WD from other liver diseases and healthy heterozygous subjects, representing a promising family screening marker[2,42].

Urinary copper excretion

Urinary copper excretion in 24 h reflects the amount of circulating non-ceruloplasmin

copper and, therefore, represents the excess copper excreted in the urine. In children, a value greater than 0.64 mmol/24 h or 40 g/24 h is suggestive of WD, while the cut-off for adults is 1.6 mmol/24 h (100 g/24 h)[16]. However, in up to 16%-23%, especially in asymptomatic children and siblings, urinary copper excretion may be lower than the values set[34,43]. After D penicillamine (DPA) administration (1,000 mg administered in two doses), urinary copper excretion consists of measuring urinary copper excretion within 24 h on the same day. It has been proven that urinary copper excretion values > 160 µg/24 h is compatible with WD in children[44]. However, this test is not standardized in adults, so it is not currently recommended in that population.

The determination of urinary copper excretion is challenging in some scenarios, such as the presence of renal failure and an incomplete or inadequate collection of urine. In addition, patients with autoimmune hepatitis, cholestatic diseases, acute liver failure, or asymptomatic heterozygous patients can show elevated urinary copper excretion[45].

Liver biopsy

Liver biopsy is a non-risk-free invasive technique; thus, it is not easy to perform in asymptomatic patients. Its use is limited to patients with compatible clinical or biochemical findings but without a definite diagnosis.

WD has no specific histological changes, although there are suggestive changes. Mild steatosis may be observed in patients without risk factors (alcohol, overweight, diabetes mellitus, or dyslipidemia) who are often mistaken to have non-alcoholic fatty liver disease. Furthermore, staining of metallothionein (protein-bound to intrahepatic copper) by orcein or lysosomal copper complexes, using rodamine or rubenic acid, show liver copper deposits[35]. The sensitivity of these stains increases when the sample is deposited in xylol for 24 h[46]. Despite this, the hepatic accumulation of copper cannot be ruled out with histochemistry as staining only reveals copper deposits in less than 10% of patients. Thus, intrahepatic copper quantification is essential for the diagnosis of WD after a hepatic biopsy. For the determination of copper in dry weight, it is necessary to obtain a significant sample (at least 1 cm) and its placement in a copper-free and dry container. Values greater than 250 µg/g (4 mmol/g) are diagnostic, while values less than 50 µg/g (0.8 µmol/g) make the diagnosis highly unlikely. The major problem of the intrahepatic quantification of copper is the heterogeneity of distribution of liver copper deposits (which could be unrepresentative), as well as the elevation of intrahepatic copper deposits in cholestatic diseases[47].

Neurological and psychiatric assessment

Patients with WD, even if they have predominantly hepatic involvement, should be evaluated neurologically. The neurological symptoms in WD are varied, and include Parkinsonian motor alterations and psychiatric symptoms[18]. Magnetic resonance imaging (MRI) shows structural abnormalities with a hyperintensity in the T2 sequence in the basal ganglia, tectum, spinal bulb, thalamus, and brainstem. Also, there is a decreased intensity in the T1 sequence in the basal ganglia[48]. During MRI, the "giant panda face" sign, found in 14% of patients, is characterized by hyperintensity of the tegmentum of the midbrain, especially around the red nucleus, which maintains its normal hypointensity on T2-weighted imaging axial sections of the brain. This sign, along with the tectal and center-protuberance plaque's hyperintensity and the simultaneous involvement of the basal ganglia, thalamus, and brainstem, are practically pathognomonic of WD[49].

Genetic testing

Direct sequencing of the *ATP7B* gene provides the greatest efficiency in clinical molecular diagnosis. The most common mutation (H1069Q) is present in 40%-50% of patients in Western countries; however, 17% of patients with a diagnosis established by the Leipzig criteria do not have any identifiable *ATP7B* gene mutation[50]. This may be explained by the inability of genetic testing to distinguish disease-specific mutations from polymorphisms of the gene and the absence of analyzing the non-coding regions of the gene, which can also affect gene expression. However, next-generation sequencing is becoming a very useful, reliable, time-saving, and cost-effective tool for diagnostic testing in the future.

How is the diagnosis established?

As previously described, a single test does not allow a definite diagnosis of WD. For this reason, a scoring system that combines clinical parameters with biochemical and

imaging tests, known as the Leipzig criteria[7,13], is needed for patients (Table 3)[51]. More than 4 points are required to establish the diagnosis of WD according to these criteria, while an alternative diagnosis should be considered in individuals showing less than 4 points. Therefore, liver biopsy and the genetic assessment may not be needed if other test results add up to at least 4 points. However, the Leipzig criteria show some weaknesses that have to be taken into account, such as the lack of definition of the upper limit of normality of urinary copper excretion or the importance attributed to urinary copper excretion in 24 h after stimulation with DPA [52,53].

TREATMENT

Lifelong treatment is necessary even in asymptomatic patients. There are several treatments for WD, including DPA, trientine, and zinc salts. Figure 1 summarizes the therapeutic approach for patients with WD. Once treatment is indicated for WD, it should be monitored in terms of efficacy (including adherence to treatment) and side effects. Briefly, urinary copper excretion should be assessed every two weeks within the first 4-6 wk and every 2-3 mo during the next 6-12 mo[10,54]. The objectives of copper excretion, according to the drug, are described in Table 4. Similarly, side effects of treatment should also be monitored using blood tests and the liver profile, as well as copper and serum ceruloplasmin[13].

DPA

DPA is the first-line drug for WD, and its mechanism involves chelation of circulating copper which will subsequently be excreted in the urine. DPA reduces copper's affinity for proteins by facilitating the removal of copper from tissues, and it induces the synthesis of metallothionein in the liver, a cysteine-rich protein with a high affinity for metal ions. It is metabolized in the liver and is mostly excreted in the urine.

DPA is administered orally, and its absorption is 40%-70% of the administered dose. The dose in adults is 750-1500 mg, and in children is 20 mg/kg/d, given in 2 or 3 divided doses in both cases. DPA should not be taken with food, antacids, or iron supplements because they decrease its absorption. Notably, pyridoxine supplementation should be recommended during treatment with DPA[7].

Up to 90% of patients under DPA therapy have hepatic improvements. However, the efficacy of DPA in neurologic WD is less satisfactory, with an improvement rate of 55%[55]. On the other hand, DPA has numerous adverse reactions; many of them can be severe (Table 5). In those situations, DPA should be discontinued and replaced with another drug. One of the most concerning scenarios is the severe and irreversible neurological worsening at the start of treatment, which can occur in 10%-50% of patients with previous neurological symptoms[56].

Although neurological worsening typically occurs with DPA treatment, it has also been demonstrated with trientine and to a lesser extent with zinc salts[16,57]. Free copper induces oxidative stress which damages brain tissue. Consequently, the chelating agent should be started at a low dose (125 mg/d) and should be increased every 3-4 d.

Trientine

Trientine or triethylenetetramine dihydrochloride is a chelating agent with a similar mechanism of action to DPA. The efficacy of trientine is similar to DPA. It forms a complex with four nitrogen atoms and copper to be excreted in the urine. It is administered orally, and is poorly absorbed from the gastrointestinal tract. The usual dose is 900 to 2700 mg/d for the initial chelation phase and 750 to 1500 mg/d for the maintenance phase in adults, while 20 mg/kg/d is recommended in children (always divided into two or three doses a day). Similar to DPA, trientine should also be administered separately from food and other drugs. Recent studies propose administering a single daily dose of 15 mg/kg, which would significantly improve adherence to treatment[58]. A particular challenge in trientine treatment is its instability as it must be kept cold (2°C-8°C). On the other hand, trientine is a well-tolerated chelating agent that decreases the discontinuation rate up to 4 times compared to DPA, but higher rates of neurological deterioration have been observed than with PDA therapy [55] (Table 5).

Zinc salts

Zinc induces metallothionein synthesis in enterocytes, binding to copper and

Table 3 Leipzig scoring for Wilson's disease

Typical clinical signs and symptoms		
Kayser-Fleischer ring		
Present	2	
Absent	0	
Neurologic symptoms or typical abnormalities on MRI		
Severe	2	
Mild	1	
Absent	0	
Serum ceruloplasmin		
Normal (> 0.2 g/L)	0	
0.1-0.2 g/L	1	
< 0.1 g/L	2	
Coombs negative hemolytic anemia		
Present	1	
Absent	0	
Other tests		
Liver copper ¹		
> 4 µmol/g	2	
0.8-4 µmol/g	1	
< 0.8 µmol/g	-1	
Rhodamine positive granules ²	1	
Urinary copper excretion ³		
Normal	0	
1-2 times ULN	1	
> 2 times ULN	2	
5 times ULN after penicillamine	2	
Mutation analysis detected		
Both chromosomes	4	
One chromosome	1	
No mutations	0	
Total Leipzig score		
Score	Evaluation	
≥ 4	Diagnosis established	
3	Diagnosis possible	
≤ 2	Diagnosis very unlikely	

¹In the absence of cholestasis.²If no quantitative liver copper available.³In the absence of acute hepatitis. MRI: Magnetic resonance imaging; ULN: Upper limit of normal.

preventing its absorption into the portal circulation. It is then excreted in feces due to the natural flaking of enterocytes. Zinc also induces metallothionein synthesis in hepatocytes by neutralizing copper in the liver[59,60]. The recommended dose is 150 mg/d, divided into three doses, while 75 mg is adequate for children lower than 50 kg, at least 30 min before meals. In combination with some chelating agents, zinc should

Table 4 Monitoring urinary copper excretion in the treatment of Wilson's disease

Treatment	Initial treatment	Maintenance treatment	Undertreatment or non-compliance	Overtreatment or non-compliance
D penicillamine	> 500 µg/24 h	200-500 µg/24 h	> 500 µg/24 h	< 200 µg/24 h
Trientine	> 500 µg/24 h	200-500 µg/24 h	> 500 µg/24 h	< 100 µg/24 h
Zinc	> 100-500 µg/24 h	< 75 µg/24 h	> 15 µg/24 h	< 5 µg/24 h

Table 5 Adverse effects of medical therapy used in the treatment of Wilson's disease

Medication	Side effects
D penicillamine	<p>Early (1-3 wk):</p> <p>Fever, cutaneous eruptions, myelosuppression, lymphadenopathy, proteinuria</p> <p>Late: (> 3 wk-yr)</p> <p>Renal: Nephrotoxicity, nephrotic syndrome</p> <p>Lungs: Goodpasture syndrome</p> <p>Bone marrow: Aplasia</p> <p>Eye: Optic neuritis, retinitis</p> <p>Skin: Pemphigus, pemphigoid lesions, aphthous stomatitis, hair loss</p> <p>Autoimmunity: Lupus erythematosus, myasthenia gravis, polymyositis, immunoglobulin A depression</p> <p>Dose-dependent:</p> <p>Pyridoxine deficiency</p> <p>Mammary hypertrophy</p> <p>Skin: Elastosis serpiginosa, lichen planus, progeria-like skin changes</p> <p>Neurological deterioration (10%-50%)</p>
Trientine	<p>Few side effects:</p> <p>Bone marrow depression</p> <p>Sideroblastic anemia</p> <p>Hemorrhagic gastritis, loss of taste, and skin rash</p> <p>Neurological deterioration is less common</p>
Zinc	<p>Very few side effects:</p> <p>Gastric irritation</p> <p>Elevation of serum amylase and lipase</p> <p>Bone marrow depression</p> <p>Neurological deterioration is very uncommon</p>
Tetrathiomolybdate	<p>Few side effects:</p> <p>Bone marrow suppression</p> <p>Increased serum aminotransferase levels</p> <p>Anemia</p> <p>No neurological deterioration</p>

be administered separately to avoid neutralization of salts. Evidence shows that zinc salts have few side effects, with gastric irritation being the most common side effect. Zinc salts are not recommended as the initial treatment, particularly in acute liver failure. Therefore, it should be used as first-line therapy only in asymptomatic patients or as maintenance treatment after initiation with chelating agents[61,62].

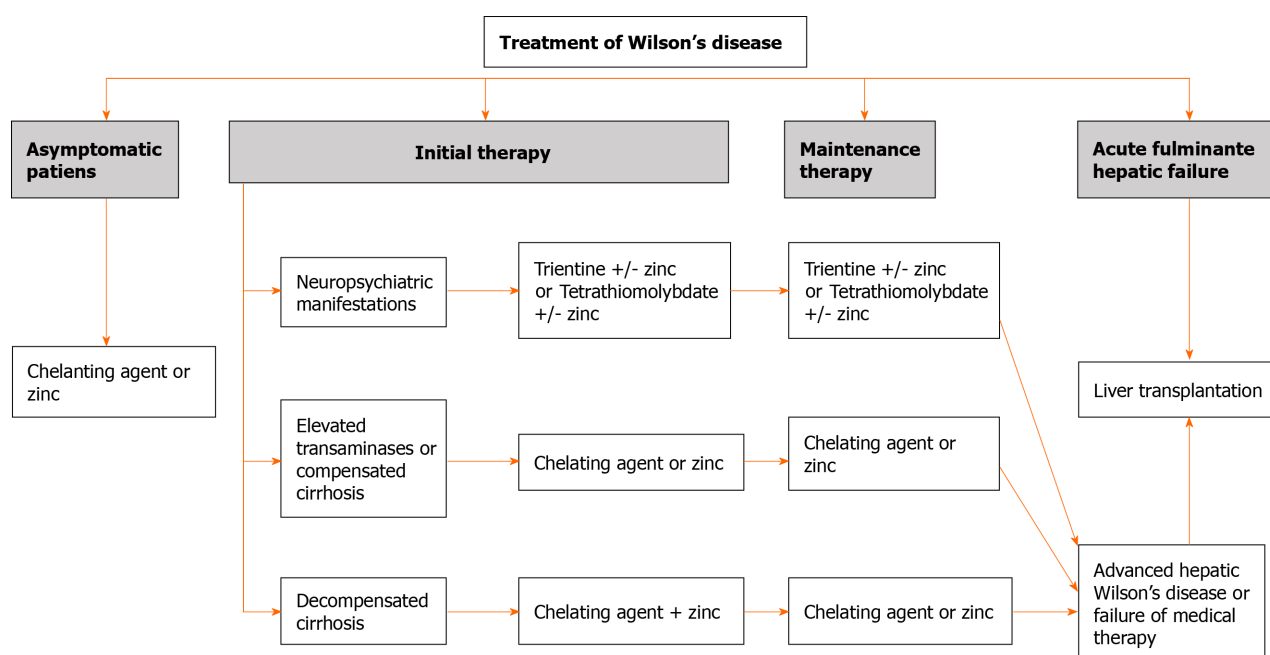


Figure 1 Therapeutic approach for Wilson's disease.

New treatment options

Trientine tetrahydrochloride is a new drug that is being studied in clinical trials. Compared with conventional trientine, it is stable at normal temperature. On the other hand, tetrathiomolybdate ammonium (TTM), a potent decoppering drug, reduces intestinal absorption of copper and forms a tripartite complex with proteins and copper that is subsequently excreted in bile. In contrast to chelating agents, TTM is not associated with neurological deterioration; thus, it can be used in the neurological phenotype of WD[63]. However, it has been associated with other side effects such as myelosuppression, anemia, and elevation of transaminases. Notably, the ammonium salt of TTM is unstable, although a new complex (Bis-choline TTM) is being developed to solve this issue[64]. Finally, methanobactins are a novel approach that is being investigated with positive results in WD treatment. They can remove copper from the mitochondria, avoiding cell toxicity and acute liver failure[65].

Is dietary copper restriction necessary?

As excessive accumulation of copper causes WD, it has been proposed that copper should be restricted in the diet. Significantly, foods to avoid are chocolate, fruits, nuts, mushrooms, liver, and seafood. Both AASLD and EASL guidelines recommend avoiding the intake of high-concentration copper foods or water, particularly within the first year of diagnosis[7,13]. Nevertheless, copper absorption depends on the content of copper in the diet, showing a self-regulatory mechanism. In fact, diets with a high copper concentration result in lower absorption by enterocytes and a higher copper excretion[66]. Thus, copper-rich foods should be consumed to generate excessive copper intake.

LT

LT has a particularly good survival rate in the WD setting[67]. It is indicated mainly in two situations: acute liver failure and end-stage liver disease. WD has a particular score (King's score) that should be used to decide on LT in the setting of acute liver failure, as an index greater than 11 is associated with a high risk of death without LT [68,69]. LT provides functionality for hepatic ATP7B, resulting in normalization of copper metabolism and removal; consequently, chelation therapy may be discontinued after LT. Although LT is controversial as a treatment for the neurological phenotype of WD, an improvement in neurological involvement has been documented[70,71].

Treatment in special situations: Pregnancy

Treatment should not be discontinued in pregnant patients as the risk is higher than with maintenance therapy, with acute liver failure cases described in patients after withdrawal of treatment[72]. Although DPA has teratogenic potential, a clear increase

in risk has not been observed in patients with this treatment, similar to trientine and zinc salts[73,74]. On the other hand, copper deficiency could have a teratogenic effect, so it is advised to reduce chelating therapy by 25%-50% during pregnancy.

PROGNOSIS

In the absence of adequate treatment, the prognosis of WD is fatal[7], but with treatment, this entity has an excellent prognosis. However, we should consider that severe neurological alterations may not be improved, although most patients show significantly improved neurological involvement. Similarly, psychiatric manifestations also improve and can even disappear. On the other hand, patients with cirrhosis often remain compensated and do not have cirrhosis complications, although patients with WD and liver cirrhosis should be screened for HCC[54].

FUTURE TREATMENT APPROACHES FOR WD

To date, treatments for WD are based on removing excess copper from the body or LT. Currently, many clinical trials are investigating new treatments with higher efficacy and tolerance, but only a few studies have focused on copper metabolism restoration.

Liver-targeted gene therapy represents an attractive treatment option for many liver conditions[75,76]. Recently, Murillo *et al*[77] demonstrated that the use of recombinant adeno-associated viral vector (rAAV8), containing complementary DNA encoding copper transporting ATPase2, normalized soluble haloceruloplasmin, and hepatic parenchymal copper levels for more than six months after a single administration, in an animal model[77]. Related to these results, a phase I/II study in sixteen adult WD patients will start in 2021 (clinical.gov. Identifier: NCT04537377), where a single intravenous dose of a rAAV liver tropic capsid containing a single-stranded DNA genome carrying a shortened version of the *ATP7B* gene will be used.

The regenerative medicine field has progressed in the past two decades. The role of hepatocytes in liver repair is well known. In fact, hepatocyte transplantation has been proposed as an alternative approach to LT, but has some disadvantages such as weak viability in cell culture, the complexity of hepatocyte source, and the vulnerability to cryopreservation[78]. In this sense, stem-cell therapy has been shown to be a potential therapeutic approach in several liver diseases[79,80]. The differentiation potential of mesenchymal cells into hepatocytes has been demonstrated in several studies[81,82]. Indeed, mesenchymal cells can be easily isolated from visceral fat or bone marrow, expanded without losing their differentiation potential, and can migrate to injured areas[83]. The potential to ameliorate liver injury in preclinical and clinical studies has been previously described[84,85]. Recently, induced Pluripotent Stem Cells (iPSCs) have dominated the field of regenerative medicine. These cells have been isolated from patients with different liver diseases showing specific genotypes[86,87]. iPSCs can be isolated by non-invasive methods[88], providing a hepatocyte source for genetic disorders, protein dysfunction, and subsequent cellular defects responsible for specific diseases. A previous study described the generation of iPSCs from WD donor fibroblasts (skin samples) that bear the R778L mutation in the *ATP7B* gene and their differentiation into hepatocyte-like cells with defective copper transport[89]. They reported gene correction using a lentiviral vector. In the future, hepatocyte-like cells from similarly genetically corrected iPSCs could be an option for autologous transplantation in WD patients. In summary, the expanding tools of gene editing and cell therapy with promising results in other monogenic liver diseases provide a new approach in WD, which could improve the quality of life of these patients by restoring copper metabolism.

CONCLUSION

The knowledge on WD is increasing. The diagnosis of this entity is based on clinical features, biochemical parameters and genetic testing, although new biomarkers are on the horizon. The development of new and effective treatments, including gene therapy, is promising for the future treatment of this disease.

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Balloon-occluded retrograde transvenous obliteration for treatment of gastric varices

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Author contributions: Waguri N wrote the manuscript; Osaki A drew the illustrations; Watanabe Y reviewed the paper.

Conflict-of-interest statement: There are no conflicts of interest.

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Manuscript source: Invited manuscript

Specialty type: Gastroenterology and hepatology

Country/Territory of origin: Japan

Peer-review report's scientific quality classification

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Abstract

Rupture of gastric varices (GVs) can be fatal. Balloon-occluded retrograde transvenous obliteration (BRTO), as known as retrograde sclerotherapy, has been widely adopted for treatment of GVVs because of its effectiveness, ability to cure, and utility in emergency and prophylactic treatment. Simplifying the route of blood flow from GVVs to the gastroduodenal shunt is important for the successful BRTO. This review outlines BRTO indications and contraindications, describes basic BRTO procedures and modifications, compares BRTO with other GVVs treatments, and discusses various combination therapies. Combined BRTO and partial splenic embolization may prevent exacerbation of esophageal varices and shows promise as a treatment option.

Key Words: Gastric varices; Balloon-occluded retrograde transvenous obliteration; Balloon-occluded antegrade transvenous obliteration; Partial splenic embolization; Transjugular intrahepatic portosystemic shunt; Plug- and coil-assisted retrograde transvenous obliteration

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Core Tip: Gastric varices (GVs) are a common complication of liver cirrhosis and their rupture is often fatal. Balloon-occluded retrograde transvenous obliteration (BRTO) has been widely adopted for treatment of GVVs because of its effectiveness, ability to cure, and utility in emergency and prophylactic treatment. Various modifications of BRTO and combinations with other therapies are also beneficial. Combined BRTO and partial splenic embolization shows promise as a treatment option.

Citation: Waguri N, Osaki A, Watanabe Y. Balloon-occluded retrograde transvenous obliteration for treatment of gastric varices. *World J Hepatol* 2021; 13(6): 650-661

Grade A (Excellent): A, A
 Grade B (Very good): B
 Grade C (Good): C
 Grade D (Fair): D
 Grade E (Poor): 0

Received: February 25, 2021

Peer-review started: February 25, 2021

First decision: March 29, 2021

Revised: April 12, 2021

Accepted: May 22, 2021

Article in press: May 22, 2021

Published online: June 27, 2021

P-Reviewer: Chiu KW, Simón-Talero M, Xiong B

S-Editor: Liu M

L-Editor: A

P-Editor: Wang LL



URL: <https://www.wjgnet.com/1948-5182/full/v13/i6/650.htm>

DOI: <https://dx.doi.org/10.4254/wjh.v13.i6.650>

INTRODUCTION

Gastric fundal varices (GVs) and esophageal varices (EVs) are two of the main presentations of cirrhosis-induced portal hypertension. Although the bleeding risk of GV is relatively low, their rupture is associated with high mortality (14%–45%)[1–4], because of their larger shunt diameter and higher flow. Hemodynamically, the two types of varices are completely different. The left and right gastric veins comprise the inflow of EVs, with the azygos vein system serving as the outflow. In contrast, the short and posterior gastric veins comprise the main inflow of GV, although the left gastric vein may also be involved; the gastrosplenic shunt (GRS), which drains blood to the left renal vein *via* the descending branch of the left inferior phrenic veins (80%–85%), and the gastrocaval shunt (GCS), which runs below the diaphragm and drains into the inferior vena cava (10%–15%) serve as outflow[5]. Eradication of GV is difficult endoscopically because of the large diameter and high flow velocity of the shunts. Balloon-occluded retrograde transvenous obliteration (BRTO), developed by Kanagawa in 1996, is a sclerotherapy technique that approaches the varices from the outflow side of the GRS[6]. Since then, BRTO has been widely accepted in Japan[7–9], Asia, and the United States[10,11] as an effective treatment for GV. In Europe, however, BRTO is not well recognized and not a treatment option for GV[12,13]. In this review, we outline the indications and contraindications for BRTO, describe basic BRTO procedures and modifications, compare BRTO with other GV treatments, and discuss various combination therapies.

INDICATIONS AND CONTRAINDICATIONS FOR BRTO

According to Saad *et al*[14], the two clinical indications for BRTO are bleeding GV (active, current, prior, and impending) and refractory hepatic encephalopathy involving the portosystemic shunt that forms GV. Contraindications include: (1) Severe uncontrollable coagulopathy associated with liver failure; (2) Splenic vein thrombosis; (3) Portal vein thrombosis; and (4) Uncontrolled bleeding from EV. In the case of uncontrolled bleeding from EV, BRTO is contraindicated as a sole procedure; combined transjugular intrahepatic portosystemic shunt (TIPS) and BRTO or balloon-occluded antegrade transvenous obliteration (BATO) *via* the TIPS route are recommended instead.

We use BRTO for both emergency and elective treatment of ruptured GV as well as prophylactic treatment according to the criteria described below[15,16]. Indications for prophylactic treatment of GV include nodular form and red color spot lesions[17], increasing size over time, and hepatic encephalopathy. However, we do not treat patients with severe hepatic dysfunction (total bilirubin ≥ 4.0 mg/dL, Child-Pugh score ≥ 13), renal dysfunction (eGFR < 30 mL/min/1.73 m²), or other serious diseases with poor prognosis as well as those without a portosystemic shunt amenable to a retrograde approach[15]. We consider the presence of contrast agent flowing freely from the GRS into the portal vein on balloon-occluded retrograde venography (BRTV) a relative contraindication[16].

ADVANTAGES OF BRTO OVER OTHER TREATMENTS

Although beta blockers are widely used to prevent bleeding in esophagogastric varices, based on a great deal of evidence[13,18], this review omits a description of them as its focus is interventional procedures.

TIPS is widely used in Western countries to treat portal hypertension in patients with esophagogastric varices and refractory ascites[19–24]. TIPS significantly reduces GV rebleeding compared with pharmacotherapy and endoscopic treatments such as endoscopic variceal band ligation[19–21]. Although TIPS reduces portal venous pressure (PVP), GV rebleeding and stent dysfunction are common[19–21,25]. Additionally, post-TIPS mortality is relatively high due to serious complications such as intraperitoneal hemorrhage, hemobilia, sepsis, hepatic failure, congestive heart

failure, and others[25,26]. However, the use of polytetrafluoroethylene-covered stents has improved the TIPS patency rate[27] and the complication rate has decreased in conjunction with more widespread use. Preemptive TIPS is also recommended to prevent esophagogastric varices rebleeding[13,28].

Endoscopic injection of n-butyl-2-cyanoacrylate (CA) has also been widely used to treat GV[29]. In patients with acute bleeding, CA injection is reportedly more effective than pharmacotherapy alone[30,31] and is the therapy of choice[32,33]; however, CA injection for elective treatment is not recommended and only used when no other treatment is available[32,33].

BRTO is highly effective to eradicate GV[6-8,15,34] and can be effective for prophylactic[7-9,34] as well as emergency bleeding treatment[15,35,36]. Several studies have shown that BRTO is superior to endoscopic interventions in terms of bleeding control and prognosis in patients with GV[35,37,38]. Furthermore, several comparative studies have reported that BRTO has a slight advantage over TIPS in terms of rebleeding, hepatic encephalopathy, hepatic functional reserve, and survival[39-44]. These studies are summarized in Tables 1-4. Table 1 shows the study design and sample size. Table 2 summarizes the sclerosant used for BRTO, types of stents used for TIPS, and the technical success rate of each procedure. Table 3 shows the rebleeding rates of GV and EV. Table 4 shows the notable complications after each procedure. Recent meta-analyses[45-47] have concluded that BRTO in patients with GV bleeding is associated with lower rates of rebleeding and postprocedural hepatic encephalopathy, as well as better survival than TIPS. Although BRTO is effective in eradicating GV, it is associated with complications such as postprocedural EV, ectopic varices, and intractable ascites. Further debate over the relative superiority of BRTO or TIPS is not constructive. Rather, clinicians should fully understand the characteristics, risks, and benefits of each and use them suitably according to individual patient therapeutic needs. Clinicians should also consider using them in various therapeutic combinations.

CONVENTIONAL BRTO PROCEDURE

BRTO drug preparation and procedures have been described in detail by Hirota *et al* [16]. In Japan, BRTO using ethanolamine oleate with iopamidol (EOI) became covered by insurance in 2018 after publication of a prospective multicenter clinical trial[48].

Our conventional BRTO method is described as follows[15,49]: GRS is diagnosed by computed tomography (CT). An 8 Fr long shepherd hook-shaped (Asato; Medikit, Tokyo, Japan) or cobra-shaped (S-one sheath; Terumo Clinical Supply Co., Gifu, Japan) sheath introducer is advanced into the left renal vein *via* the right femoral or internal jugular vein, respectively. A 6 Fr catheter with a 20 mm diameter balloon or 5.2 Fr catheter with a 9 mm diameter balloon (Selecon MP Catheter; Terumo Clinical Supply Co.) is then advanced into the GRS through the introducer in a retrograde fashion. BRTV (Figure 1) is then performed to identify shunts and their inflow and outflow. Before sclerosing the GRS, the route from the GV to the GRS needs to be simplified. We use the down-grading method [50], selective coil embolization of the minor accessory draining veins[51], and/or the stepwise injection method [51] to down-grade the target shunt vessels to a relatively simple Hirota grade 1 or 2[52] (Figure 2A-D). If the coexisting GCS has a large diameter and selective coil embolization of the left inferior phrenic vein is impossible, the GCS is occluded with another balloon catheter [53] (Figure 2E). Under temporary balloon occlusion, contrast medium is injected *via* the balloon catheter to confirm stagnation of variceal flow for ≥ 10 min and evaluate the required volume of sclerosing solution. When stagnation of the contrast medium is confirmed, the same volume (10-40 mL) of 5% EOI is injected and remains stagnant in the vessels with overnight balloon occlusion. Human haptoglobin (4000 units) is administered prior to EOI injection to prevent acute kidney injury secondary to hemolysis caused by EOI[54]. The catheter is removed after overnight occlusion. Thrombosis of the GV-GRS outflow (therapeutic effect) and thrombus formation elsewhere in the portal system (adverse effect) are confirmed by CT 3 to 7 d after BRTO. Eradication of GV is confirmed by endoscopy after 2 to 3 mo.

BRTO MODIFICATIONS

BRTO is commonly performed overnight to prevent the outflow of sclerosant into the systemic circulation[15,16,48]. Alternatively, a vascular plug[55] or microcoils[56] can

Table 1 The studies comparing balloon-occluded retrograde transvenous obliteration and transjugular intrahepatic portosystemic shunt

Ref.	Journal	Country	Study design	Number of cases	
				BRTO	TIPS
Choi <i>et al</i> [39]	KJR 2003	South Korea	RCT, Single institution	8	13
Ninoi <i>et al</i> [40]	AJR 2004	Japan	Retrospective, Single institution	77 (BRTO: 49 / PTS: 28)	27
Sabri <i>et al</i> [41]	JVIR 2014	United States	Retrospective, Single institution	23	27
Kim <i>et al</i> [42]	KJR 2017	United States	Retrospective, Single institution	25	27
Lee <i>et al</i> [43]	JGH 2017	South Korea	Retrospective, Two institutions	95	47
Gimm <i>et al</i> [44]	Gut and Liver 2018	South Korea	Retrospective, Single institution	157	19

BRTO: Balloon-occluded retrograde transvenous obliteration; TIPS: Transjugular intrahepatic portosystemic shunt; RCT: Randomized controlled trial; PTS: Percutaneous transhepatic sclerotherapy.

Table 2 Materials used and technical success rate

Ref.	BRTO	Tips	Technical success rate	
	sclerosant	Stent type	BRTO	TIPS
Choi <i>et al</i> [39]	EO	Bare	8/8	13/13
Ninoi <i>et al</i> [40]	EO	Bare	49/58	27/27
Sabri <i>et al</i> [41]	STS	Covered	21/23	27/27
Kim <i>et al</i> [42]	EO, STS	Covered	22/25	27/27
Lee <i>et al</i> [43]	EO, STS, polidocanol	Covered	106/123	49/60
Gimm <i>et al</i> [44]	EO, STS	Bare, covered	159/166	19/22
Total			365/403	162/176
			90.6% ¹	92.0% ¹

¹Not significant. BRTO: Balloon-occluded retrograde transvenous obliteration; TIPS: Transjugular intrahepatic portosystemic shunt; EO: Ethanolamine oleate, STS: Sodium tetradecyl sulfate.

be placed to occlude the GRS instead, allowing catheter system removal as soon as the treatment is complete (a single day procedure). The original methods of plug-associated retrograde transvenous obliteration (PARTO)[55] and coil-associated retrograde transvenous obliteration (CARTO)[56] (Figure 3A and B) emphasized their advantage of not requiring balloon catheters, sclerosants, or a long period of postprocedural bed rest and monitoring. However, these techniques have the disadvantage of high cost. By embolizing the small drainage vessels with gelatin particles, the selective coil embolization procedure can be omitted, and the procedure becomes easy and effective[55,57]. However, recurrence of GVs is lower when a surfactant such as sodium tetradecyl sulfate is used as a sclerosant in PARTO compared with use of gelatin alone[57]. Recurrence might be due to recanalization through the gelatin sponge which does not provide the permanent endothelial injury and thrombosis caused by sclerosants[58]. Injected gelatin has no direct effect on blood clot formation. Once the injected gelatin particles flow into the systemic circulation, they become emboli to the micro-vessels elsewhere. In contrast, sclerosant has a thrombus-forming effect on small drainage vessels, even in small amounts. However, if a small amount of sclerosant flows into the systemic circulation, it is often diluted with a large amount of blood and the effect of vascular endothelial damage can be ignored. Therefore, we believe that sclerosant should be used in BRTO rather than gelatin sponge alone.

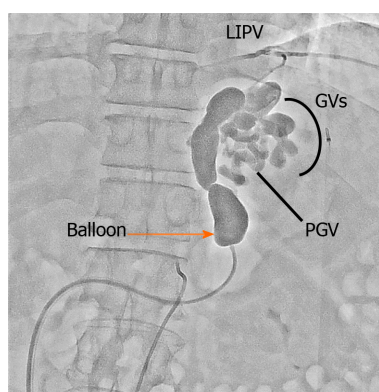
Instead of downgrading by advancing the balloon catheter, a modified CARTO[59] in which embolization is performed using microcoils and sclerosant is injected upstream to the GVs has also been described (Figure 3C). Yamamoto *et al*[60] described CARTO-II, in which sclerosant is injected from a balloon catheter in the

Table 3 Rebleeding rate from gastric varices and esophageal varices

Ref.	Rebleeding rate from GV's		Rebleeding rate from EV's	
	BRTD	TIPS	BRTD	TIPS
Choi <i>et al</i> [39]	0/8	1/13	0/8	0/13
Ninoi <i>et al</i> [40]	1/77	6/27	3/77	2/27
Sabri <i>et al</i> [41]	0/23	3/27	0/23	0/27
Kim <i>et al</i> [42]	2/25	2/27	1/25	0/27
Lee <i>et al</i> [43]	7/95	6/47	4/95	7/47
Gimm <i>et al</i> [44]	8/157	3/19		
Total	18/385	21/160	8/228	9/141
	4.7% ¹	13.1% ¹	3.5% ²	6.4% ²

¹*P* = 0.0005.²Not significant. GV's: Gastric varices; EV's: Esophageal varices; BRTD: Balloon-occluded retrograde transvenous obliteration; TIPS: Transjugular intrahepatic portosystemic shunt.**Table 4** Complications after balloon-occluded retrograde transvenous obliteration or transjugular intrahepatic portosystemic shunt

Ref.	LF		HE		Ascites		EV's aggravation	
	BRTD	TIPS	BRTD	TIPS	BRTD	TIPS	BRTD	TIPS
Choi <i>et al</i> [39]	0/8	1/13	0/8	1/13	0/8	0/13	1/8	0/13
Ninoi <i>et al</i> [40]	3/77 ¹	10/27 ¹	0/77	5/27	6/77		14/77	
Sabri <i>et al</i> [41]	0/23	0/27	0/23	6/27				
Kim <i>et al</i> [42]	0/25	0/27	0/25	6/27	1/25	1/27	1/25	0/27
Lee <i>et al</i> [43]	0/95	1/47	0/95	14/47	13/95	2/47		
Gimm <i>et al</i> [44]	0/157	0/19	4/157	0/19	48/157	1/19	22/157	1/19

¹Including long-term events. BRTD: Balloon-occluded retrograde transvenous obliteration; TIPS: Transjugular intrahepatic portosystemic shunt; LF: Liver failure; HE: Hepatic encephalopathy; EV's: Esophageal varices.**Figure 1** Balloon-occluded retrograde transvenous venography. When the gastrosplenic shunt is balloon-occluded (arrow) and retrogradely imaged, the posterior gastric vein, which is the inflow vessel, is visualized via the gastric varices. A part of the left inferior phrenic vein as an outflow vessel is also demonstrated. PGV: Posterior gastric vein; GV: Gastric varices; LIPV: Left inferior phrenic vein.

same manner as conventional BRTD, coil-embolization is performed just above the balloon (Figure 3D), and the balloon catheter is finally removed. In CARTO-II, thrombosis has already occurred due to vascular endothelial damage caused by the sclerosant, and coil-embolization is performed to prevent the thrombus from flowing

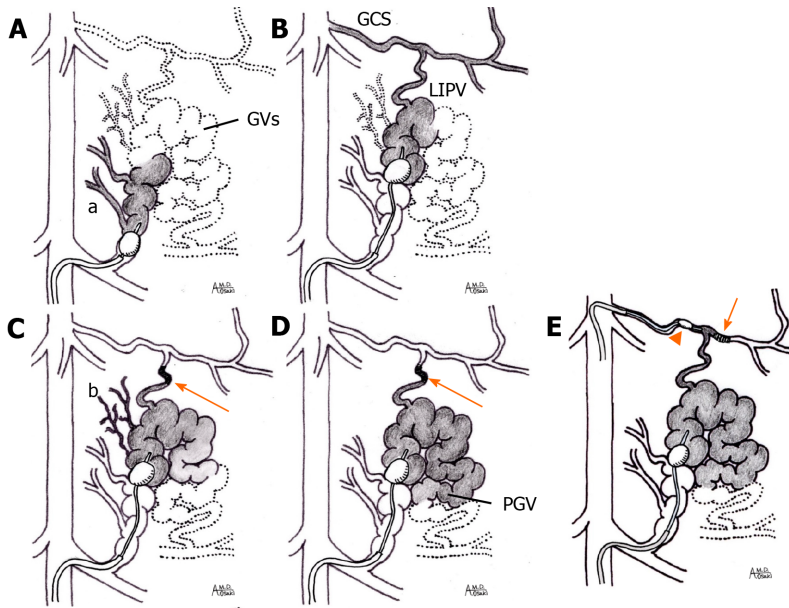


Figure 2 Illustration of the balloon-occluded retrograde transvenous obliteration procedure. A: Balloon-occluded retrograde transvenous venography (BRTV). The initial BRTV does not visualize the main body of the gastric varices (GVs) because multiple draining vessels are present (a); B: When the balloon catheter is advanced beyond the small drainage vessels (downgrading method), the relatively large diameter left inferior phrenic vein (LIPV) becomes visualized as another drainage route to the gastrocaval shunt (GCS); C: GV become visualized when selective coil embolization (arrow) of the LIPV is performed. As small amounts of sclerosant are injected sequentially over time, the smaller drainage vessels (b) are gradually embolized (stepwise injection method); D: After stepwise injection, BRTV demonstrated the GV in their entirety as well as the inflowing posterior gastric vein; E: If selective coil embolization of the LIPV is impossible, the GCS should be occluded with another balloon catheter for balloon-occluded retrograde transvenous obliteration (BRTO) (dual-BRTO). Selective coil embolization of the LIPV branch (arrow) is performed through the catheter via the GCS. PGV: Posterior gastric vein; GV: Gastric varices; LIPV: Left inferior phrenic vein; GCS: Gastrocaval shunt.

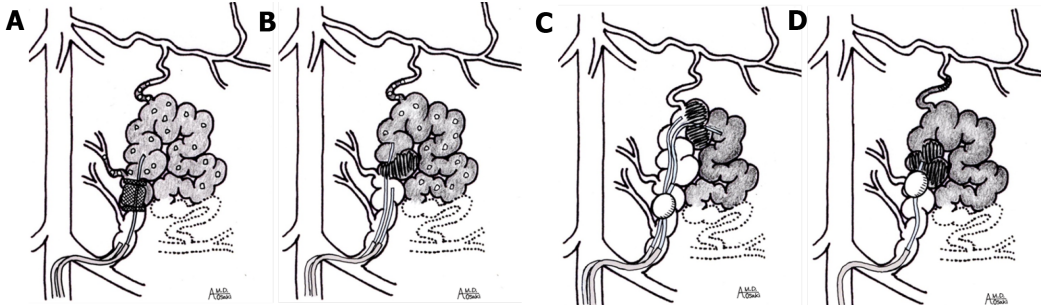


Figure 3 Schema of balloon-occluded retrograde transvenous obliteration modified variants. A: In plug-assisted retrograde transvenous obliteration, a vascular plug is placed instead of a balloon catheter to block shunt blood flow. In the original method, gelatin sponge suspension is injected instead of sclerosant; B: In coil-assisted retrograde transvenous obliteration (CARTO), shunt blood flow is blocked using microcoils and gelatin sponge suspension is injected to embolize the gastric varices; C: In modified CARTO, instead of downgrading by advancing the balloon catheter, embolization is performed using microcoils and sclerosant is injected upstream into the gastric varices; D: In CARTO-II, sclerosant is injected from a balloon catheter in the same manner as conventional balloon-occluded retrograde transvenous obliteration, coil embolization is performed just above the balloon, and the balloon catheter is finally removed.

to the systemic circulation after removing the balloon catheter. The same group also reported the utility of a mixture of low-dose gelatin sponge particles and 5% EOI in retrograde transvenous obliteration (GERTO)[61]. GERTO combines the advantages of gelatin particles and sclerosant, blocking small drainage vessels and causing reliable thrombosis *via* vascular endothelial damage.

Although these various BRTO modifications have appeared, their advantages and disadvantages have not yet been thoroughly evaluated. However, an advantage of both PARTO and CARTO is short indwelling balloon time; their disadvantage is high cost.

COMBINED TREATMENT

Various additional treatments have been performed in combination with BRTO. If BRTO alone is difficult, additional embolization of gastric vein inflow may be used to completely obliterate the GV. Percutaneous transhepatic obliteration (PTO) may be used when conditions are unsuitable for BRTO, such as GVs without GRS[40,50,62]. Combined BRTO and PTO can obstruct both the feeding and draining veins of GVs to completely retain the sclerosant within GVs, which may provide better control of variceal blood flow than either procedure alone[63]. However, the drawback of shunt embolization, including BRTO and PTO, is an increase in PVP. Although BRTO is associated with a lower rate of GVs rebleeding than TIPS[39-44] or endoscopic intervention[37,38], the increased PVP may cause enlargement of EVs[64-66]. Saad *et al* [67] therefore proposed use of BATO *via* the TIPS route, combined TIPS and BRTO, or combined BATO and BRTO, depending on the clinical situation. A recent study[68] has proposed a modified method, balloon-assisted antegrade transvenous obliteration (BAATO), in which balloon occlusion of the GRS is performed in retrograde fashion followed by antegrade trans-TIPS catheter injection of CA rather than sclerosant. The distribution of CA in GVs can be controlled by modifying blood flow velocity *via* balloon size adjustment. Thus, BAATO might be a valuable alternative option as well. Although, TIPS certainly offsets the increase in PVP caused by BATO and/or BRTO, it can cause hepatic encephalopathy. Partial splenic embolization (PSE) also has a PVP-reducing effect, although weaker than TIPS, and combination with BRTO can be effective[69]. We previously reported that PSE can diminish the increase in PVP after BRTO[49] and that combined BRTO and PSE is a safe and effective treatment for GVs [15]. PSE is technically easier than TIPS and can be performed rapidly. Furthermore, the incidence of EVs exacerbation is lower and improvement in hepatic functional reserve is greater after combined BRTO and PSE than BRTO alone[15]. Increased portal venous flow after BRTO leads to improvement in the hepatic functional reserve [65,70] and is mainly due to increased splenic venous blood flow (Figure 4A and B) without a substantial increase in hepatopetal mesenteric venous blood flow. We speculate that hepatopetal mesenteric venous blood flow increases after PSE decreases the splenic venous blood flow (Figure 4C), which results in improved hepatic functional reserve. PSE has a PVP-reducing effect and can prevent exacerbation of EVs after BRTO. However, PSE-related complications may occur. According to a systematic review of 30 articles[71], the incidence of post-embolic syndrome, pleural effusion, ascites, thrombosis (mainly portal thrombosis), splenic abscess/bacterial peritonitis, and death after PSE is 73.4%, 9.4%, 8.1%, 2.4%, 1.3%, and 1.0%, respectively. Underlying liver dysfunction and splenic infarction rate (infarcted splenic volume/total splenic volume) greater than 70% may be risk factors for major complications[71,72].

CONCLUSION

GVs rupture is potentially fatal. Although various GV treatments have been reported, BRTO is widely used because of its effectiveness, ability to cure, and utility for both emergency and prophylactic treatment. Recent BRTO modifications and combinations with other therapies are also beneficial. Although BRTO combined with TIPS and BRTO combined with PSE seem promising, randomized trials have not been performed and serious complications may occur. Their use should be approached with caution.

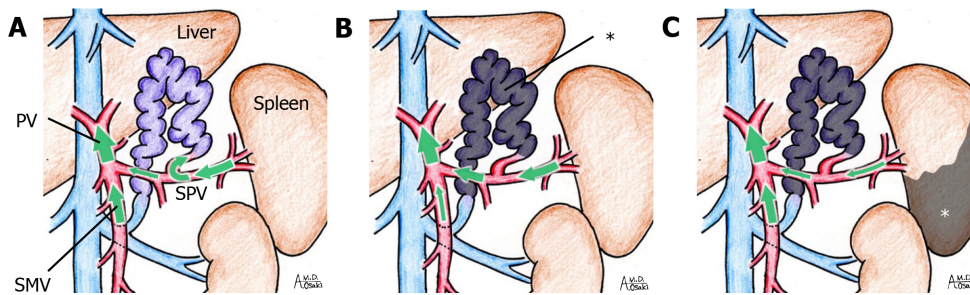


Figure 4 Schema of changes in portal hemodynamics due to combined balloon-occluded retrograde transvenous obliteration and partial splenic embolization. A: Before treatment, most of the splenic blood flow is short-circuited to the systemic circulation via the gastrorenal shunt (GRS); B: The GRS is embolized by balloon-occluded retrograde transvenous obliteration (BRT) (black asterisk). The increase in portal venous flow after BRT is mainly caused by increased splenic venous blood flow without a substantial increase in hepatopetal mesenteric venous blood flow; C: The lower half of the spleen is infarcted by partial splenic embolization (PSE) (white asterisk). Hepatopetal mesenteric venous blood flow increases after splenic venous blood flow is decreased by PSE. PV: portal vein, SPV: splenic vein, SMV: superior mesenteric vein.

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Role of chromosome 1q copy number variation in hepatocellular carcinoma

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Author contributions: Norton PA designed the project; Jacobs NR and Norton PA performed the research and wrote the paper.

Conflict-of-interest statement: The authors declare no conflicts of interests for this article.

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Manuscript source: Invited manuscript

Specialty type: Gastroenterology and hepatology

Country/Territory of origin: United States

Peer-review report's scientific

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Abstract

Chromosome 1q often has been observed to be amplified in hepatocellular carcinoma. This review summarizes literature reports of multiple genes that have been proposed as possible 1q amplification drivers. These largely fall within 1q21-1q23. In addition, publicly available copy number alteration data from The Cancer Genome Atlas project were used to identify additional candidate genes involved in carcinogenesis. The most frequent location for gene amplification was 1q22, consistent with the results of the literature search. The genes *TPM3* and *NUF2* were found to be candidates whose amplification and/or mRNA up-regulation was most highly associated with poorer hepatocellular carcinoma outcomes.

Key Words: Liver cancer; Chromosomal amplification; Hepatocellular carcinoma; The Cancer Genome Atlas

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Core Tip: A list of candidate chromosome 1q amplification driver genes was compiled from the existing literature by PubMed search. Bioinformatics tools were used to identify additional candidates using publicly available genomics and transcriptomics data. Genes identified this way were largely distinct from those identified from the literature. Thus, these two strategies can be used in a complementary manner.

Citation: Jacobs NR, Norton PA. Role of chromosome 1q copy number variation in hepatocellular carcinoma. *World J Hepatol* 2021; 13(6): 662-672

URL: <https://www.wjgnet.com/1948-5182/full/v13/i6/662.htm>

DOI: <https://dx.doi.org/10.4254/wjh.v13.i6.662>

quality classification

Grade A (Excellent): 0
 Grade B (Very good): B
 Grade C (Good): 0
 Grade D (Fair): 0
 Grade E (Poor): 0

Received: February 24, 2021

Peer-review started: February 24, 2021

First decision: May 3, 2021

Revised: May 13, 2021

Accepted: June 4, 2021

Article in press: June 4, 2021

Published online: June 27, 2021

P-Reviewer: Gomez-Quiroz L

S-Editor: Gao CC

L-Editor: A

P-Editor: Wang LL



INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer related deaths worldwide. Liver cancers are the fourth most common cause of cancer related deaths (the sixth most commonly diagnosed type of cancer), and HCC accounts for between 75% and 85% of primary liver cancer cases[1]. About 54% of HCC cases worldwide are attributed to the hepatitis B virus (HBV) while 31% of cases are attributed to hepatitis C virus (HCV) infections[2]. Given the fact that chronic HBV infection presents as a significant risk factor for HCC, vaccination against HBV is recommended as a way to prevent HCC[3].

COMMON GENOMIC ALTERATIONS IN HCC

More recently, technological advances have permitted the sequencing of the genomes and transcriptomes of numerous cancers. Mutations in several genes have been detected repeatedly in HCC[4]. Common somatic changes include mutations to beta-catenin and p53, resulting in activation of the Wnt signaling pathway and dysregulation of the cell cycle, respectively. Mutations activating *TERT* gene expression are also common. Patterns of genetic alterations in individual tumors have been examined with the goal of classifying them, to predict outcome and potentially guide therapeutic decisions[5].

Over the past few decades, a significant amount of research has shown an association between HCC and specific chromosomal abnormalities. In particular, chromosomal gains have been noted for 1q, 6p, 8q, 17q, and 20q. Similarly, chromosomal losses have been detected for 1p, 4q, 6q, 8p, 13q 16p, and 17q[6-8]. Amplification of chromosome 1q21-23 has been identified as the most frequent chromosomal alteration associated with HCC[9]. Thus, we were interested in considering the evidence for which gene or genes is critical for driving this chromosomal abnormality.

AMPLIFICATION OF CHROMOSOME 1Q GENES

During the past two decades, several genes within or near the 1q21-23 range have been highlighted as potentially significant to HCC[10]. Many of these are highlighted in Table 1. In 2003, Wong *et al*[11] studied the 1q21-1q22 region using positional mapping by interphase cytogenetics. They identified significantly increased levels of gene expression of the *JTB*, *SHC1*, *CCT3*, and *COPA* genes in five cases of HCC compared to paired adjacent non-malignant liver tissues, and they concluded that these genes may represent targets in HCC progression[11]. More recently, *JTB* (Jumping Translocation Breakpoint) has been identified as a protein that negatively regulates the apoptotic process by affecting the activation of caspase 9[12]. *SHC1* is involved in signal transduction from receptor tyrosine kinases to various downstream proteins and has been identified in mitogenic signaling[13-15]. *CCT3* is involved in cell cycle regulation[16]. *COPA* is the α -subunit of the coatamer protein complex I which plays a role in retrograde protein trafficking from the Golgi to the endoplasmic reticulum[17].

In 2004, Midorikawa *et al*[8] used an expression imbalance map analysis [which they confirmed using genomic quantitative real-time polymerase chain reaction (qPCR)] to demonstrate amplification of the 1q21-12 region in HCC tumor samples. Moreover, they identified two new genes (*HAX-1* and *CKS1B*) as being as being highly expressed in HCC tissue compared with noncancerous tissues. They also described the amplification of *SHC1* and *CCT3* (previously identified by Wong *et al*[11]). *HAX-1* (*HCLS1* associated protein X-1, gene name *HAX1*) has been associated with activation of tyrosine kinases[18]. Like *CCT3*, *CKS1B* (CDC28 protein kinase regulatory subunit) plays an essential role in mediating a cell's progression through the cell cycle[19]. To further support the conclusions of Midorikawa *et al*[8], Shen *et al*[20] demonstrated that HCC cells had increased levels of *CKS1B* mRNA and protein compared to adjacent non-tumor liver tissue. Elevated *CKS1B* expression was also positively associated with poor differentiation features[20].

In 2008, Inagaki *et al*[21] analyzed a 700-kb DNA region located at 1q21 in 19 HCC-derived cell lines. Using high-density SNP microarray analysis, fluorescence in situ hybridization (FISH), and real-time quantitative PCR, they identified a significant increase in copy number at the 1q21 region. Using reverse transcriptase PCR, they identified three genes (*CREB3L4*, *INTS3*, and *SNAPAP*) that were significantly overex-

Table 1 Amplified genes within/near 1q21-23 that have been associated with hepatocellular carcinoma

Gene ¹	Location ²	Description of protein product	Ref.
<i>JTB</i>	1q21.3	Promotes cell resistance to apoptosis	Wong <i>et al</i> [11] and Kanome <i>et al</i> [12]
<i>SHC1</i>	1q21.3	Downstream signaling from receptor tyrosine kinases	Wong <i>et al</i> [11], Midorikawa <i>et al</i> [8], Pelicci <i>et al</i> [13], Kavanaugh and Williams[14], and van der Geer <i>et al</i> [15]
<i>CCT3</i>	1q22	Associated with cell cycle regulation	Wong <i>et al</i> [11], Midorikawa <i>et al</i> [8], Won <i>et al</i> [16]
<i>COPA</i>	1q23.2	Assists in retrograde vesicular transport from Golgi to endoplasmic reticulum	Wong <i>et al</i> [11] and Vece <i>et al</i> [17]
<i>CKS1B</i>	1q21.2	Associated with cell cycle regulation	Midorikawa <i>et al</i> [8] and Ganoth <i>et al</i> [19]
<i>HAX-1 (HAX1)</i>	1q21.3	Plays a role in the activation of receptor tyrosine kinases	Midorikawa <i>et al</i> [8] and Suzuki <i>et al</i> [18]
<i>CREB3L4</i>	1q21.3	Associated with androgen receptor signaling	Inagaki <i>et al</i> [21] and Qi <i>et al</i> [22]
<i>INTS3</i>	1q21.3	Associated with RNA polymerase II	Inagaki <i>et al</i> [21] and Baillat <i>et al</i> [24]
<i>SNAPAP (SNAPIN)</i>	1q21.3	Part of SNARE complex (docking and fusion of synaptic vesicles)	Inagaki <i>et al</i> [21] and Ilardi <i>et al</i> [25]
<i>ALC1 (CHD1L)</i>	1q21.1	Facilitates DNA synthesis and cell cycle when over expressed	Ma <i>et al</i> [26]
<i>ASH1L</i>	1q22	Histone methyltransferase involved in gene expression	Elseman <i>et al</i> [27] and An <i>et al</i> [29]
<i>METTL13 (EEF1AKNMT)</i>	1q24.3	Regulates protein synthesis in cancer cells; promotes tumor growth and metastasis	Elseman <i>et al</i> [27]; Liu <i>et al</i> [30], and Li <i>et al</i> [31]
<i>TARBP1</i>	1q42.2	Double-stranded RNA binding protein; promotes HIV-1 and -2 and HCV replication	Elseman <i>et al</i> [27], Zhang <i>et al</i> [50], and Christensen <i>et al</i> [32]
<i>SMYD2</i>	1q32.2	Part of the protein lysine methyltransferase family of enzymes	Elseman <i>et al</i> [27] and Leinhardt and Brown[34]
<i>SMYD3</i>	1q44	Part of the protein lysine methyltransferase family of enzymes	Elseman <i>et al</i> [27] and Leinhardt and Brown[34]

¹Alternative gene designation provided in parentheses (see text).

²Chromosome locations are as found on the Genome Browser at <http://genome.ucsc.edu>[65]. HIV: Human immunodeficiency virus; HCV: Hepatitis C virus.

pressed in samples taken from HCC tumors[21]. Based on these findings, they concluded that these three genes are likely targets for the amplification mechanism, and they may be involved in HCC progression. *CREB3L4* (cyclic amplification responsive element binding protein 3-like 4) is part of the CREB/ATF family of transcriptional factors, and it is primarily expressed in the prostate gland in humans as well as prostate and breast cancer cell lines[22]. *CREB3L4* has been shown (by immunostaining) to have a higher expression level in cancerous prostate cells than in adjacent noncancerous cells[22] and it has also been shown to contribute to the progression of breast cancer[23]. *INTS3* (integrator complex subunit 3) is part of the Integrator complex which is associated with the C-terminal domain of RNA polymerase II[24]. *SNAPAP* (snare-associated protein, gene name *SNAPIN*) is part of the SNARE complex of proteins that is involved in the docking and fusion of synaptic vesicle[25]. At this point, little is known about the relationship of either *INTS3* or *SNAPAP* with tumorigenesis.

Later in 2008, Ma *et al*[26] used microdissected DNA from 1q21 and hybrid selection to isolate *ALC1* (also known as *CHD1L*) as a candidate oncogene. After confirming the amplification of *ALC1* using FISH, they transfected it into human liver cell lines resulting in the cells being able to form more colonies than vector-transfected cells when grown in soft agar[26]. They also demonstrated that *ALC1* overexpression plays a role in facilitating DNA synthesis, down-regulating p53 expression, promoting G1/S phase transition, and inhibiting apoptosis.

More recently, in 2016 Elseman *et al*[27] were interested in S-adenosylmethionine (SAME) which has been described by Lu *et al*[28] as playing a significant role in hepatic diseases including HCC. SAME is synthesized from ATP and methionine by methionine adenosyl transferase genes including *MAT1A* which is significantly

downregulated in HCC. Elsemman *et al*[27] analyzed reactions containing SAME, and using copy number variation analysis they identified five methyltransferase genes (*ASH1L*, *METTL13*, *TARBP1*, *SMYD2*, and *SMYD3*) located on chromosome 1q, all of which were amplified in samples of HCC relative to the healthy tissue samples. *ASH1L* is a histone methyltransferase protein which is involved in the regulation of gene expression[29]. *METTL13* (gene name *EEF1AKNMT*) has been shown repeatedly to promote tumor growth and metastasis and is negatively associated with survival among lung and pancreatic cancer patients[30,31]. *TARBP1* is a double-stranded RNA binding protein that promotes the replication of human immunodeficiency virus-1 and -2 as well as HCV[32]. It has also been directly correlated with decreased survival rates in patients with HCC[33]. *SMYD2* and *SMYD3* are both members of the protein lysine methyltransferase family of proteins[34], and each has been associated with a variety of cancer types. *SMYD2* has been shown to be overexpressed in esophageal squamous carcinoma, gastric cancer, and pediatric acute lymphoblastic leukemia[35-37]. *SMYD3* is overexpressed in cancers including breast, liver, and colorectal cancer[38,39].

ANALYSIS OF GENOMIC AND TRANSCRIPTOMIC DATA

We were interested in what more recent genomic and transcriptomic studies have revealed about chromosome 1q amplification and HCC. The Cancer Genome Atlas (TCGA) Project has accumulated an important, publicly available genomic and mRNA expression data set which includes multiple cancers types including HCC (data set Liver Hepatocellular Carcinoma, LIHC)[40]. There is also a more recent version of this data, which is part of TCGA Pan-Cancer Clinical Data Resource[41], a subset of the LIHC data set that has been curated to include four major clinical outcome endpoints. We chose to use this data set to try to identify additional candidate amplification driver genes. This version of the LIHC patient cohort (PanCan-LIHC) has the following patient characteristics: 251 males/121 female with 241 living, and 131 deceased. Most individuals had a total of 10-140 mutations genome wide; 23 had 140-190, 18 had greater than 190, and 2 had fewer than 10 (14 did not have data available). Most PanCan-LIHC individuals exhibited genome alterations, with gains in 1q being the most common alteration: 225 individuals (60.5%) exhibited 1q gains, with 23.7% called as diploid and 15.9% with data not available).

The original publication reporting the LIHC cohort analyses identified copy number alterations (CNAs) in several likely driver genes spread across several chromosomes [40]. However, the only driver gene listed for 1q is *MCL1* at 1q21.3. They also reported a short stretch of four genes that were significantly amplified at 1q22, but no candidate genes were indicated. In a report on the analysis of aneuploidy across TCGA cancer types, strong 1q amplification was noted in the PanCan-LIHC cohort (as well as in other epithelial breast and lung tumors)[42]. Using the OncoPrint tool at the cBioPortal for Cancer Genomics (<https://www.cbioportal.org/>), we could see that all of the genes listed in Table 1 were amplified in 7%-13% of tumors, with mRNAs overexpressed in 9%-41% of tumors (data not shown), consistent with the earlier reports described above.

STRATEGY TO IDENTIFY ADDITIONAL CANDIDATE DRIVER GENES

To further explore possible 1q amplification driver candidates, the frequency of CNAs in the Pan-Cancer version of LIHC sample set was explored using the cBioPortal suite of tools[43,44]. First, the CNA data set for all genes in the PanCan-LIHC was downloaded and imported into an Excel spreadsheet. Second, all genes that had been scored as having an amplification or homozygous deletion with a frequency of at least 5% of tumor samples were sorted from those with lower frequency. This resulted in a list of 1871 genes meeting these criteria. Finally, this set of 1871 altered genes was sorted by chromosome and further restricted to those that were annotated as Cancer genes according OncoKB[45].

These steps produced a list of 49 candidate genes localized to chromosome 1q (not shown). These fell into two groups, a centromere proximal group spanning intervals 1q21.2-1q25.2 (28 genes), and a second group covering the distal interval of 1q31.1-1q44 (21 genes). Across the 1q region, the gene amplified in the highest percentage of tumors was *MUC1* located at 1q22 (11.7% amplification). This might correspond to the short stretch identified at 1q22 by the TCGA-LIHC paper referred to above. The overall frequency of amplification was greater in the proximal group of genes (mean of

10.29%, range of 8.2%-11.7%) *vs* the distal set (mean of 6.41%, range of 5.4%-7.4%). Of the 15 genes listed in Table 1, only two were present in the list of 49, *CKS1B* and *SMYD3*.

ANALYSIS OF NEW CANDIDATES

Using the OncoPrint visualization tool at cBioPortal, all 49 genes were examined to determine the putative CNAs from GISTIC2.0 calls[46], as well as the presence of non-synonymous mutations and altered mRNA expression (z-score threshold of ± 2.0 relative to diploid samples). The total alteration percentages ranged from $< 10\%$ to 50% for the individual genes, with few non-synonymous mutations (not shown). The total number of genes under consideration was narrowed down to 12 by focusing on those with at least 25% of samples with one or more of the various alterations (Figure 1). All but one of these genes was derived from the centromere proximal half of the 1q arm (the exception was *PARP1* at 1q42.12). All 12 genes exhibited numerous instances of mRNA upregulation, both with and without DNA amplification. Note that *COP1* in Figure 1 at 1q25.1 is not the same as *COPA* at 1q23.3 (Table 1).

Each of the 12 genes was examined individually using the cBioPortal Comparison and Survival tools to determine whether the presence of alterations was associated with survival outcomes. There were only two genes where amplification, or mRNA increase, or both were associated with reduced survival compared with the samples without either type of alteration. These two were *TPM3* at 1q21.3 and *NUF2* at 1q23.3 (Table 2, scores designated “all”). However, when the CNAs were examined separately from increased mRNA levels, amplification alone was not associated with any survival or outcome measure (not shown). Instead, the mRNA elevations clearly had a more significant correlation with patient outcome, as can be seen from the Logrank test q-values (Table 2, “mRNA”). Patients with *TPM3* mRNA elevation had an overall median survival of 25.15 mo *vs* 80.74 mo for those without the elevation. Patients with *NUF2* mRNA elevation had an overall median survival of 23.38 mo *vs* 70.06 mo for the unaltered group. Thus, altered expression of these two genes may contribute to clinical outcome.

COMPARING THE FREQUENCY OF *TPM3* AND *NUF2* ALTERATIONS IN HCC WITH OTHER CANCERS

We were interested whether *TPM3* and *NUF2* alterations were common in other types of cancer besides HCC. To explore the alteration frequencies in other cancer types, the entire Pan-Cancer patient cohort was analyzed using the cBioPortal suite of tools[41]. All 32 cancer types included in the Pan-Cancer sample set were selected, and the *TPM3* and *NUF2* genes were searched individually. The Cancer Types Summary produced a display showing the frequency of gene alterations (amplifications, deep deletions, non-synonymous mutations, structural variants) in all 32 types of cancer as well as the types of alterations identified (Figure 2). The PanCan-LIHC HCC dataset had the second highest percentage of *TPM3* alterations and the third highest percentage of *NUF2* alterations. In the case of both genes, amplification of *TPM3* and *NUF2* was the most common type of alteration seen in the HCC patient sample. Interestingly, *NUF2* had a relatively higher frequency of mutations than amplifications in some cancer types.

PREVIOUSLY REPORTED ASSOCIATION BETWEEN *TPM2* OR *NUF2* AND HCC

Despite the low q-values, it remains possible that the association between *TPM3* and *NUF2* gene expression and patient survival is random. Therefore, we searched the literature to find whether either *TPM3* or *NUF2* genes had been associated previously with HCC. Kim *et al*[47] examined chromosomal alterations in 76 HCC, finding frequent gain of 1q. They found *TPM3* mRNA was elevated in tumors compared to normal tissue, and proposed that it might represent an oncogene in HCC, consistent with our analysis. A follow up study found that knock down of *TPM3* in HCC cells reduced migration and invasion capabilities[48].

Table 2 Correlation between *TPM3* and *NUF2* alterations and prognosis

Gene	n (affected)	Overall ¹	Disease-specific ¹	Progression free ¹	Disease free ¹
<i>TPM3</i> (all)	92	1.283e-3	1.263e-3	0.108	0.0242
<i>NUF2</i> (all)	87	4.931e-3	2.357e-4	6.231e-4	4.931e-3
<i>TPM3</i> (mRNA)	78	4.046e-5	4.046e-5	1.231e-3	2.238e-3
<i>NUF2</i> (mRNA)	65	3.898e-4	1.441e-5	3.374e-4	1.520e-3

¹All Logrank test survival *P* and *q* values were generated using the Comparison/Survival tool at cBioPortal to compare survival rates between groups of patients. Various types of survival values were calculated for individuals with any type of alteration (all) or only those with altered mRNA levels (mRNA). The *q*-values shown were derived from the initially *P* values using the Benjamini-Hochberg False Discovery Rate correction procedure.

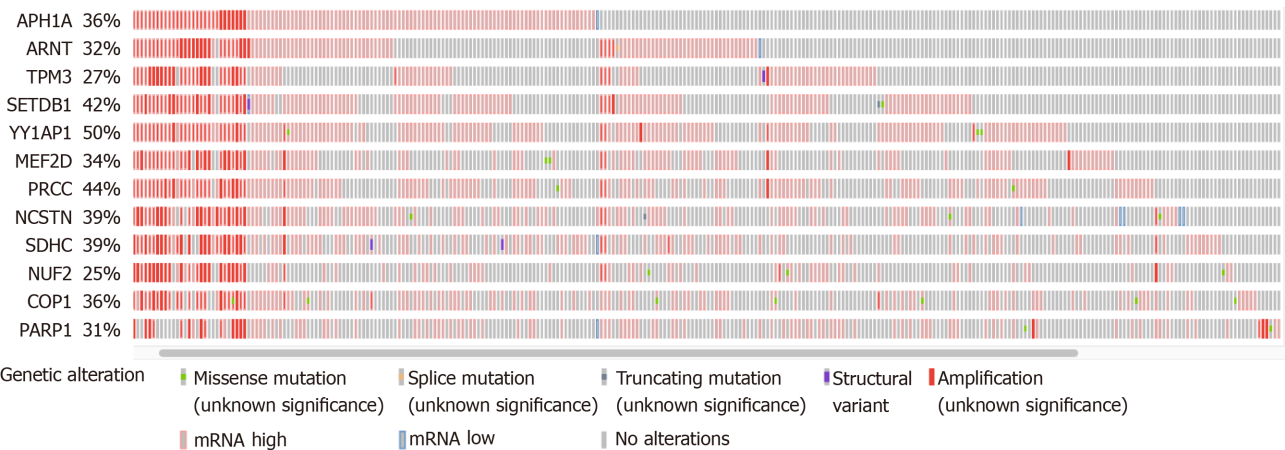


Figure 1 Oncoprint of genetic alterations and mRNA elevations. The alterations to the 12 genes identified by the amplification driver gene identification strategy were visualized using the cBioPortal Oncoprint tool. The nature of the alterations is indicated by the key below the Oncoprint. Note that some individuals display both amplification (solid red) plus elevated mRNA (red outline). Each vertical set of lines reflects the alterations occurring in a single hepatocellular carcinoma patient. Individuals with no alterations detected in any of the 12 genes are not shown.

NUF2 elevation was reported in micro-dissected malignant hepatocytes derived from HBV-associated tumors[49]. Analysis of the Gene Expression Omnibus HCC data also revealed upregulation of *NUF2* in HCC compared with healthy colon epithelial cells[50]. An analysis of the original TCGA-LIHC data set, which has substantial overlap with the PanCan-LIHC samples that we explored, also found that *NUF2* was overexpressed compared with normal liver samples[51], and that overexpression was significantly associated with overall median survival. Other independent analyses of the same data set also reported *NUF2* upregulation and association with poorer prognosis[52-54]. It has been suggested that *NUF2* may represent a biomarker for early recurrence after HCC resection[55], and that it might represent a potential therapeutic target[56].

IMPLICATIONS OF *TPM3* AND/OR *NUF2* OVEREXPRESSION

The product of the *TPM3* gene is tropomyosin3, an actin binding protein. The four *TPM* genes produce 40 distinct protein isoforms by use of alternative promoters and extensive alternative mRNA splicing[57]. Changes in isoform production have been associated with cellular transformation[48,58]. The specific role of increased *TPM3* in cancer cells is unclear, as the protein is involved in numerous activities related to the actin cytoskeleton. Despite this, it is worth noting that small molecules that block the binding of isoform *TPM3.1* to actin showed promise in perturbing the growth of cancer cells[59,60].

The protein encoded by the *NUF2* gene, along with those encoded *NDC80*, *SPC24* and *SPC25* form the Nuclear Division Cycle 80 complex. This complex plays an important role in mitotic spindle formation and chromosome segregation[61]. Over expression of other complex members, especially *NDC80*, has also been observed

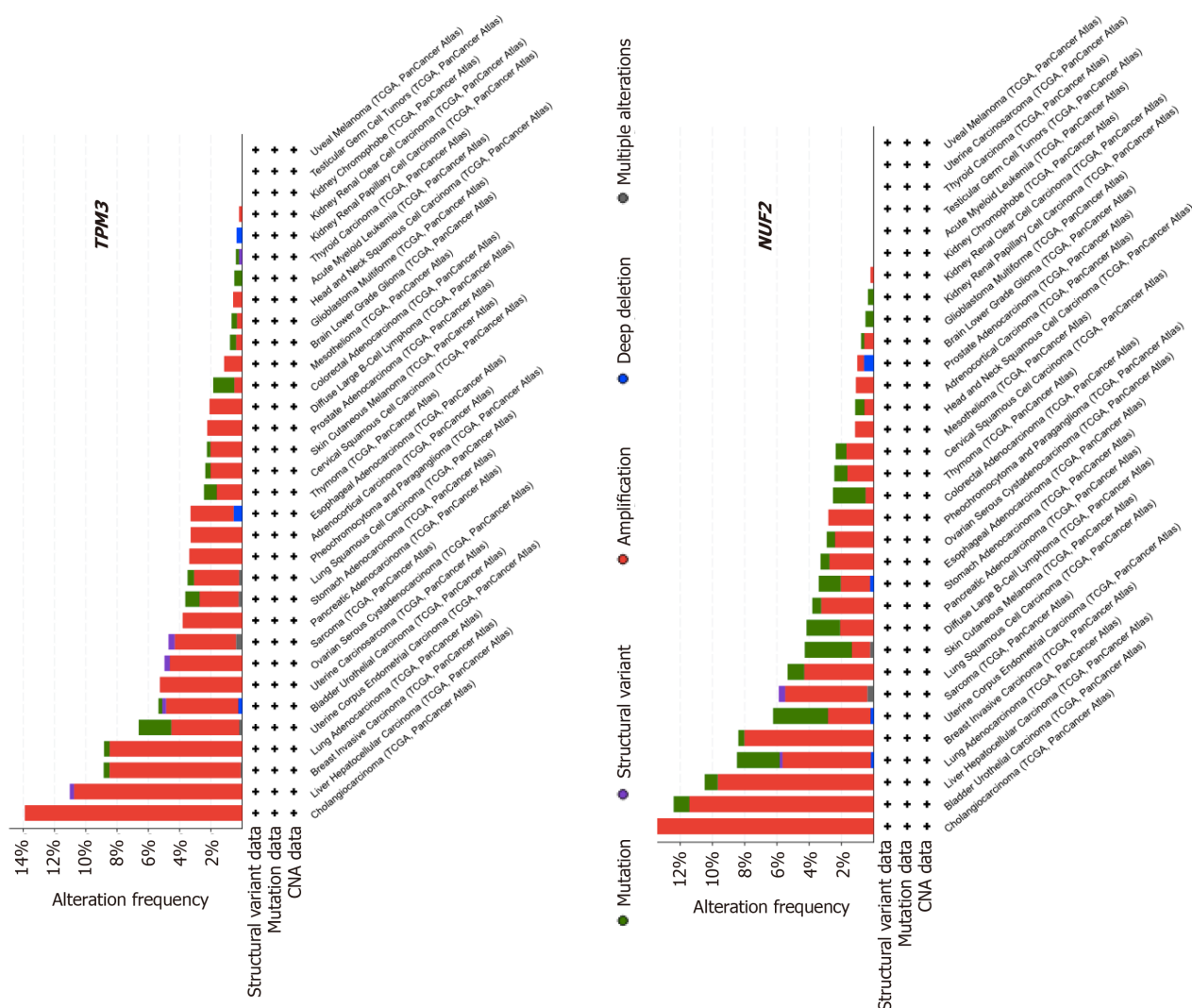


Figure 2 Frequency of *TPM3* or *NUF2* alterations in other cancers. Analyses from cBioPortal show the percent of cancer cases that include a *TPM3* gene alteration (upper plot) or a *NUF2* gene alteration. The results were generated by first selecting all 32 of The Cancer Genome Atlas PanCancer Atlas studies[41] from the cBioPortal Query page, and then searching for DNA alterations in the *TPM3* gene and *NUF2* gene individually. Graphs shown in this figure were taken from the Cancer Types Summary results page that is produced by cBioPortal following this search. The colors above represent the following: green, mutation; purple, fusion; red, amplification; blue, deep deletion; grey, multiple alterations.

frequently in multiple cancers, and it has been proposed that overexpression of NDC80 complex proteins leads to defective mitosis and may promote aneuploidy[62]. Screening in epithelial ovarian carcinoma cells of an siRNA library has identified *NUF2* as one of four genes that reduced cell viability and increased apoptosis when knocked down[63]. This study also found a correlation between *NUF2* mRNA elevation and poorer prognosis in ovarian carcinoma patients. *NDC80* (also known as Hec1) interacts directly with *NUF2* and may represent a therapeutic target. A screen of a small molecule library for inhibitors of the interaction between *NDC80* and mitotic kinase Nek2 identified a compound named INH1 as being able to disrupt the protein-protein interaction[64]. This study also showed that INH1 decreased proliferation of breast cancer cells in culture and in a mouse xenograft assay.

CONCLUSION

In conclusion, our review of the literature and independent analysis of the TCGA-LIHC PanCancer data set identified two non-overlapping sets of genes that reside on chromosome 1q and frequently undergo amplification in HCC (compare [Figure 1](#) and [Table 1](#)). We found what appears to be a significant correlation between amplification and/or increased expression of *TPM3* and *NUF2* and poorer prognosis, which is consistent with previous reports in the literature. Amplification of 1q also is observed

frequently in other cancers. One limitation to our strategy to identify additional driver genes is that only genes previously identified as involved in cancer by OncoKB were considered. The absence of many genes in [Table 1](#) suggests more candidate genes may still be identified. In the case of large chromosomal CNAs such as seen with 1q, it is truly challenging to identify the critical driver mutations involved. Further studies will be needed to understand the contributions of numerous genes amplified on chromosome 1q so as to effectively target therapeutics.

ACKNOWLEDGEMENTS

We thank Danielle Gerken for some of the initial research that led to this project. We are also grateful for the tools provided by the cBioPortal for Cancer Genomics (<https://www.cbioportal.org/>) for the analysis of the TCGA data.

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Retrospective Cohort Study

Impact of donor-specific antibodies on long-term graft survival with pediatric liver transplantation

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Institutional review board

statement: Our study was approved by the responsible ethics committee.

Informed consent statement:

Informed written consent was obtained from the patient for publication of this report and any accompanying images.

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Abstract

BACKGROUND

In a previous paper, we reported a high prevalence of donor-specific antibody (DSA) in pediatric patients with chronic rejection and expressed the need for confirmation of these findings in a larger cohort.

AIM

To clarify the importance of DSAs on long-term graft survival in a larger cohort of pediatric patients.

METHODS

We performed a retrospective analysis of 123 pediatric liver transplantation (LT) recipients who participated in yearly follow-ups including Luminex testing for DSA at our center. The cohort was split into two groups according to the DSA status (DSA-positive $n = 54$, DSA-negative $n = 69$). Groups were compared with regard to liver function, biopsy findings, graft survival, need for re-LT and immunosuppressive medication.

RESULTS

DSA-positive pediatric patients showed a higher prevalence of chronic rejection ($P = 0.01$), fibrosis ($P < 0.001$) and re-transplantation ($P = 0.018$) than DSA-negative patients. Class II DSAs particularly influenced graft survival. Alleles DQ2, DQ7, DQ8 and DQ9 might serve as indicators for the risk of chronic rejection and/or

Conflict-of-interest statement: The authors declare no conflicts of interest.

Data sharing statement: No additional data are available.

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

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Manuscript source: Invited manuscript

Specialty type: Transplantation

Country/Territory of origin: Germany

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

Received: January 8, 2021

Peer-review started: January 8, 2021

First decision: March 29, 2021

Revised: April 12, 2021

Accepted: May 21, 2021

Article in press: May 21, 2021

Published online: June 27, 2021

P-Reviewer: Ferrarese A

S-Editor: Zhang H

L-Editor: A

P-Editor: Li JH

allograft fibrosis. Mean fluorescence intensity levels and DSA number did not impact graft survival. Previous episodes of chronic rejection might lead to DSA development.

CONCLUSION

DSA prevalence significantly affected long-term liver allograft performance and liver allograft survival in our cohort of pediatric LT. Screening for class II DSAs in combination with assessment of protocol liver biopsies for chronic antibody-mediated rejection improved early identification of patients at risk of graft loss.

Key Words: Donor-specific antibodies; Graft rejection; Liver transplantation; Fluoro-immunoassay; Pediatrics; Graft dysfunction; Fibrosis

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Core Tip: This was a retrospective study to evaluate the impact of donor-specific antibodies (DSAs) on graft survival with pediatric liver transplantation. Graft fibrosis and graft loss was significantly higher in patients with DSAs. Screening for DSAs should be included in follow-ups to avoid delayed identification of patients at risk of graft loss (rejection), and may be even more relevant for patients with early histological signs of possible allograft dysfunction (fibrosis). Moreover, patients with DSAs may be poor candidates for reduction of initial immunosuppression or even weaning.

Citation: Schotters FL, Beime J, Briem-Richter A, Binder T, Herden U, Grabhorn EF. Impact of donor-specific antibodies on long-term graft survival with pediatric liver transplantation. *World J Hepatol* 2021; 13(6): 673-685

URL: <https://www.wjgnet.com/1948-5182/full/v13/i6/673.htm>

DOI: <https://dx.doi.org/10.4254/wjh.v13.i6.673>

INTRODUCTION

The impact of anti-human leucocyte antigen (HLA) donor-specific antibodies (DSAs) on graft survival and graft function in kidney and heart transplantation is crucial[1-3]. However, their impact on liver transplantation (LT) is still controversial: Some research suggests that the overall impact on graft and patient outcome is limited[4], but other studies found that it may be an independent risk factor for patient death and graft loss[5-7].

For a long time, DSAs were suspected to have a minor influence on liver allografts [8,9], based on low vascular expression of HLA class II antigens, weak HLA class I expression on hepatocytes and a large endothelial surface diluting soluble antibodies and antigens[10]. However, HLA class II expression is present, especially on perivascular dendritic cells and endothelial cells of the septal venule, sinusoidal and central vein[11,12].

There is growing (but limited) data for pediatric LT suggesting that DSAs might have an impact on long-term graft survival by influencing the development of portal inflammation, portal fibrosis[13-15], perivascular or perisinusoidal fibrosis[15-18], or obliterative portal venopathy[19] and might lead to chronic antibody-mediated rejection (cAMR)[10,19,20].

According to Demetris *et al*[10,21-23] chronic rejection (CR) is characterized by a slow process based on alloreactivity. Histopathological findings of T-cell-mediated rejection (TCMR) include lesions or loss of small bile ducts, portal inflammation, venous endothelial inflammation, obliterative arteriopathy and low-grade necroinflammation. Since obliterative arteriopathy is rarely found in a percutaneous needle biopsy, ductopenia and signs of necroinflammation tend to be used for the diagnosis of chronic rejection in biopsy specimens[23]. According to O'Leary *et al*[24], ductopenia, biliary strictures and fibrosis are associated with DSAs in adult LT.

Initially, AMR was only suspected after ABO-incompatible transplantation, but it has since been reported in ABO-compatible LT as well[19,25,26]. It has also been



suggested that TCMR and cAMR are linked, and that any form of TCMR might channel cAMR by increasing the presentation of intra- and extracellular donor antigens on dendritic cells, which would then stimulate the production of DSAs (second-hit hypothesis)[10,23,24,27,28].

This paper reports the results of a retrospective cross-sectional study of the influence of DSAs developed after LT on long-term graft survival in pediatric LT recipients.

MATERIALS AND METHODS

Study design and study population

From 1993 to 2015, 765 pediatric LTs were performed at our tertiary center. Testing of DSAs started in 2013, mostly with the ELISA technique. We performed a cross-sectional retrospective chart analysis of all patients coming for check-ups at our pediatric department between 2013 and 2017. All charts were checked for DSA measurement with the Luminex technique (single antigen bead assay) as the most sensitive test, as well as donor HLA typing and complete laboratory values. We included 123 patients in the present study after exclusions due to change of residency, follow-up in other transplant centers, missing donor typing and missing Luminex testing as well as deaths within the first year after LT.

DSA testing usually took place as part of the yearly follow-up routine at 1 to 19 years (mean 8.9 years) after first LT. There were 55 female (44.7%) and 68 male (55.3%) participants. The main diagnosis leading to LT was biliary atresia ($n = 40$, 32.5%), followed by metabolic disorders ($n = 40$, 32.5%), acute liver failure ($n = 10$, 8.1%), Alagille syndrome ($n = 9$, 7.3%) and others ($n = 64$, 52%). LT was performed either as full-graft ($n = 22$) or technically modified as a split graft ($n = 74$), reduced-size graft ($n = 10$) or living donor LT ($n = 14$). The majority of patients are still living with the first transplant ($n = 87$, 70.7%); 26 patients were retransplanted once (21.1%), 8 patients twice (6.5%) and two patients three times (1.6%). The main cause for re-transplantation was chronic rejection with chronic graft dysfunction; see baseline characteristics in Table 1 for more details.

Yearly follow-ups included physical examination of the child, an ultrasound evaluation of the graft, extensive laboratory diagnostics (including detection of DSAs) and histopathological examination of liver biopsies if available.

Since we perform DSA detection with luminex testing as part of our routine clinical practice and because of the retrospective study design, our study was readily approved by the ethics committee.

Histological graft examination

In our center, we perform routine protocol liver biopsies every three to five years after LT. Liver biopsy is also indicated if there are laboratory signs of allograft dysfunction or if rejection is suspected. The histological features that we assessed are shown in Table 2. In case of fibrosis or rejection, grading and rating was performed using the Desmet score for fibrosis, the rejection activity index and Banff scores for chronic liver transplant rejection by experienced in-house pathologists.

HLA typing and luminex

We described the technique of HLA typing and HLA antibody testing in our previous paper[14]. In this study, we also used luminex single antigen class I and class II beads (One Lambda Inc., LABScreen®) for retrospective detection of anti-HLA-antibodies (A, -B, -Cw, -DR, -DQ, and -DP). Normalized mean fluorescence intensity (MFI) > 1500 was regarded as positive.

Statistical analysis

All data were statistically analyzed using IBM SPSS Statistics® software, Version 25. Due to their mainly Fisher's exact non-Gaussian character, variables were analyzed with Pearson's chi-square, Cramer and Phi, Mann-Whitney *U* and the Wilcoxon test. Freedom from events (graft loss and rejection) was estimated by the Kaplan-Meier method and was compared across groups with the log-rank test. Graft survival was computed from the date of LT to re-transplantation, or to biopsy-proven rejection. A *P* value < 0.05 was considered statistically significant. We performed binary correlation analysis and evaluation of odds ratios (95%CI, *P* < 0.05). Significant correlations (*P* < 0.02) were included to form predictive models for DSA development and chronic

Table 1 Baseline characteristics

	'DSA-positive' group 1 (n = 54)	'DSA-negative' group 2 (n = 69)
Age		
At LT (yr)	3.3 (1 mo-17 yr)	4.0 (1 mo-17.8 yr)
At follow-up (yr)	13.8 (2-23)	12.6 (1-24)
Gender		
Female	n = 21	n = 35
Male	n = 33	n = 35
Main diagnosis		
Biliary atresia	n = 20	n = 20
Alagille syndrome	n = 1	n = 8
Acute liver failure	n = 6	n = 4
Metabolic disorders ¹	n = 14	n = 26
Others ²	n = 13	n = 11
Donor source		
LdlT	n = 9	n = 11
DdlT	n = 45	n = 58
Full-graft	n = 8	n = 12
Split size	n = 30	n = 43
Reduced size	n = 7	n = 3
Cold ischemic time (min)	543.3 (122-949)	572.5 (145-943)
RelT	n = 22	n = 14
Graft loss due to		
Cr	n = 23	n = 7
Alv	n = 7	n = 3
Thrombosis	n = 3	n = 3
Rond	n = 2	n = 0
Ssc	n = 2	n = 0
Time to DSA-testing		
Years after current LT	9.75 (1-19)	7.98 (1-19)
Anti-HLA antibodies	n = 54	n = 32
Previous episodes		
Of acute rejection	n = 12	n = 7
Of chronic rejection	n = 10	n = 3

¹Such as carbamoyl phosphate synthetase defects, ornithine transcarbamylase deficiency, primary hyperoxaluria, alpha1-antitrypsin-deficiency, glycogen storage disease, maple syrup urine disease, citrullinemia, Wilson disease, others.

²Such as idiopathic cirrhosis, autosomal recessive polycystic kidney disease, Crijgler-Najar syndrome, progressive familial intrahepatic cholestasis, malignancies, vascular dysfunction, neonatal hepatitis, primary sclerosing cholangitis, autoimmune hepatitis.

LDLT: Living donor liver transplantation; DDLT: Dead donor liver transplantation; relt: Retransplantation; HLA: Human leucocyte antigen; DSA: Donor-specific antibodies; CR: Chronic rejection; ALV: Acute liver failure; ROND: Recurrence of native disease; SSC: Secondary sclerosing cholangitis.

rejection with binary logistic regression and Cox regression analysis.

Group formation

The population was divided into two groups according to DSA status (group 1 = DSA-positive, n = 54; group 2 = DSA-negative, n = 69). If DSAs against a certain HLA locus

Table 2 Biopsy characteristics

	Group 1: 'DSA-positive' (n = 38)	Group 2: 'DSA-negative' (n = 34)	P value
Fibrosis ¹	n = 24	n = 6	
Low-grade	n = 23	n = 6	< 0.001
High-grade	n = 1	n = 0	0.5
Cirrhosis	n = 3	n = 0	0.5
Steatosis	n = 6	n = 6	0.8
Portal inflammation	n = 28	n = 18	0.005
Perivenular/perisinusoidal inflammation	n = 11	n = 2	0.011
Ductular inflammation	n = 13	n = 3	0.004
Endothelitis	n = 6	n = 2	0.1
Biliary lesions/ductulopenia	n = 6	n = 0	0.009
Ductular cholestasis	n = 12	n = 4	0.01
Biliary tract strictures	n = 9	n = 6	0.03
Single cell necrosis	n = 5	n = 0	0.02
Chronic rejection ²	n = 7	n = 0	0.009
Possible camr ³	n = 9	n = 0	0.002

¹Fibrosis grading according to Desmet *et al.*

²Chronic rejection according to Banff criteria.

³Chronic antibody-mediated rejection (camr) according to Banff 2016 criteria, with absent C4d staining.

DSA: Donor-specific antibodies.

were found in more than four patients ($n \geq 5$), they were analyzed separately to determine whether a single HLA locus was a common target for DSA formation or if it might be associated with histopathological changes, chronic rejection or retransplantation. To assess whether the number of DSAs influenced graft survival, we compared graft survival of patients with a single DSA ($n = 26$) with those who had multiple DSAs ($n = 28$). To determine whether high MFI levels influenced graft performance, we compared patients with very high MFI levels [$\text{MFI} > 10000$ ($n = 24$)] to patients with lower MFI levels [$\text{MFI} < 10000$ but > 1500 ($n = 30$)].

Not every patient with luminex testing received a liver biopsy, so we could not include every participant for histopathological analysis.

Immunosuppressive medication

Initial immunosuppression (IS) within the first year and also maintenance therapy 1 year post-LT has already been described by our group[14]. In the present study population, patients mainly received immunosuppressive therapy with CNI (group 1: CSA $n = 27$; Tac $n = 24$; group 2: CSA $n = 29$; TAC $n = 36$) which was either monotherapy (group 1: 53.7%; group 2: 56.5%) or in combination with other medications. Detailed information is provided in Table 3. IS was modified if there were side effects or rejection episodes.

RESULTS

HLA analysis and DSAs

There were 123 patients in the study. HLA antibodies were found in 74.1% of all patients ($n = 106$), and 43.9% ($n = 54$) presented with DSAs. The mean number of HLA antibodies per patient in group 1 was 10.9 (minimum of $n = 1$, maximum of $n = 63$, SD = 10.6) whereas group 2 had only 2.9 HLA antibodies per patient (minimum of $n = 0$, maximum of $n = 33$, SD = 6.2). The mean number of DSAs was 2.2 with a maximum of up to 6 DSAs per patient and graft (SD = 1.4).

Table 3 Immunosuppressive therapy

Group 1 'DSA-positive'		Group 2 'DSA-negative'
Monotherapy	<i>n</i> = 29	<i>n</i> = 39
CSA	<i>n</i> = 17	<i>n</i> = 20
Trough level	109.5 µg/L (23-674 µg/L) median 73 µg/L	58.0 µg/L (29-106 µg/L)
TAC	<i>n</i> = 10	<i>n</i> = 17
Trough level	4.4 µg/L (1.0-7.3 µg/L)	5.5 µg/L (2.6-7.7 µg/L)
EVE		<i>n</i> = 1
Trough level		10.3 µg/L
SIR	<i>n</i> = 1	
Trough level	5.2 µg/L	
MMF	<i>n</i> = 1	<i>n</i> = 1
Combined therapy	<i>n</i> = 25	<i>n</i> = 30
CSA		
+ EVE	<i>n</i> = 3	<i>n</i> = 4
+ MMF	<i>n</i> = 3	<i>n</i> = 3
+ PRED	<i>n</i> = 2	<i>n</i> = 1
+ EVE + PRED		<i>n</i> = 1
+ MMF + PRED	<i>n</i> = 2	
Trough level	54.7 µg/L (11-89 µg/L)	72.7 µg/L (23-137 µg/L)
TAC		
+ EVE	<i>n</i> = 3	<i>n</i> = 6
+ MMF	<i>n</i> = 5	<i>n</i> = 7
+ PRED	<i>n</i> = 5	<i>n</i> = 3
+ MMF + PRED	<i>n</i> = 1	<i>n</i> = 3
Trough level	6.1 µg/L (3.0-9.9 µg/L)	6.3 µg/L (2.1-15.1 µg/L)
EVE		
+ MMF		<i>n</i> = 1
+ MMF + PRED	<i>n</i> = 1	
Trough level	3.7 µg/L (1.0-5.0 µg/L)	4.3 µg/L (1.8-7.8 µg/L)
SIR		
+ MMF		<i>n</i> = 1 (trough levels, NA)
Adherence rates		
CSA	81.5%	72.4%
TAC	87.5%	88.9%
EVE	75%	84.9%
SIR	100%	100%

Trough levels given as mean (and range). MMF: Mycophenolate mofetil; CSA: Cyclosporin A; TAC: Tacrolimus; EVE: Everolimus; PRED: Prednisolone; SIR: Sirolimus; NA: Not available; DSA: Donor-specific antibodies.

All DSA-positive patients except one had DSAs of HLA class II (*n* = 53), while 14.8% (*n* = 8) had DSAs of both classes. Only one patient had DSAs exclusively in class I.

A detailed analysis of DSA HLA allele distribution showed that mainly HLA class II alleles, especially DR (*n* = 26 out of 54) and DQ (*n* = 39 out of 54) alleles were targeted. DP-HLA alleles could not be evaluated, because HLA donor typing was missing or

incomplete for the majority of DP alleles. We could count these as HLA antibodies, but could not determine donor specificity. Nevertheless, the DSA-positive group showed a 46.3% prevalence of anti-HLA DP antibodies ($n = 25$ of 54), while the prevalence of these antibodies was only 14.5% ($n = 10$ of 69) in the DSA-negative group (Figure 1).

Liver biopsies

Liver biopsies (group 1 $n = 38$; group 2 $n = 34$) were mostly performed as protocol biopsies (67.3% in group 1 *vs* 85.7% in group 2), followed by suspected rejection (16.3% *vs* 10.7%). Recurrence of native disease was suspected in four children and confirmed in three (PFIC2 $n = 1$; AIH $n = 1$; PSC $n = 1$).

Comparing both groups, we found that fibrosis, portal inflammation, perivenular or perisinusoidal inflammation, ductular inflammation, biliary lesions/ductulopenia, ductular cholestasis, biliary tract strictures, single cell necrosis and chronic rejection were significantly more common in the DSA-positive group. Fibrosis was significantly correlated to class II HLA-DSAs ($P < 0.001$), especially to alleles DQ2 ($P = 0.03$), DQ7 ($P < 0.001$), DQ8 ($P = 0.02$) and DQ9 ($P = 0.007$). Low-grade fibrosis (F1 and F2) in particular was significantly higher in DSA-positive patients ($P < 0.001$) and was found in 17 routine protocol biopsies in group 1 (F1 and F2), whereas only 3 protocol biopsies showed signs of low-grade fibrosis (F1) in group 2.

We also found a higher incidence of high-grade fibrosis (F3), cirrhosis and endothelitis in group 1, although the difference was not significant. Steatosis, hepatocyte ballooning and other signs of toxic damage to the graft were either comparable or more likely to be found in group 2 (Table 2).

Correlation analysis showed a significant connection between biopsy-proven rejection and DSAs of HLA class II ($P = 0.005$), in particular against DQ2 ($P = 0.02$), DQ8 ($P = 0.02$) and DR52 ($P = 0.03$).

Overall graft survival according to Kaplan-Meier estimates was significantly lower for patients with DSAs (Mantel-Cox test: $P = 0.04$, Figure 2).

Clinical evaluation of liver enzymes, liver synthesis parameters and ultrasound criteria in CR-positive patients was not consistent with the histopathological status. Elevated levels of ALT and AST were only found in 10%-20% of CR-positive patients; γ GT- and GLDH-elevation occurred in 50%-66%. None of these enzyme elevations reached statistical significance, nor did aberration of liver function parameters (albumin, bilirubin, international normalized ratio).

Histopathological indicators of possible cAMR were found in 9 patients of Group 1 (5 male, 4 female). Of these, three patients received monotherapy (CSA $n = 2$, MMF $n = 1$), while the other patients were treated with CNI in combination with EVE or MMF. Combined therapy was introduced if renal function was impaired or if there were previous signs of rejection. Trough levels were in the therapeutic range in all but two patients. Liver enzymes were normal except for elevated γ GT in four patients. Alleles most targeted by DSAs belonged to HLA class II. DSAs against DQ alleles were particularly prevalent (DQ2 $n = 2$; DQ7 $n = 4$; DQ8 $n = 4$, DQ9 $n = 3$).

There was a significantly higher incidence of previous episodes of chronic rejection in our DSA-positive patients ($P = 0.015$). The rate of previous episodes of acute rejection was comparable in both groups.

Influence of DSA number and MFI levels

There was no correlation between DSA number and MFI levels with chronic rejection or re-LT. The Mantel-Cox test showed no significant influence of the number of DSAs on graft survival ($P = 0.7$; Figure 3).

Single MFI levels ranged from 1668 to 31309 (cumulated MFI from 1678 to 120181; SD = 22333). Fibrosis, biopsy-proven chronic rejection, re-LT and numbers of re-LT were not significantly influenced by high MFI levels (MFI >10000 *vs* MFI > 1500 but < 10000). Graft survival was not significantly decreased in patients with high MFI levels (Mantel-Cox $P = 0.7$, Figure 4).

DSA-influence on re-LT

Comparing both groups, retransplantation was significantly more common in the DSA-positive group ($n = 22$ *vs* $n = 14$; $P = 0.018$). 79.9% of group 2 patients were able to maintain their first graft, but only 61.1% of group 1 maintained their initial grafts. The overall number of LTs was significantly higher in group 1 (1 to 4 LTs) than in Group 2 (1 to 3 LTs; $P = 0.015$). Also, the number of LTs was directly correlated with the presence of DSAs ($P = 0.002$). Retransplantation due to chronic rejection was significantly more common in group 1 ($n = 9$) than in group 2 ($n = 2$; $P = 0.04$).

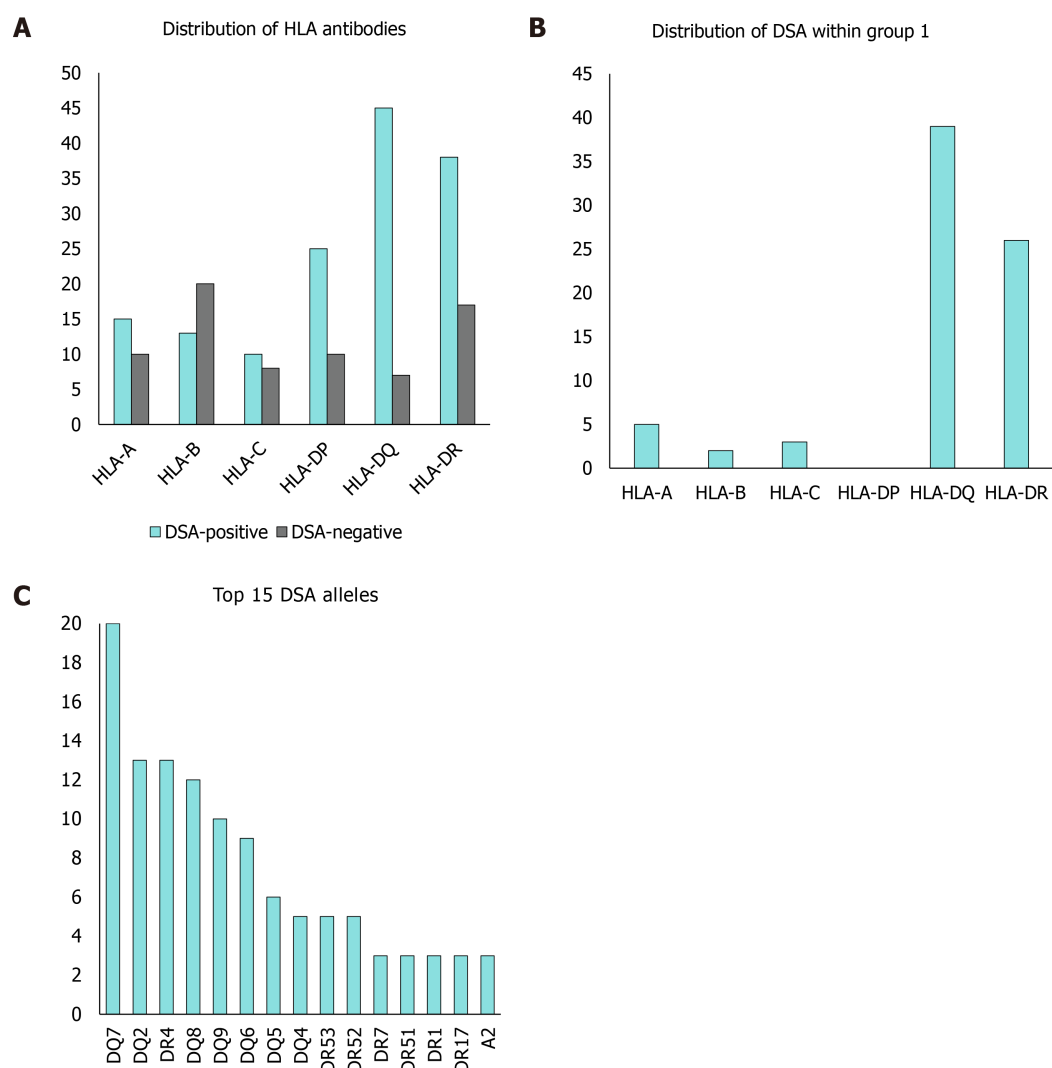


Figure 1 Allele distribution. A: Distribution of human leucocyte antigen (HLA) antibodies; B: Distribution of donor-specific antibodies (DSAs) within group 1; C: Top 15 DSA alleles. Vertical axis: numbers, quantitative; horizontal axis: categories of anti-HLA antibodies and subcategories of DSAs; columns: DSA-positive patients shown in green, DSA-negative patients shown in light grey. HLA: Human leucocyte antigen; DSA: Donor-specific antibody.

DISCUSSION

Rejection

The role of DSAs in the pathogenesis of chronic rejection in pediatric LT recipients has been subject to various studies but is still not fully understood. In a previous study, our group reported a higher prevalence of DSAs in patients with CR, although the statistical significance could not be determined due to the small cohort[14]. CR in DSA-positive patients was also described later by Wozniak *et al*[27] in a cross-sectional study of 50 pediatric patients.

The results of the present study show that histological indicators of CR have a significantly higher prevalence in DSA-positive patients, confirming O'Leary's findings in a pediatric population and reaffirming the results of our previous study in a larger pediatric cohort. Furthermore, in all cases of biopsy-proven CR, patient sera were positive for DSAs. We also identified nine DSA-positive patients who possibly suffered from chronic antibody-mediated allograft rejection. Even biopsies of DSA-positive patients who received routine protocol biopsies and had no laboratory signs of impaired allograft function exhibited histological signs of fibrosis or rejection. This shows that correlating the aberration of laboratory parameters and CR or fibrosis is not a reliable clinical procedure. Ohlsson *et al*[29] recently confirmed the value of protocol biopsies in detecting silent immune-mediated allograft injuries, regularly associated with the presence of DSAs.

As this study had a cross-sectional design, we could not examine the development of DSAs over the full study period, especially after rejection episodes. Nevertheless,

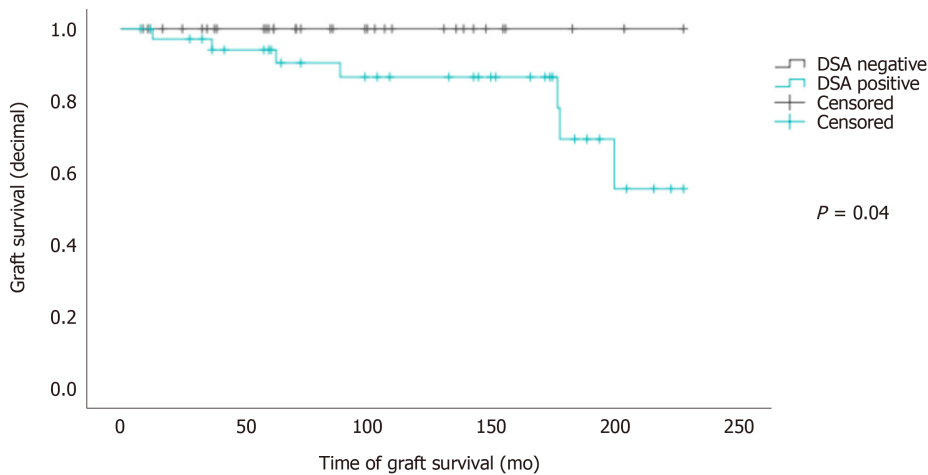


Figure 2 Donor-specific antibody presences on graft survival. Kaplan-Meier survival plot; vertical axis: Graft survival (decimal); horizontal axis: Time of graft survival (months); graphs: Donor-specific antibody (DSA)-negative patients are shown in dark grey, numbers at risk $n = 34$; DSA-positive patients are shown in green, numbers at risk $n = 38$. DSA: Donor-specific antibody.

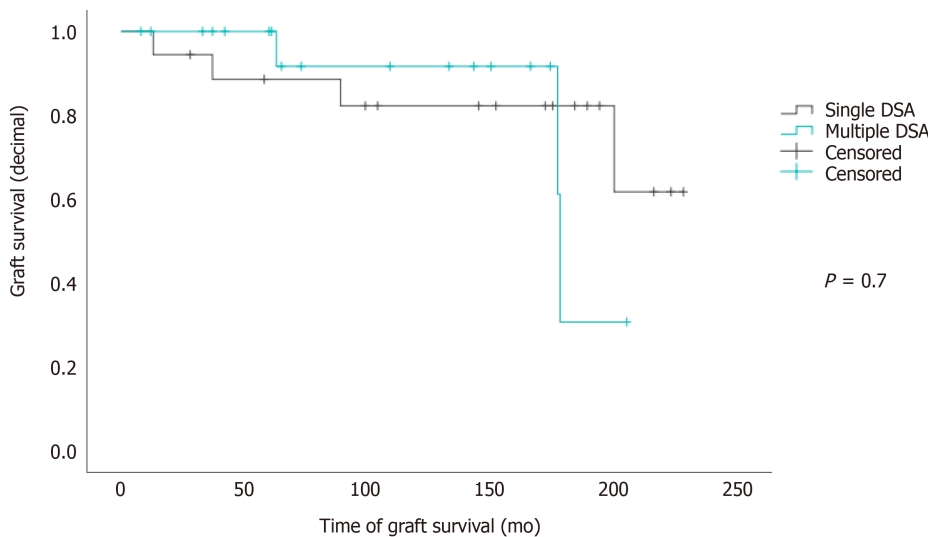


Figure 3 Number of donor-specific antibodies on graft survival. Kaplan-Meier survival plot; vertical axis: Graft survival (decimal); horizontal axis: Time of graft survival (months); graphs: Patients with single donor-specific antibody (DSA) are shown in a dark grey, numbers at risk $n = 19$; patients with multiple DSAs are shown in green, numbers at risk $n = 19$. DSA: Donor-specific antibody.

previous episodes of CR with the same graft (prior to the current follow-up, which did not lead to graft loss or re-LT) were significantly more common in our DSA-positive patients ($P = 0.015$).

Although there is growing evidence that DSAs impact graft survival, it is still unclear whether DSA number or quality matter. While Couchonnal *et al*[30] reported poorer long-term graft survival in patients with high MFI (> 10000), Wozniak *et al*[27] described a significantly higher impact of the overall presence of DQ-DSAs on graft survival, as opposed to the presence of any DSAs with high MFI levels (threshold > 13000).

We also used MFI levels of > 10000 to identify strong DSA effects, but we found no statistically significant impact of MFI levels on biopsy-proven CR, graft survival or need for re-transplantation. However, anti-HLA class II antibodies and especially anti-HLA-DQ2 and -DQ8-antibodies were significantly correlated with graft survival. We therefore support Demetris' 'second-hit' hypothesis and regard it as probable that class II HLA-DSAs influence TCMR, cAMR and probably the need for reLT. This is supported by a significant influence of the presence of DSAs on graft survival in survival analysis. The use of anti-HLA-DQ2 and -DQ8-antibodies as screening markers needs to be assessed with further prospective studies.

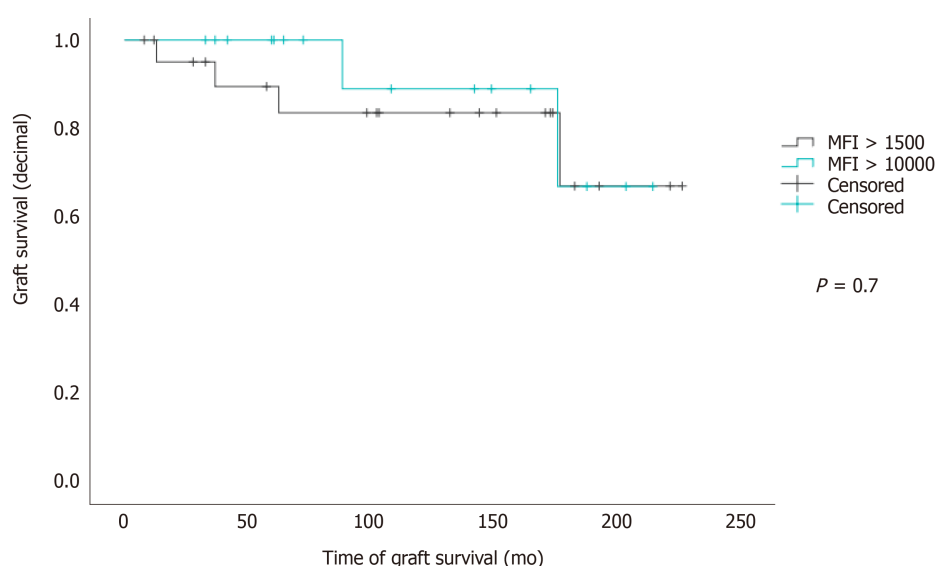


Figure 4 Mean fluorescence intensity levels on graft survival. Kaplan-Meier survival plot; vertical axis: Graft survival (decimal); horizontal axis: Duration of graft survival (months); graphs: Patients who have donor-specific antibodies (DSAs) with mean fluorescence intensity (MFI) above 1500 are shown in dark grey; numbers at risk $n = 22$; patients who have DSAs with MFI above 10000 are shown in green; numbers at risk $n = 16$. MFI: Mean fluorescence intensity.

Fibrosis

Mild to moderate allograft fibrosis is a common finding in protocol biopsies obtained 5-10 years post-LT. So far, low IS trough levels or even weaning off are thought to promote the development of such fibrosis by enabling alloreactivity that leads to allograft inflammation[31] and fibrosis, with possible later development of cirrhosis and graft failure[17,20,32,33]. According to Briem-Richter *et al*[31], increasing IS dosage results in the resolution of histopathological signs of rejection and severity of fibrosis. In this present study, we found that portal, perivenular or perisinusoidal inflammation is very common in DSA-positive patients. Also, mild to moderate allograft fibrosis (grade 1-2) was significantly more common in DSA-positive patients. We therefore consider DSA presence as a symptom of such alloreactivity; this might help to identify children who are poor candidates for reducing IS levels.

Correlation between DSA and cirrhosis did not reach statistical significance, probably because of the small number of cirrhosis patients confirmed with histopathology (DSA-positive patients: $n = 3$ vs DSA-negative patients: $n = 0$).

The presence of HLA class II DSAs, especially anti-HLA-DQ antibodies, coincided significantly with allograft fibrosis. Furthermore, we were able to identify four specific HLA-DQ alleles that might serve as serological markers or have predictive value: DQ2, DQ7, DQ8 and DQ9.

Limitations

As a liver biopsy was not performed for every study participant, one might argue that there is selection of patients with poorer graft performance, leading to a biased correlation of DSA presence with CR. However, the general group of poor allograft performance is still relatively small, thus reversing the suspected bias with an overall representative selection of the study population. Other limitations of this study were the incomplete HLA typing of DP alleles, errors in sampling, and missing HLA donor typing in general, which led to exclusion of participants. Also, C4d staining was not performed on liver biopsies that were taken prior to the updated Banff criteria in 2016, so that these biopsies could not be fully included in the evaluation. As these are parts of the general restrictions of retrospective studies, we plan to conduct a prospective clinical trial to assess the new issues that this study has raised.

CONCLUSION

Long-term allograft survival is even more valuable in pediatric LT than in adult LT, and with the decreased graft survival and increased prevalence of allograft dysfunction and retransplantation in DSA-positive patients, this important subject should not be underestimated.

Screening for DSA must be included in follow-ups to ensure identification of patients at risk of potential graft loss (rejection), and may be even more relevant for patients with early signs of allograft dysfunction (fibrosis). Moreover, patients with DSA might not be good candidates for reduction of IS or even weaning. According to our results, in the presence of DSA, selected patients should be considered for additional graft biopsies including assessment with Banff chronic cAMR criteria after C4d-staining, since routine laboratory parameters are not sufficiently accurate for monitoring the allograft status and cannot identify patients with silent immune-mediated allograft injuries. Whether the latter could be detected by ultrasound elastography could be the subject of a future clinical trial.

Since HLA class I DSAs are less common and have less impact on allograft fibrosis or rejection, screening could be limited to HLA class II DSA (-DQ, -DR -DP).

ARTICLE HIGHLIGHTS

Research background

An impact of donor-specific antibodies (DSAs) on long-term liver allograft survival was found previously in a small cohort of pediatric patients, but the statistical significance was unclear.

Research motivation

The aim of this study was to clarify the importance of DSAs on long-term graft survival in a larger cohort of pediatric patients.

Research objectives

The objective of this study was to emphasize the importance of comprehensive follow-up examinations in clinical practice after pediatric liver transplantation (LT) and contribute to optimizing and standardizing LT follow-ups.

Research methods

This was a cross-sectional retrospective cohort study that compared the outcomes of two patient groups after pediatric LT.

Research results

Our study showed that DSAs significantly impact liver allograft survival. The presence of human leucocyte antigen class II DSAs is associated with chronic rejection, chronic antibody-mediated rejection, graft fibrosis, graft failure, graft loss and re-LT.

Research conclusions

Screening of DSAs and protocol liver biopsies including C4d immunostaining should be standard practice in follow-ups after pediatric LT.

Research perspectives

Further prospective studies should be conducted to explore whether certain DQ-DSAs could be used as a serological marker for the risk of graft loss.

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Retrospective Cohort Study

Mortality and health care burden of Budd Chiari syndrome in the United States: A nationwide analysis (1998-2017)

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Institutional review board

statement: Since the data used for this study were de-identified, IRB approval was not required as per local hospital IRB requirements.

Informed consent statement: Being a de-identified database study, consent form is not applicable.

Conflict-of-interest statement: No conflict of interest.

Data sharing statement: NIS datasets are available to everyone at a nominal fee.

Open-Access: This article is an open-access article that was selected by an in-house editor and

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Abstract

BACKGROUND

The Budd Chiari syndrome (BCS) is a rare and potentially fatal disease, but there is a paucity of data on the in-hospital mortality as well its economic burden on the health care system.

AIM

To evaluate trends in mortality, length of hospital stays and resource utilization among inpatients with BCS.

METHODS

Data on all adult patients with a diagnosis of BCS were extracted from the National Inpatient Sample (NIS) from 1998 to 2017. To make inferences regarding the national estimates for the total number of BCS discharges across the study period, sample weights were applied to each admission per recommendations from the NIS.

RESULTS

During the study period, there were 3591 (8.73%) in-patient deaths. The overall in-hospital mortality rates among BCS patients decreased from 18% in 1998 to 8% in 2017; the mortality decreased by 4.41% ($P < 0.0001$) every year. On multivariate analysis, older age, higher comorbidity score, acute liver failure, acute kidney injury, acute respiratory failure, hepatic encephalopathy, hepatorenal syndrome, inferior vena cava thrombosis, intestinal infarct, sepsis/septic shock and cancer were associated increased risk of mortality. The average of length of stay was 8.8 d and it consistently decreased by 2.04% (95% CI: -2.67%, -1.41%, $P < 0.001$) from 12.7 d in 1998 to 7.6 d in 2017. The average total charges after adjusted for Medical Care Consumers Price Index to 2017 dollars during the time period was \$94440

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Manuscript source: Unsolicited manuscript

Specialty type: Gastroenterology and hepatology

Country/Territory of origin: United States

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: February 18, 2021

Peer-review started: February 18, 2021

First decision: March 16, 2021

Revised: March 27, 2021

Accepted: May 20, 2021

Article in press: May 20, 2021

Published online: June 27, 2021

P-Reviewer: Alvarez-Bañuelos MT

S-Editor: Wang JL

L-Editor: A

P-Editor: Wang LL



and the annual percentage change increased by 1.15% (95%CI: 0.35%, 1.96%, $P = 0.005$) from \$95515 in 1998 to \$103850 in 2017.

CONCLUSION

The in-hospital mortality rate for patients admitted with BCS in the United States has reduced between 1998 and 2017 and this may be a reflection of better management of these patients.

Key Words: National Inpatient Sample; Budd Chiari syndrome; Mortality; Length of stay; Total charges

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Core Tip: Using a large administrative database, we were able to analyze the mortality and socioeconomic impact of Budd Chiari syndrome hospitalizations in the United States over a 19-year period with a high degree of granularity. We were able to show that while the mortality rate and length of stay has declined significantly, total charges continue to show an upward trend.

Citation: Alukal JJ, Zhang T, Thuluvath PJ. Mortality and health care burden of Budd Chiari syndrome in the United States: A nationwide analysis (1998-2017). *World J Hepatol* 2021; 13(6): 686-698

URL: <https://www.wjgnet.com/1948-5182/full/v13/i6/686.htm>

DOI: <https://dx.doi.org/10.4254/wjh.v13.i6.686>

INTRODUCTION

The Budd Chiari syndrome (BCS) is a rare but potentially fatal disorder that results from partial or complete obstruction of the hepatic venous outflow in the absence of right heart failure. Unlike Asian countries, the incidence and prevalence of BCS in Western countries is thought to be lower, but there are no large epidemiological studies[1]. BCS is a heterogeneous disease with a protean clinical presentation ranging from asymptomatic or chronic forms to fulminant liver failure[2,3]. Prognosis of BCS is highly variable and studies from large academic centers have reported mortality rates ranging anywhere between 13%-36%[4-9]. These wide ranges of mortality are more likely related to small sample size, variability in follow up period and publication selection bias. Risk factors such as ascites, hepatic encephalopathy, coagulopathy, elevated creatinine or bilirubin are considered to be independent risk factors for mortality[4-8]. A stepwise management approach consisting of anticoagulation, endovascular venoplasty, transjugular intrahepatic portosystemic shunts (TIPS) and liver transplantation (LT) has been proposed for the management of BCS[3,9-12]. However, this approach may not be applicable to all patients because of varying severity of presentation, the extent of venous occlusion and other serious comorbidities.

There are multiple studies that had investigated the mortality and economic burden of decompensated liver cirrhosis in the United States, but there is a paucity of data regarding the mortality burden and health care utilization for patients with BCS. The primary objective of our study was to assess the trends in in-hospital mortality, length of stay (LOS) and resource utilization among inpatients with BCS using the National Inpatient Sample (NIS) database.

MATERIALS AND METHODS

Study design and data source

This was a retrospective study where data were extracted from the NIS from 1998 to 2017. The NIS is the largest publicly available all-payer inpatient administrative database developed by the Agency for Healthcare Research and Quality (AHRQ) for

the Health Care cost and Utilization Project (HCUP). It represents approximately 20% stratified sample of discharges from community hospitals, but excludes long term acute care hospitals and rehabilitation facilities and contains information of more than 7 million hospital discharges annually. The number of states participating in the NIS grew from 8 in 1988 to 48 in 2017. The database captures information about primary and secondary diagnoses during each hospital stay as well as information about procedures. NIS also contains other valuable information such as severity and comorbidity measures, hospital characteristics (size, region, bed size, teaching/non-teaching), payment source (Medicare/Medicaid/private), total charges and length of hospital stay. In 2012, NIS revised the sample design so as to represent a sample of discharges rather than a sample of hospitals. This new strategy is expected to make the estimates more precise by reducing the sampling error. Starting October 1, 2015 all hospitals in the United States adopted International Classification of Diseases (ICD) 10 codes for disease classification as well as for procedures. The calendar year for 2016 and 2017 which is included in this study uses ICD 10 CM/PCS codes.

Population

Data were extracted from the NIS to identify patients ≥ 18 years of age using all listed diagnosis (primary or secondary diagnosis) of BCS from 1998 to 2017. The diagnosis of BCS was captured using the codes 453.0 (ICD-9) and I82.0 (ICD-10).

Variables

We obtained information on patient demographics (age, sex, race) and hospital characteristics (region of the country, bed-size, teaching status), patient disposition and insurance status (Medicare, Medicaid and private insurance). Study outcome included changes in inpatient mortality, LOS and total charges with time. We investigated if important complications such as acute liver failure, acute kidney injury, cirrhosis, ascites, hepatic encephalopathy, esophageal varices, portal vein thrombosis, inferior vena cava (IVC) thrombosis and spontaneous bacterial peritonitis had an impact on outcome. We also analyzed inpatient procedures such as liver biopsy, upper gastrointestinal endoscopy, paracentesis, TIPS and LT using appropriate ICD codes (Supplementary Table 1 shows the list of ICD-9 and ICD-10 codes). Severity of illness was measured using the Elixhauser comorbidity index after excluding liver diseases and this included 29 major Elixhauser comorbidity conditions[13].

Statistical analyses

Descriptive statistics are used to summarize patients' characteristics, hospital characteristics and utilization, comorbidities, complications, procedures and the outcome by using the weighted survey methods. Data are presented as mean and standard error for continuous variables, percentage and standard error for categorical variables. Standard errors of percentage or mean were estimated using Taylor series linearization method. To make inferences regarding the national estimates for the total number of BCS discharges across the study period, sample weights were applied to each admission per recommendations from the NIS. For the years from 1993-2011, AHRQ had developed discharge trend weights, specifically the NIS Trend Weight Files. Therefore, in our study for trend analyses spanning 2012 and earlier, NIS data trend weights were used to make estimates comparable to the new 2012 NIS design. We used the trend weight in place of the original discharge weights to create national estimates for trend analysis to make the data similar for the entire study period. For 2012 or later data, no trend weights were necessary and the discharge weight supplied on the NIS files were used directly[14]. We calculated BCS discharges rate per 1000000 US populations by dividing the estimated total BCS discharges by projected US population from the Census Bureau.

The annual percentage change (APC) was derived to compare the patients' characteristics, hospitals' characteristics and outcomes over time by using Poisson regression for categorical variables and linear regression with natural logarithm transformation for continuous variables. *P* value for APC was used to determine if the trends in the annual percentage change was significantly different from zero, the change was considered as statistically significant with *P* value of 0.05 or less.

The hierarchical generalized linear mixed model with hospitals as random effects was performed to evaluate the effects of potential associations between outcomes (mortality, length of stay and total charges) and patients' demographics (age, gender, and race), patient-level hospitalization variables (primary payer, disposition of patient), hospital-level variables (hospital region, bed size, location and teaching status), comorbidities, complications and procedures separately. Since race was not

available in some states, a dummy variable was created for missing data in the models to prevent the observation from being dropped. For mortality, binomial distribution and logit link was used. For length of stay, negative binomial distribution and log links were used. When analyzing the total charges, final total charges were adjusted to 2017 dollars based on medical care Consumer Price Index in US city average provided by the Bureau of Labor Statistics. We specified the models using gamma distributions and log links. A variable with *P* value of 0.05 or less was retained in the model and considered as statistically significantly associated with outcomes. All analyses were performed with SAS version 9.4 (SAS Institute, Cary, NC, United States)

RESULTS

Between 1998 and 2017, we identified a total of 8435 hospitalizations related to BCS. The mean age of the cohort was 50.5 years, 55% were women and 56 % were white. Nearly half (52%) the patients were covered by government funded health insurance (Medicare and Medicaid) (Table 1). A majority of the patients (59%) were discharged home, and an additional 13.5% were discharged with home health services. While the number of routine home discharges remained the same, there was a 3.31% increase in utilization of home health services ($P < 0.0001$) (Table 2).

Hospital mortality

Between 1998 and 2017, the in-hospital mortality was 8.74% ($n = 737$). Using the sample weights provided by HCUP, this corresponded to 3591 deaths (Table 2). Despite a significant increase in the comorbidity score during the time period, overall, in-hospital mortality rate among BCS patients decreased significantly by 4.41% per year ($P < 0.0001$) from 18% in 1998 to 8% in 2017, with the mortality rate being the lowest in 2015 (5%) (Figure 1A). There were no gender differences in mortality, but those who died were older than those who were discharged from the hospital (mean age 58.7 years *vs* 49.7 years, $P < 0.001$). Of the patients who died, 53% were Caucasians, 13% were African Americans and 10% were Hispanics. Most deaths occurred in large hospitals (73%) or urban teaching hospitals (71%) (Supplementary Table 2). On multivariate analysis, older age, higher comorbidity score, acute liver failure, acute kidney injury (AKI), acute respiratory failure, hepatic encephalopathy, hepatorenal syndrome, intestinal infarct, IVC thrombosis, sepsis/septic shock and cancer were associated increased risk of mortality (Table 3).

Length of stay

The average of LOS was 8.8 days and it consistently decreased by 2% (95%CI: -2.67%, -1.41%, $P < 0.001$) per year from 12.7 d in 1998 to 7.6 d in 2017 (Figure 1B). The LOS in patients who died was longer compared to those who survived (13.54 d *vs* 8.38 d, $P < 0.0001$). On multivariate analysis primary payer, and hospital characteristics had impact on LOS. Important complications that had impact on LOS included AKI, acute liver failure, acute respiratory failure, ascites, spontaneous bacterial peritonitis, IVC thrombosis, comorbidity score and cancer (Table 4). Compared to the West, hospitals in the North East, Midwest and South had longer inpatient stays. LOS in urban teaching hospitals was significantly higher than urban non-teaching hospitals ($P < 0.0001$) (Supplementary Table 3).

Hospital costs

The average total charges after adjusted for Medical Care Consumers Price Index to 2017 dollars during the time period was \$94440, and the APC increased by 1.15% (95%CI: 0.35%, 1.96%, $P = 0.005$) per year from \$95515 in 1998 to \$103850 in 2017 (Figure 1C). The hospital charge was higher in patients who died compared to those who survived (\$190724 *vs* \$85071, $P < 0.0001$). The charge was also higher in urban teaching hospitals than urban non-teaching hospitals ($P < 0.0001$). When stratified by different regions of the country, the charges were higher in the West compared to every other region in the country ($P < 0.001$, Supplementary Table 4). On multivariate analysis, race, hospital characteristics, number of procedures, length of stay, and comorbidity score were associated with total charges. Important complications that had an effect on total charges included AKI, acute respiratory failure, HRS, IVC thrombosis, cancer, and anemia due to acute blood loss (Table 5).

Table 1 Characteristics of patients and hospitals and individual effects on mortality, length of stay and total charges

Study time period	1998-2017	Individual effect (Type III test, <i>P</i> value)		
		Mortality	Length of stay	Total charges
BCS patients' characteristics				
Age	50.50 (0.19)	< 0.001	0.052	< 0.001
Female	55.19 (0.54)	0.003	0.001	< 0.001
Race		0.138	0.013	< 0.001
1: White	56.03 (0.54)			
2: Black	13.26 (0.37)			
3: Hispanic	9.56 (0.32)			
4: Asian/Pacific Islander	2.56 (0.17)			
6: Other	3.65 (0.2)			
9: Unknown	14.93 (0.39)			
Primary payer		< 0.001	0.002	< 0.001
1: Medicare	33.17 (0.52)			
2: Medicaid	18.42 (0.42)			
3: Private insurance	40.20 (0.54)			
6: Other	8.21 (0.3)			
Hospital characteristics				
Hospital size		0.014	< 0.001	< 0.001
1: Small	9.81 (0.32)			
2: Medium	18.92 (0.43)			
3: Large	71.27 (0.49)			
Hospital location and teaching status		0.195	< 0.001	< 0.001
1: Rural	6.89 (0.28)			
2: Urban nonteaching	24.24 (0.47)			
3: Urban teaching	68.87 (0.51)			
Hospital region		0.533	0.010	< 0.001
1: Northeast	21.84 (0.45)			
2: Midwest	22.15 (0.46)			
3: South	33.45 (0.52)			
4: West	22.57 (0.46)			
Clinical characteristics				
Ascites	29.93 (0.5)	< 0.001	< 0.001	< 0.001
Acute kidney injury	18.84 (0.43)	< 0.001	< 0.001	< 0.001
Hepatic cirrhosis with no mention of alcohol	18.65 (0.43)	0.901	0.031	0.838
Cancer	17.26 (0.41)	< 0.001	0.002	0.010
Portal hypertension	16.57 (0.41)	0.029	0.898	0.000
Hepatic encephalopathy	9.59 (0.32)	< 0.001	< 0.001	< 0.001
Portal vein thrombosis	7.92 (0.3)	0.006	0.372	0.073
Esophageal varices without bleeding	7.44 (0.29)	0.002	0.324	0.091
Acute respiratory Failure	7.03 (0.28)	< 0.001	< 0.001	< 0.001
HCC	6.93 (0.28)	< 0.001	< 0.001	0.543

Acute blood loss anemia/hemorrhagic	6.62 (0.27)	0.008	< 0.001	< 0.001
IVC thrombosis	6.39 (0.27)	< 0.001	< 0.001	< 0.001
Sepsis	6.10 (0.26)	< 0.001	< 0.001	< 0.001
Alcoholic cirrhosis	5.73 (0.25)	0.113	0.731	0.140
Acute liver failure	5.60 (0.25)	< 0.001	< 0.001	< 0.001
Hepatorenal syndrome	3.29 (0.2)	< 0.001	< 0.001	< 0.001
Variceal bleeding	3.20 (0.19)	0.107	0.160	0.001
Spontaneous bacterial peritonitis	2.83 (0.18)	< 0.001	< 0.001	< 0.001
Intestinal infarct/acute vascular insufficiency	2.11 (0.16)	< 0.001	< 0.001	< 0.001
Elixhauser Comorbidity Score excluding liver disease	9.38 (0.12)	< 0.001	< 0.001	< 0.001

All data are presented as percentage (SE) for categorical variables and mean (SE) for continuous variables. BCS: Budd Chiari syndrome; HCC: Hepatocellular carcinoma IVC: Inferior vena cava.

Utilization of procedures

During their in-patient stay, patients underwent an average of 2.64 procedures per hospitalization. Paracentesis was the most frequent procedure (18.4%) followed by upper gastrointestinal endoscopy (10.9%), liver biopsy (6.2%), TIPS (3.6%) and LT (1.9%) (Table 2). Subgroup analysis showed that out of the 307 patients who underwent TIPS, 145 (47%) had LT.

While total number of procedures performed remained stable during the study period, there was a significant and notable reduction in the number of liver biopsies (APC: -4.01%, 95%CI: -5.42%, -2.58%, $P < 0.0001$), TIPS (APC: -4.95%, 95%CI: -6.78%, -3.09%, $P < 0.0001$) and LT (APC: -2.68%, 95%CI: -5.26%, -0.02%, $P = 0.05$). Hispanics underwent more procedures than Caucasians ($P < 0.001$) and Blacks ($P < 0.001$). Patients admitted to urban teaching hospitals underwent more procedures than urban non-teaching hospitals ($P < 0.0001$) and rural hospitals ($P < 0.0001$) (Supplementary Table 5).

DISCUSSION

In this large population-based study from the United States, we found that the overall in-patient mortality rate for an unselected group of patients with BCS was 8%. The mortality rates and LOS reduced significantly from 1998 to 2017, but total hospital charges, however, increased during the study period. The patients who survived hospitalization were younger than those who died (49.7 years *vs* 58.6 years), but race, hospital teaching status and hospital region did not impact survival. The reduction in mortality was multifactorial and possibly could be related to earlier detection of BCS, advances in therapeutic options and a better overall inpatient care.

To our knowledge, there are no prior studies that have exclusively analyzed inpatient mortality secondary to BCS, but multicenter studies in the recent era that investigated prognosis of BCS have reported improvement in survival rates with time [9,10]. A European study that consisted of 157 BCS patients, who were managed using a stepwise treatment algorithm over a median duration of 50 mo reported a mortality of 23% [9]. A majority of these patients succumbed to liver failure (33%) and the median time to death for the cohort was 10 mo. The study found that age, bilirubin and creatinine were independent risk factors for survival. Most patients (88.5%) in their study were on long term anticoagulation and those who did not respond to medical management were treated with percutaneous angioplasty/thrombolysis ($n = 22$), TIPS ($n = 62$) and LT ($n = 20$) in a step wise manner. Due to inherent limitations of the NIS dataset we were unable to determine how many patients in our study were on anticoagulation.

Overall, less than 5% of the patients underwent invasive procedures such as TIPS and LT. There were no significant differences in mortality between patients who underwent these procedures and those who did not. However, 89% of patients who underwent TIPS and 92% who had LT during their inpatient stay survived hospitalization. We also noticed a downward trend in the number of TIPS and LT in hospitalized BCS patients, perhaps because these procedures were done after patients were

Table 2 Trends in outcomes of interest

	1998-2017 (unweighted: 8435, weighted: 41119)	1998 (unweighted: 262, weighted: 1367)	2017 (unweighted: 680, weighted: 3400)	APC (95%CI)	P value for APC
Procedures					
Number of procedures	2.64 (0.03)	3.09 (0.20)	2.42 (0.13)	-0.51% (-1.09%, 0.06%)	0.082
Paracentesis	18.41 (0.42)	28.56 (2.82)	16.47 (1.42)	-1.67% (-2.53%, -0.81%)	0.000
Upper endoscopy	10.94 (0.34)	13.08 (2.06)	11.91 (1.24)	-0.17% (-1.31%, 0.97%)	0.766
Liver biopsy	6.24 (0.26)	10.35 (1.9)	5.15 (0.85)	-4.01% (-5.42%, -2.58%)	< 0.0001
Portosystemic shunt/TIPS	3.63 (0.2)	6.12 (1.54)	2.94 (0.65)	-4.95% (-6.78%, -3.09%)	< 0.0001
Liver transplantation	1.9 (0.15)	1.29 (0.75)	2.06 (0.54)	-2.68% (-5.26%, -0.02%)	0.048
Disposition of patient					
1: Discharged to home or selfcare	58.8(0.54)	50.71 (3.12)	55.96 (1.91)	-0.28% (-0.77%, 0.21%)	0.262
6: Home health care	13.49 (0.37)	12.04 (2.04)	17.23 (1.45)	3.31% (2.20%, 4.43%)	< 0.0001
5: Transfer: other type of facility	11.12 (0.34)	10.21 (1.89)	12.08 (1.25)	1.87% (0.70%, 3.06%)	0.002
20: Died in hospital	8.74 (0.31)	18.17 (2.44)	7.66 (1.02)	-4.31% (-5.50%, -3.10%)	< 0.0001
2: Transfer: short-term hospital	6.8 (0.28)	8.25 (1.71)	5.6 (0.88)	-1.26% (-2.66%, 0.17%)	0.084
7: Against medical advice	1 (0.11)	0.61 (0.43)	1.47 (0.46)	2.94% (-1.02%, 7.07%)	0.148
Outcomes					
Number of deaths	737 (Unweighted); 3591 (Weighted)	46 (Unweighted); 249 (Weighted)	52 (Unweighted); 260 (Weighted)		
Mortality rate per 1000000 United States populations		0.9	0.8	-0.29% (-0.86%, 0.27%)	0.309
Mortality rate per 1000000 inpatients		8.87	8.55	0.34% (-0.23%, 0.92%)	0.243
Mortality rate among BCS inpatients	0.09	0.18	0.08	-4.41% (-4.95%, -3.88%)	< 0.0001
Length of stay (d)	8.84 (0.13)	12.73 (1.01)	7.64 (0.36)	-2.04% (-2.67%, -1.41%)	< 0.0001
Average total charges in 2017 dollars	94440.04 (1996.06)	95515.01 (9483.24)	103850.98 (8183.79)	1.15% (0.35%, 1.96%)	0.005

All data are presented as percentage (SE) for categorical variables and mean (SE) for continuous variables. Annual percentage change (APC) > 0 means increasing, < 0 means decreasing. *P* value for APC measures if APC is significantly different from zero. *P* value ≤ 0.05 means the change is significant. BCS: Budd Chiari syndrome; APC: Annual percentage change.

discharged and hence was not captured by the NIS database. Nearly half (47%) the patients who had TIPS underwent LT, and it possible that TIPS was done in these patients as a bridge to LT, or perhaps they had more complications such as variceal bleeding or refractory ascites.

A management strategy that consists of a stepwise invasive treatment algorithm guided by response to prior treatment have resulted in better short- and long-term outcome in BCS patients[3,9-12,15]. This consists of early and prompt initiation of anticoagulation with low molecular weight heparin to prevent extension of thrombosis, referral to a hematologist for treatment of specific underlying clotting disorders and treatment of portal hypertension related complications. Patients who

Table 3 Multivariate model on mortality

	Response	Beta estimate	Standard error	P value for beta	Odds ratio (95%CI)
Age		0.024	0.003	< 0.0001	1.024 (1.019, 1.029)
Acute respiratory Failure	Yes (reference = No)	1.652	0.109	< 0.0001	5.219 (4.211, 6.468)
Intestinal infarct/acute vascular insufficiency	Yes (reference = No)	1.422	0.201	< 0.0001	4.143 (2.795, 6.142)
Acute liver failure	Yes (reference = No)	1.286	0.119	< 0.0001	3.617 (2.864, 4.567)
Hepatorenal syndrome	Yes (reference = No)	1.123	0.147	< 0.0001	3.072 (2.302, 4.101)
Cancer	Yes (reference = No)	0.882	0.098	< 0.0001	2.415 (1.993, 2.927)
Acute kidney injury	Yes (reference = No)	0.803	0.092	< 0.0001	2.232 (1.862, 2.675)
Sepsis/severe sepsis/septic shock	Yes (reference = No)	0.635	0.122	< 0.0001	1.886 (1.484, 2.398)
Hepatic encephalopathy	Yes (reference = No)	0.280	0.117	0.020	1.323 (1.052, 1.662)
Elixhauser Comorbidity Score excluding liver disease		0.026	0.004	< 0.0001	1.027 (1.019, 1.034)

deteriorate despite optimal medical management are considered for percutaneous or transhepatic angioplasty, TIPS and/or LT. The NIS data set did not include data on venoplasty or stenting perhaps because many of these procedures are done in the outpatient setting. Several studies have reported excellent outcome following LT in patients with BCS. In a previous study, using United Network of Organ Sharing (UNOS) datasets, we had reported 85% 3-year survival in patients with BCS who underwent LT in the United States[16]. Our group recently analyzed outcome of LT in 55 BCS patients who presented with fulminant hepatic failure using the UNOS database and found that expeditious LT in this subset of patients was associated with excellent long-term patient and graft survival. We also found that despite the presence of 3 or more organ failures, LT in these patients was associated with good outcome. They also achieved excellent post LT functional status as determined by the Karnofsky performance status scores[17]. A European series that investigated outcome of LT in 248 patients report actuarial survival of 76% at 1 year, 71% at 5 years and 68% at 10 years, with majority of the deaths occurring in the first 3 mo[18].

In our study we found that the average LOS was 9 d and this reduced consistently with an APC of 2% during the 19-year period. The reduction is consistent with nationwide efforts to reduce LOS for hospitalized patients. Multivariate analysis showed significant association between LOS and complications such as AKI, acute liver failure, acute respiratory failure, SBP and IVC thrombosis. The LOS was longer in medium and large sized hospitals compared to smaller hospitals probably because these hospitals were tertiary care centers and BCS patients admitted in those hospitals were perhaps more sicker requiring prolonged inpatient stay. This would also explain why urban hospitals had a longer LOS compared to hospitals in rural areas. Longer LOS in such hospitals was associated with higher total charges as expected. We also noticed a geographical variation in the LOS, as hospitals in the North East, Midwest and South had longer inpatient stays compared to the West. Although it is difficult to explain this particular finding, a similar observation was made by the HCUP report on US hospital LOS variation by region in 2016 and could be related to physician practice patterns, access to health care services, treatment preferences and cost of living that varies by geographic location in a diverse country like United States[19].

The average total costs for BCS hospitalizations between 1998 and 2017 was \$94440 and this continued to show a significant upward trend. We found that compared to the West, hospitals in the Northeast, Midwest and South of United States had lower total charges. We do not have a good explanation for this finding. The increasing financial burden of BCS hospitalizations to the US health care system in our study, despite a reduction in the average LOS, is consistent with other studies that have analyzed the economic impact of hospitalizations related to decompensated cirrhosis and can be attributed to the increasing hospitalization rate as well as increasing severity of disease burden as indicated by comorbidity score[20,21].

Our study has a few limitations most of which are inherent to the use of a large administrative database. The use of ICD codes to capture the diagnosis of BCS could result in coding errors potentially resulting in misclassification. We could not perform a sensitivity analysis because of the absence of patient identifiers in the datasets. Another major shortcoming is that the NIS reports every hospitalization as a separate

Table 4 Multivariate model on length of stay

	Response	Beta estimate	Standard error	P value for Beta	P value for type 3 test
Primary payer	1: Medicare (reference)	0.000	-	-	0.022
	2: Medicaid	0.053	0.037	0.144	
	3: Private insurance	0.084	0.030	0.005	
Hospital bed size	1: Small (reference)	0.000	-	-	< 0.0001
	2: Medium	0.113	0.049	0.021	
	3: Large	0.293	0.042	<.0001	
Hospital location and teaching status	1: Rural (reference)	0.000	-	-	< 0.0001
	2: Urban nonteaching	0.206	0.054	<.0001	
	3: Urban teaching	0.433	0.050	<.0001	
Hospital region	1: Northeast	0.171	0.038	<.0001	< 0.0001
	2: Midwest	0.017	0.038	<.0001	
	3: South	0.055	0.034	<.0001	
	4: West (reference)	0.000			
Complications					
Acute liver failure	Yes (reference = No)	0.223	0.057	< 0.0001	< 0.0001
Acute respiratory Failure	Yes (reference = No)	0.380	0.052	< 0.0001	< 0.0001
Acute kidney injury	Yes (reference = No)	0.255	0.035	< 0.0001	< 0.0001
Ascites	Yes (reference = No)	0.118	0.028	< 0.0001	< 0.0001
Spontaneous bacterial peritonitis	Yes (reference = No)	0.480	0.076	< 0.0001	< 0.0001
IVC thrombosis	Yes (reference = No)	0.138	0.052	0.008	0.008
Intestinal infarct/acute vascular insufficiency	Yes (reference = No)	0.383	0.088	< 0.0001	< 0.0001
cancer	Yes (reference = No)	-0.278	0.036	< 0.0001	< 0.0001
Elixhauser Comorbidity Score excluding liver disease		0.019	0.001	< 0.0001	< 0.0001

IVC: Inferior vena cava.

encounter and not as a unique patient. It is possible that many of these patients were readmitted and were counted more than once. We were also unable to obtain information regarding therapeutic data with respect to anticoagulation and specific pharmacological agents used to treat underlying thrombophilia. Nonetheless, the NIS database is considered to be a powerful research tool providing robust clinical data about real world scenarios and its reliability has been extensively validated[22].

CONCLUSION

In conclusion, this is the first study from the United States to illustrate reducing mortality related to BCS hospitalizations as well as a reduction in the average LOS. While these findings are reassuring, BCS continues to have a significant economic impact as indicated by the rising healthcare costs.

Table 5 Multivariate model on total charges

	Response	Estimate	Standard error	P value for beta	P value for type 3 test
Race	1: White (reference)	0.000	-	-	< 0.0001
	2: Black	-0.037	0.026	0.162	
	3: Hispanic	0.015	0.031	0.613	
	4: Asian/Pacific Islander	0.136	0.058	0.019	
	6: Other	0.082	0.046	0.077	
	9: Unknown	-0.185	0.025	< 0.0001	
Hospital bed size	1: Small (reference)	0.000	-	-	< 0.0001
	2: Medium	0.080	0.034	0.018	
	3: Large	0.169	0.029	< 0.0001	
Hospital location and teaching status	1: Rural (reference)	0.000	-	-	< 0.0001
	2: Urban nonteaching	0.428	0.037	< 0.0001	
	3: Urban teaching	0.552	0.034	< 0.0001	
Hospital region	1: Northeast	-0.195	0.027	< 0.0001	< 0.0001
	2: Midwest	-0.333	0.027	< 0.0001	
	3: South	-0.330	0.024	< 0.0001	
	4: West (reference)	0.000	-	-	
Complications					
Acute liver failure	Yes (reference = No)	0.078	0.039	0.044	0.044
Acute respiratory Failure	Yes (reference = No)	0.204	0.036	< 0.0001	< 0.0001
Acute kidney injury	Yes (reference = No)	0.146	0.025	< 0.0001	< 0.0001
Hepatorenal syndrome	Yes (reference = No)	-0.132	0.050	0.008	0.009
IVC thrombosis	Yes (reference = No)	0.075	0.035	0.035	0.035
Acute blood loss anemia/ hemorrhagic	Yes (reference = No)	0.155	0.035	< 0.0001	< 0.0001
Cancer	Yes (reference = No)	-0.052	0.025	0.037	0.037
Elixhauser Comoridity Score excluding liver disease		0.005	0.001	< 0.0001	< 0.0001
Other variables					
Number of procedures		0.118	0.004	< 0.0001	< 0.0001
Length of stay		0.054	0.001	< 0.0001	< 0.0001

IVC: Inferior vena cava.

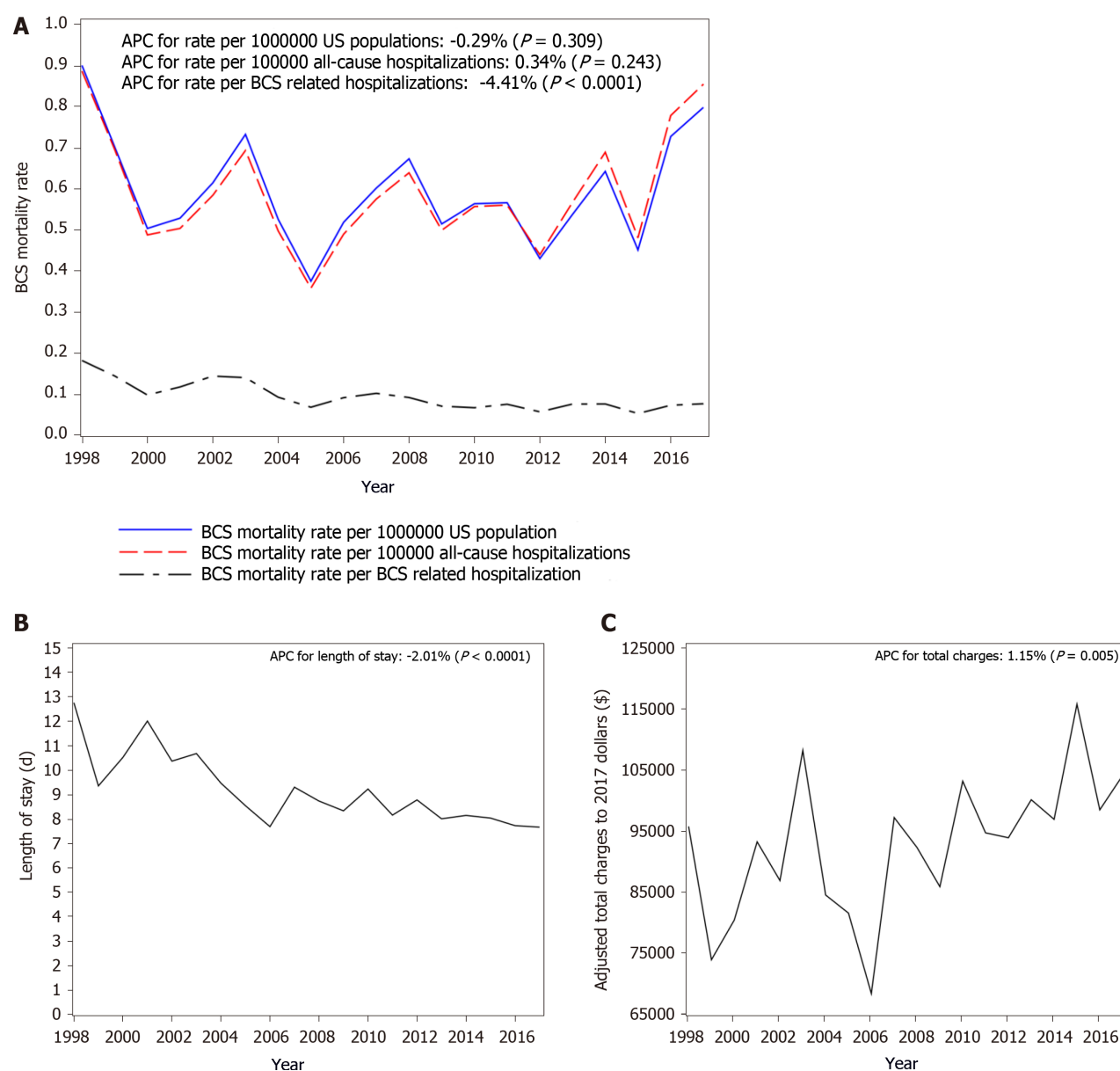


Figure 1 Annual percentage changes. A: Annual percentage change for mortality in patients with Budd Chiari syndrome (BCS) (per 1000000 United States population, per 100000 all cause hospitalizations and BCS related hospitalizations); B: Length of hospital stay for patients with BCS from 1998 to 2017; C: Adjusted (charges adjusted to 2017 dollars) hospital charges from 1998 to 2017 in patients with BCS. APC: Annual percentage change; BCS: Budd Chiari syndrome; US: United States.

ARTICLE HIGHLIGHTS

Research background

The Budd Chiari syndrome (BCS) is a rare disorder that results from partial or complete obstruction of the hepatic venous outflow in the absence of right heart failure.

Research motivation

There is a paucity of data on the in-hospital mortality of BCS as well its economic impact on the United States health care system.

Research objectives

This study aimed to evaluate trends in mortality, length of hospital stays and resource utilization among inpatients with BCS.

Research methods

Retrospective study where data were extracted from the National Inpatient Sample

(NIS) from 1998 to 2017. To make inferences regarding the national estimates for the total number of BCS discharges across the study period, sample weights were applied to each admission per recommendations from the NIS.

Research results

During the study period, there were 3591 (8.73%) in-patient deaths. The overall in-hospital mortality rate among BCS patients decreased from 18% in 1998 to 8% in 2017; the mortality decreased by 4.41% every year. The average of length of stay was 8.8 d and it consistently decreased by 2.04% from 12.7 d in 1998 to 7.6 d in 2017. The average total charges during the time period was \$94440 and the annual percentage change increased by 1.15%.

Research conclusions

The in-hospital mortality rate for patients admitted with BCS in the United States has reduced between 1998 and 2017 while total charges continued to increase.

Research perspectives

Using a large national database, we analyzed the mortality and socioeconomic impact of BCS hospitalizations in the United States with a high degree of granularity.

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Retrospective Study

Comparison of unenhanced magnetic resonance imaging and ultrasound in detecting very small hepatocellular carcinoma

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Author contributions: Tarao K summarized the data and wrote the paper; Nozaki A, Komatsu H, Komatsu T, Tanaka K, Chuma M, Numata K, and Maeda S followed up the patients; Taguri M conducted statistical analysis; Yoshida T and Koyasu H made suggestions regarding the MRI technique.

Institutional review board

statement: The study was reviewed and approved by the Ethics Committee of Yokohama Municipal Citizen's Hospital Institutional Review Board (Approval No. 21-02-01).

Informed consent statement: This study was performed after approval by the respective institutional review boards. The

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Abstract

BACKGROUND

In hepatocellular carcinoma (HCC), detection and treatment prior to growth beyond 2 cm are important as a larger tumor size is more frequently associated with microvascular invasion and/or satellites. In the surveillance of very small HCC nodules (≤ 2 cm in maximum diameter, Barcelona clinical stage 0), we demonstrated that the tumor markers alpha-fetoprotein and PIVKA-II are not so useful. Therefore, we must survey with imaging modalities. The superiority of magnetic resonance imaging (MRI) over ultrasound (US) to detect HCC was confirmed in many studies. Although enhanced MRI is now performed to

study used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement:

There are no conflicts of interest to disclose.

Data sharing statement:

No additional data are available.

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Manuscript source: Invited manuscript

Specialty type: Gastroenterology and hepatology

Country/Territory of origin: Japan

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

Received: February 4, 2021

Peer-review started: February 4, 2021

First decision: February 24, 2021

Revised: March 9, 2021

Accepted: May 20, 2021

Article in press: May 20, 2021

Published online: June 27, 2021

P-Reviewer: Elsayed M, Yang L

S-Editor: Gao CC

L-Editor: Wang TQ

P-Editor: Wang LL



accurately diagnose HCC, in conventional clinical practice for HCC surveillance in liver diseases, unenhanced MRI is widely performed throughout the world. While, MRI has made marked improvements in recent years.

AIM

To make a comparison of unenhanced MRI and US in detecting very small HCC that was examined in the last ten years in patients in whom MRI and US examinations were performed nearly simultaneously.

METHODS

In 394 patients with very small HCC nodules, those who underwent MRI and US at nearly the same time (on the same day whenever possible or at least within 14 days of one another) at the first diagnosis of HCC were selected. The detection rate of HCC with unenhanced MRI was investigated and compared with that of unenhanced US.

RESULTS

The sensitivity of unenhanced MRI for detecting very small HCC was 95.1% (97/102, 95% confidence interval: 90.9-99.3) and that of unenhanced US was 69.6% (71/102, 95% confidence interval: 60.7-78.5). The sensitivity of unenhanced MRI for detecting very small HCC was significantly higher than that of unenhanced US ($P < 0.001$). Regarding the location of HCC in the liver in patients in whom detection by US was unsuccessful, S_{7-8} was identified in 51.7%.

CONCLUSION

Currently, unenhanced MRI is a very useful tool for the surveillance of very small HCC in conventional clinical follow-up practice.

Key Words: Comparison of magnetic resonance imaging and ultrasound; Surveillance of very small hepatocellular carcinoma; Magnetic resonance imaging; Ultrasound; Unenhanced magnetic resonance imaging

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Core Tip: Recent technological development of magnetic resonance imaging (MRI) scanners has been excellent. The 3.0-tesla (T) MR scanner with a higher field strength has been increasingly used because improved lesion detection can be expected as a result of the increased signal-to-noise ratio, which is theoretically twice with 3.0-T compared with 1.5-T. Another important improvement in MRI is the practical use of diffusion-weighted imaging. In this study, a comparison of unenhanced MRI and ultrasound in detecting very small hepatocellular carcinoma (2 cm in maximum diameter) was made. The sensitivity of unenhanced MRI for detecting very small hepatocellular carcinoma was as high as 95.1% as compared with 69.6% of unenhanced ultrasound ($P < 0.001$).

Citation: Tarao K, Nozaki A, Komatsu H, Komatsu T, Taguri M, Tanaka K, Yoshida T, Koyasu H, Chuma M, Numata K, Maeda S. Comparison of unenhanced magnetic resonance imaging and ultrasound in detecting very small hepatocellular carcinoma. *World J Hepatol* 2021; 13(6): 699-708

URL: <https://www.wjgnet.com/1948-5182/full/v13/i6/699.htm>

DOI: <https://dx.doi.org/10.4254/wjh.v13.i6.699>

INTRODUCTION

If hepatocellular carcinoma (HCC) tumors are growing up to more than 2 cm in diameter, they are often associated with microvascular invasion and/or satellites, which are major predictors of recurrence after initial effective treatments[1]. The same tendency was observed by Stravitz *et al*[2], who reported that the early detection of HCC improves the prognosis. Therefore, we must identify very small HCC nodules (\leq

2 cm in maximum diameter) in the surveillance of HCC.

Recently, we demonstrated that more than one third of patients with very small HCC nodules were dropped from surveillance using the tumor markers alpha-fetoprotein (AFP) and PIVKA-II[3]. Therefore, we must survey patients with liver diseases using imaging modalities.

Surveillance of HCC in liver diseases, especially in liver cirrhosis, has been conducted by ultrasound (US) or magnetic resonance imaging (MRI) throughout the world.

Although US was performed more popularly than MRI in the surveillance of HCC, the superiority of MRI over US has been demonstrated in many studies since 2001-2003[4,5]. Although enhanced MRI is now performed for the accurate diagnosis of HCC[5-9], in conventional clinical practice for HCC surveillance in liver diseases, unenhanced MRI is widely performed throughout the world. On the other hand, MRI has made much progress in recent years.

In this study, a comparison of unenhanced MRI and US in surveying very small HCC was made. In order to conduct precise evaluation, we selected patients in whom MRI and US were performed at about the same time.

MATERIALS AND METHODS

Study population

This was a retrospective observational study that included 403 patients with small single HCC nodules (≤ 2 cm in maximum diameter, Barcelona clinical stage 0) who visited the following three hospitals and one clinic in Yokohama City for the first time between January 2008 and September 2020: Gastroenterological Center, Medical Center, Yokohama City University; Department of Gastroenterology, Yokohama Municipal Citizen's Hospital; Department of Gastroenterology, National Hospital Organization, Yokohama Medical Center; and Tarao's Gastroenterological Clinic. Of the 403 patients with very small HCC, 102 were selected in whom MRI and US were conducted simultaneously (on the same day or at least within 14 days of one another) (Figure 1). In this series of the study, MRI and US were performed in unenhanced states because we wanted to study the usefulness to survey HCC in routine follow-up study. In the unenhanced MRI, a very small HCC usually appears as a dark spot in T_1 image and light white spot in T_2 image (see Figures 2-5). It is important that characteristics of both T_1 and T_2 images were present at the same time. In the US images, it usually appears as a dark round spot.

HCCs were diagnosed chiefly by dynamic computed tomography (CT) and abdominal angiography, which showed early enhancement and early washout. This work was performed in accordance with the Declaration of Helsinki.

Previously diagnosed HCC was excluded from the protocol. This study was performed after approval by the respective institutional review boards.

The patients were classified according to the etiologies of liver diseases (Table 1).

HCC detection

The diagnosis of HCC was confirmed by US, MRI, CT, enhanced dynamic CT, and abdominal angiography. All patients underwent abdominal angiography to confirm the single nodules. The maximum diameter of the HCC nodules was scaled by US or MRI.

Helical dynamic CT and abdominal angiography were performed in almost all patients except those with hypersensitivity to iodine and advanced kidney disease. In the helical dynamic CT, an intravenous bolus injection of contrast material and sequential scanning were performed, and an intense homogenous arterial phase (early enhancement) and early washout in the venous phase were considered to be characteristic of HCC[10-12]. Abdominal angiography was also performed to exclude the benign nodular lesions and exclude HCC patients with macrovascular invasion. Of course, the characteristic features of very small HCC in unenhanced MRI as mentioned above were taken into account.

Patients with macrovascular invasion or extrahepatic metastasis were excluded. In patients undergoing hepatectomy, the final decision on HCC was made by pathological diagnosis, and cases of benign nodules were excluded.

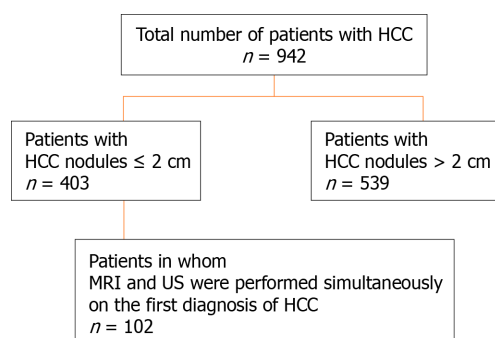
Statistical analysis

We calculated the detection rate and its 95% confidence interval (CI) for each method. We then compared the detection rates between MRI and US using McNemar's test.

Table 1 Background of hepatocellular carcinoma patients (≤ 2 cm in diameter) who underwent unenhanced magnetic resonance imaging and unenhanced ultrasound simultaneously

Background of patients	
Number of patients	102
Age (yr)	72.4 \pm 9.6
Sex (%)	
Male	52 (51.0)
Female	50 (49.0)
Etiology (%)	
HBV	13 (12.9)
HCV	61 (60.3)
Alcohol	14 (13.9)
NBNC	7 (6.9)
Autoimmune	2 (2.0)
NASH	2 (2.0)
PBC	1 (1.0)
Others	2 (2.0)

HBV: Hepatitis B virus; HCV: Hepatitis C virus; NBNC: Non-B non-C; NASH: Nonalcoholic steatohepatitis; PBC: Primary biliary cirrhosis.

**Figure 1 Patient selection.** HCC: Hepatocellular carcinoma; MRI: Magnetic resonance imaging; US: Ultrasound.

RESULTS

The sensitivity of unenhanced MRI for detecting very small HCC (≤ 2 cm in diameter) was 95.1% [97/102, 95%CI: 90.9-99.3] and that of unenhanced US was 69.6% (71/102, 95%CI: 60.7-78.5) ($P < 0.001$).

Table 2 shows the location of the HCC in the liver of patients in whom detection by US was unsuccessful. S_{7-8} was the site in 51.7% of these patients. Thus, HCC lesions in S_{7-8} may be difficult to identify by US. Representative images of four cases of very small HCC (A, B, C, and D) by unenhanced MRI are shown in Figures 2-5. In all the four cases, HCC was confirmed using hepatectomized specimens.

Moreover, the treatment methods for 102 HCC patients are shown in Table 3.

DISCUSSION

For the surveillance of very small HCC, US was hitherto performed worldwide. However, in recent years, the superiority of MRI over US to detect very small HCC has been reported in many articles.

Table 2 Location of hepatocellular carcinoma in the liver in patients for whom detection by ultrasound was unsuccessful

Location in the liver	Number of patients (%)
S ₁₋₄	6 (20.7)
S ₅₋₆	8 (27.6)
S ₇₋₈	15 (51.7)

Table 3 Treatment methods for hepatocellular carcinoma in 102 very small hepatocellular carcinoma patients

Therapy	Number of treated patients
Hepatectomy	19
RFA	58
TACE	14
TACE + RFA	2
TAI	1
Chemotherapy	2
BSC	3
Others	3

BSC: Best supportive care; RFA: Radiofrequency ablation; TACE: Transcatheter arterial chemoembolization; TAI: Transcatheter arterial infusion.

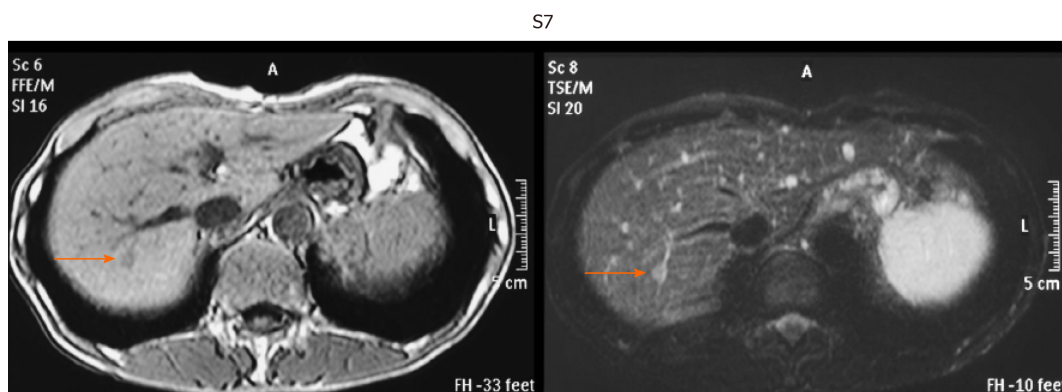


Figure 2 Representative image of very small hepatocellular carcinoma by unenhanced magnetic resonance imaging. Hepatocellular carcinoma in S7 segment. T1-weighted image (left, light dark spot). T2-weighted image (right, light white spot).

Colli *et al*[4] conducted a systemic review on this issue, and found that the pooled estimate of 14 US studies was 60.5% (95%CI: 44-76) for sensitivity[13-25], and that of 9 MRI studies was 80.6% (95%CI: 70-91) for sensitivity[9,23,24,26-31]. The difference in sensitivity between US and MRI may be due to the fact that MRI is less influenced by the operator's technique, patient's body type, and location of HCC lesions.

More recently, in 2017, Kim *et al*[5] compared MRI and US in a cohort of 407 patients with cirrhosis who underwent 1100 surveillance examinations, and found that MRI had a sensitivity of 83.7% (95%CI: 69.7-92.2) for early HCC detection, which was significantly higher than that of US (25.6%, 95%CI: 14.8-49.4).

We demonstrated in this study that 95% of cases with very small HCC can be detected by unenhanced MRI. This figure is very high compared with previous reports published between 2001 and 2003 concerning the sensitivity of unenhanced MRI for detecting very small HCC. Table 4 shows the reported sensitivity of unenhanced MRI for detecting very small HCC between 2001 and 2003 when MRI used 1.5-tesla (T) imaging. The average sensitivity in that period was 60.3% (95%CI: 52.2-68.4)[25,27,28,30,31].

Table 4 Reported sensitivity of unenhanced magnetic resonance imaging to detect very small hepatocellular carcinomas (≤ 2 cm in diameter) between 2001 and 2003

Ref.	Sensitivity (%)
Krinsky <i>et al</i> [27], 2001	7/15 (46.7)
de Lédinghen <i>et al</i> [28], 2002	33/54 (61.1)
Libbrecht <i>et al</i> [25], 2002	7/10 (70.0)
Bhartia <i>et al</i> [30], 2003	15/21 (71.4)
Burrel <i>et al</i> [31], 2003	23/41 (56.1)
Pooled estimates	85/141 (60.3)
	95%CI: 52.2-68.4

CI: Confidence interval.

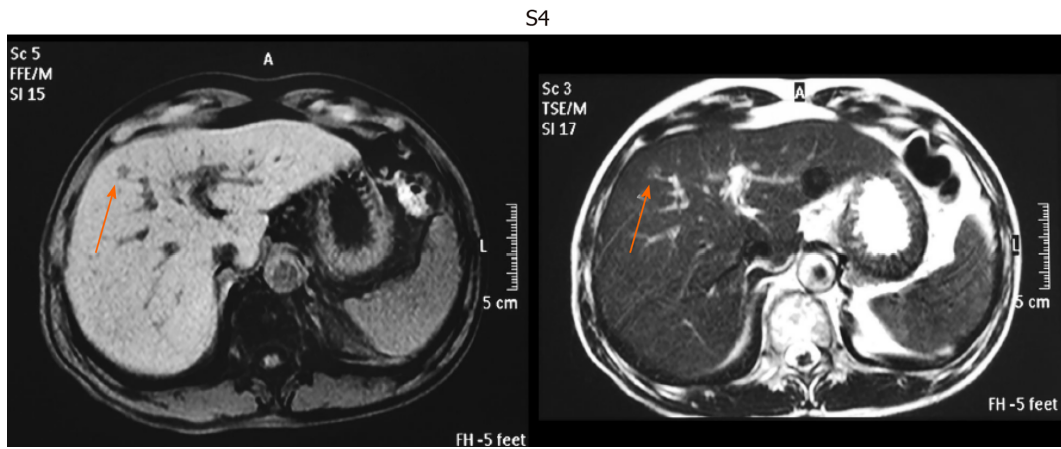


Figure 3 Representative image of very small hepatocellular carcinoma by unenhanced magnetic resonance imaging. Hepatocellular carcinoma in S4 segment. T1-weighted image (left, light dark spot). T2-weighted image (right, light white spot).

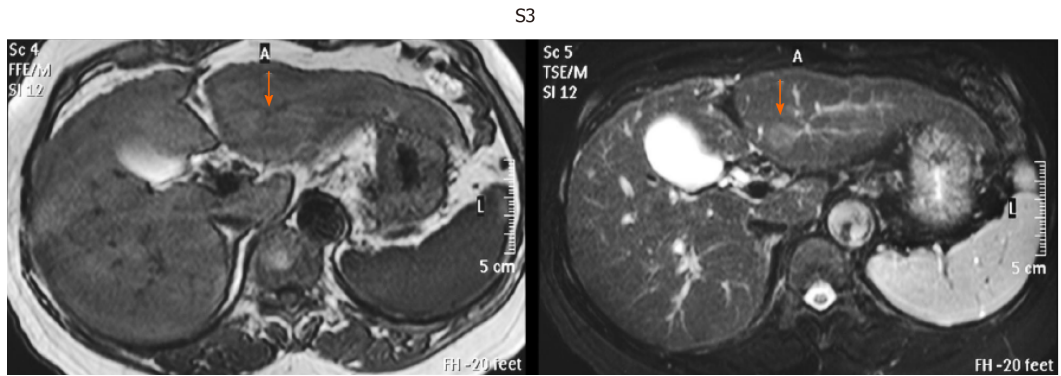


Figure 4 Representative image of very small hepatocellular carcinoma by unenhanced magnetic resonance imaging. Hepatocellular carcinoma in S3 segment. T1-weighted image (left, light dark spot). T2-weighted image (right, light white spot).

The reasons why this marked improvement appeared in the sensitivity of unenhanced MRI with regard to detecting very small HCC must be considered.

First of all, MRI has made marked progress in its ability in recent years. Recent technological development of MRI scanners has allowed high-quality multiphasic imaging of the entire liver. Since 2003-2005, the 3.0-T magnetic resonance (MR) scanner with a higher field strength has been increasingly used because improved lesion detection can be expected as a result of the increased signal-to-noise ratio (SNR), which is theoretically twice the SNR at 1.5-T[32,33]. Indeed, it was demonstrated that

S8

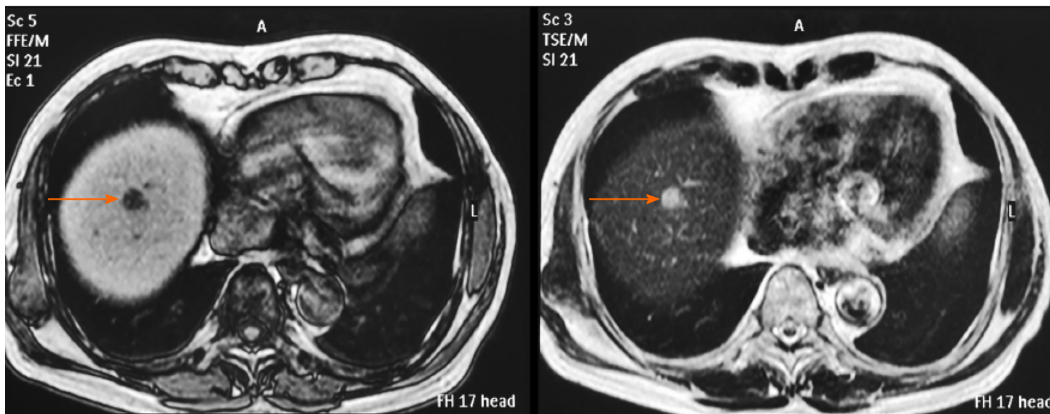


Figure 5 Representative image of very small hepatocellular carcinoma by unenhanced magnetic resonance imaging. Hepatocellular carcinoma in S8 segment. T1-weighted image (left, light dark spot). T2-weighted image (right, light white spot).

3.0-T images were superior to 1.5 T images for detecting hepatic metastases[34]. Previous misdiagnoses of HCC on MRI maybe have been due to poor patient compliance, especially the inability to suspend respiration. These problems can be resolved by the new advancements mentioned above to develop faster and motion-robust sequences.

Another important improvement of MRI is the practical use of diffusion-weighted imaging. Indeed, it was demonstrated that the sensitivity of detecting pancreatic cancer rose with the use of diffusion-weighted imaging[35].

On the other hand, the sensitivity of unenhanced US in our study for detecting very small HCC was 69.6%, which was nearly the same as those in previous reports[9,22,24,26-29]. One of the reasons for the inferiority of US may be the location of HCC in the liver. A lesion located at S₇₋₈ (the most frequent HCC lesion in the liver) may be difficult to identify by US.

Our present study indicates the importance of unenhanced MRI in detecting very small HCC, because more than one third of these patients were dropped from surveillance by tumor markers AFP and PVKA-II. However, there are two limitations of unenhanced MRI. First, it is more expensive than US. Second, in case of very tiny HCC (3-5 mm), it is difficult to find HCC by unenhanced MRI.

CONCLUSION

Considering the above-mentioned facts, unenhanced MRI is a very useful tool for detecting very small HCC in the conventional follow-up of patients with liver diseases, especially liver cirrhosis.

ARTICLE HIGHLIGHTS

Research background

Nowadays advancement of magnetic resonance imaging (MRI) has markedly improved the quality of liver imaging. We believe that a high-speed scan and diffusion-weighted imaging are two major factors that have contributed to the improved detection of hepatocellular carcinomas (HCCs). In early MRI, a respiration artifact was the most troublesome factor deteriorating the quality of images of the liver. A high-speed scan brought by the conversion from 1.5-tesla (T) to 3.0-T facilitates whole-liver MRI while patients hold their breath. Breath-holding scans reduce motion and misregistration artifacts, and create high-quality liver images. In addition, the practical use of diffusion-weighted imaging has contributed to the detection of cell-rich lesions. Tumors are proper objects of these sequences. There is a report (or several reports) that the sensitivity of detecting pancreatic cancer rose with the use of diffusion-weighted imaging. We believe that the same can be applied to detect HCC. Currently, dynamic MRI with contrast media is considered the standard procedure to diagnose HCC. However, with improved images, non-contrasted liver MRI is still a

useful modality to detect HCCs.

Research motivation

Previous reports in 2001-2003 stated that the sensitivity of unenhanced MRI to detect very small HCC (≤ 2 cm in diameter) was about 60%. Since then, there have been few reports on the sensitivity to detect very small HCC, especially in recent years.

Research objectives

Surveillance of HCC in liver diseases, especially in liver cirrhosis, has been conducted by ultrasound (US) or MRI throughout the world. Although US was performed more popularly than MRI in the surveillance of HCC, the superiority of MRI over US has been demonstrated in many studies since 2001-2003. Although enhanced MRI is now performed for the accurate diagnosis of HCC, in conventional clinical practice for HCC surveillance in liver diseases, unenhanced MRI is widely performed throughout the world. On the other hand, MRI has made marked improvements in recent years. In this study, a comparison of unenhanced MRI and US in detecting very small HCC was made. In order to conduct precise evaluation, we selected patients in whom MRI and US were performed at about the same time (on the same day whenever possible or at least within 14 d of one another).

Research methods

Out of the 403 patients with very small HCC nodules (≤ 2 cm in maximal diameter), 102 who underwent unenhanced MRI and US at nearly the same time (on the same day whenever possible or at least within 14 d of one another) at the first diagnosis of HCC were selected. The detection rate of HCC by unenhanced MRI was studied in comparison with unenhanced US.

Research results

We found that the sensitivity of unenhanced MRI for detecting very small HCC was as high as 95.1%, as compared with 69.6% by unenhanced US ($P < 0.001$).

Research conclusions

Currently, unenhanced MRI is a very important imaging modality for picking up very small HCC in usual clinical practice.

Research perspectives

As in this study, the marked superiority of unenhanced MRI to detect very small HCC as compared with unenhanced US was confirmed, and it may be desirable to perform routine surveillance of HCC in liver diseases by unenhanced MRI.

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Distant metastasis of hepatocellular carcinoma to Meckel's cave and cranial nerves: A case report and review of literature

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Author contributions: Kim SK conceived the case and wrote the manuscript; Kim SR and Komaki R observed the clinical course of the patient and made the figures; Fujii T, Okuda T, Fujii Y, Hayakumo T, Yuasa K, Takami M, Ohtani A and Saijo Y observed the clinical course of the patient; Kobayashi H conducted the radiological examinations and interpreted the imaging findings; Koma YI conducted histological examinations.

Informed consent statement: Informed written consent was obtained from the patient for publication of this report and any accompanying images.

Conflict-of-interest statement: The authors declare that they have no

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Abstract

BACKGROUND

Metastasis occurs as a late event in the natural history of hepatocellular carcinoma (HCC), and most patients die of liver failure attributed to the tumor supplanting the liver. Conversely, the brain is a less common metastatic site.

CASE SUMMARY

We describe a rare case of hepatitis C virus-related multiple HCC metastasizing to the cavernous sinus, Meckel's cave, and the petrous bone involving multiple cranial nerves in an 82-year-old woman. At admission imaging studies including Gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid-enhanced magnetic resonance imaging (MRI) revealed multiple HCC nodules in both right and left lobes. Ultrasound guided biopsy of the left lobe revealed moderately differentiated HCC. Molecular targeted therapy with Lenvatinib (8 mg/d for 94 d, *per os*) and Ramucirumab (340 mg/d and 320 mg/d, two times by intravenous injection) were administered for 4 mo, resulting in progression of the disease. Three months after the start of molecular target therapy, the patient presented with symptoms of hyperalgesia of the right face and limited abduction of the right

conflict of interest.

CARE Checklist (2016) statement:

The authors have read the CARE Checklist (2016), and the manuscript was prepared and revised according to the CARE Checklist (2016).

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Manuscript source: Unsolicited manuscript

Specialty type: Gastroenterology and hepatology

Country/Territory of origin: Japan

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C, C, C
Grade D (Fair): 0
Grade E (Poor): 0

Received: February 17, 2021

Peer-review started: February 17, 2021

First decision: March 16, 2021

Revised: March 29, 2021

Accepted: June 16, 2021

Article in press: June 16, 2021

Published online: June 27, 2021

P-Reviewer: Elpek GO, Ferreira GSA, Gelmini R

S-Editor: Fan JR

L-Editor: A

P-Editor: Wang LL



eye, indicating disturbances in the right trigeminal and abducens nerves. Brain MRI disclosed a mass involving the cavernous sinus, Meckel's cave and the petrous bone. Contrast-enhanced MRI with gadolinium-chelated contrast medium revealed a well-defined mass with abnormal enhancement around the right cavernous sinus and the right Meckel's cave.

CONCLUSION

The diagnosis of metastatic HCC to the cavernous sinus, Meckel's cave, and the petrous bone was made based on neurological findings and imaging studies including MRI, but not on histological examinations. Further studies may provide insights into various methods for diagnosing HCC metastasizing to the craniocervical area.

Key Words: Meckel's cave; Abducens nerve; Trigeminal nerve; Hepatocellular carcinoma; Magnetic resonance imaging; Case report

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Core Tip: We describe a case of hyperalgesia of the right side of the face and limited abduction of the right eye caused by hepatocellular carcinoma (HCC) metastasizing to the right cavernous sinus, the right Meckel's cave, and the right petrous bone diagnosed through neurological findings and imaging studies. Although HCC metastasizing to the cavernous sinus, Meckel's cave and the petrous bone is rare, clinicians need to be vigilant when the patients show neurological dysfunction, especially cranial nerve involvement.

Citation: Kim SK, Fujii T, Komaki R, Kobayashi H, Okuda T, Fujii Y, Hayakumo T, Yuasa K, Takami M, Ohtani A, Saijo Y, Koma YI, Kim SR. Distant metastasis of hepatocellular carcinoma to Meckel's cave and cranial nerves: A case report and review of literature. *World J Hepatol* 2021; 13(6): 709-716

URL: <https://www.wjgnet.com/1948-5182/full/v13/i6/709.htm>

DOI: <https://dx.doi.org/10.4254/wjh.v13.i6.709>

INTRODUCTION

Hepatocellular carcinoma (HCC), the most common liver cancer, is considered to bring more than 25 hundred thousand deaths worldwide every year. Metastasis is one of the most major points influencing prognosis. HCC often involves metastasis in the liver, but metastasis out of the liver to the lung, bone, and adrenal glands is less frequent, whereas the brain is commonly not connected. The authors report a case of hyperalgesia of the right side of the face and limited abduction of the right eye caused by HCC metastasizing to the right cavernous sinus, the right Meckel's cave, and the right petrous bone diagnosed through neurological findings and radiological studies.

CASE PRESENTATION

Chief complaints

An 82-year-old woman was in November 2019 admitted to Kobe Asahi Hospital for the treatment of HCC with molecular targeted therapy such as Lenvatinib (LEN) (8 mg/d).

History of present illness

She had overcome hepatitis C virus infection (HCV) 10 years earlier with interferon treatment, but still retained Child A liver cirrhosis.

History of past illness

She has suffered from chronic obstructive pulmonary disease for 20 years.

Personal and family history

Nothing particular.

Physical examination

She had no hepatomegaly and no splenomegaly.

Laboratory examinations

Laboratory examinations at admission revealed the following: Total protein 7.3 g/dL (normal 6.5-8.3), albumin 3.6 g/dL (3.8-5.3), aspartate aminotransferase 92 IU/L (10-40), alanine aminotransferase 172 IU/L (5-40), gamma-glutamyl transpeptidase 90 IU/L (< 35), alkaline phosphatase 422 IU/L (115-359), T-bil 1.3 mg/dL (0.2-1.2), NH₃ 163 µg/dL (< 130), pertussis toxin 88.3% (70-130), white blood cell $67 \times 10^3/\mu\text{L}$ (36-90), Hb 13.6 g/dL (11.5-15.0), platelets $32.0 \times 10^4/\mu\text{L}$ (13.4-34.9), hepatitis B surface antigen (-), HCVAb (+), HCV RNA (-), tumor markers were as follows: Alpha-fetoprotein (AFP) 30332.7 ng/mL (< 10.0), PIVKA-II 1395 mAU/mL (< 40) (Table 1).

Imaging examinations

Imaging examination 1: At admission imaging studies including Gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid-enhanced magnetic resonance imaging (MRI) showed multiple HCC nodules in both right and left lobes (Figure 1A). Gastrointestinal fiberscope revealed atrophic gastritis.

Imaging examination 2: Brain MRI revealed high intensity in the bilateral globus pallidus on T2-weighted images (T2WI), ascribed to elevated serum ammonia (163 µg/dL), but no findings in the cavernous sinus or Meckel's cave (Figure 1B), and marrow in the petrous bone was intact (Figure 1C).

Imaging examination 3: Brain MRI revealed a low intensity mass around the right Meckel's cave on T2WI (Figure 1D) and loss of normal fatty bone marrow signal intensity in the right petrous bone on T1-weighted images (T1WI) (Figure 1E).

Imaging examination 4: MRI revealed a low intensity mass around the right cavernous node, the right Meckel's cave, and the right petrous bone on T2WI (Figure 1F). Based on MRI findings, the rapid increase in the size of the lesions over 1 mo and the onset of neurologic dysfunction, such as impairment of right trigeminal and abducens nerves, were most likely due to the metastasizing HCC.

Histopathological examinations

Ultrasound guided biopsy of the left lobe revealed moderately differentiated HCC (Figure 1G).

FINAL DIAGNOSIS

Contrast-enhanced MRI with gadolinium-chelated contrast medium revealed a well-defined mass with abnormal enhancement around the right cavernous sinus and the right Meckel's cave (Figure 1H).

TREATMENT

Molecular targeted therapy with LEN (8 mg/d for 94 d, *per os*) and Ramucirumab (340 mg/d and 320 mg/d, two times by intravenous injection) were administered for 4 mo, resulting in progression of the disease. Two months after the start of molecular targeted therapy, tumor markers were as follows: AFP 3830 ng/mL, PIVKA-II 3782 mAU/mL.

Three months after the start of molecular targeted therapy, tumor markers were as follows: AFP 25761 ng/mL, PIVKA-II 13045 mAU/mL. The patient demonstrated hyperalgesia of the right side of the face and limited abduction of the right eye.

Four months after the start of molecular targeted therapy, tumor markers were as follows: AFP 226112 ng/mL, PIVKA-II 268638 mAU/mL, carcinoma embryonic

Table 1 Laboratory data on admission

Parameters	Results	Parameters	Results
WBC	$67 \times 10^3/\mu\text{L}$	ALP	422 IU/L
Hb	13.6 g/dL	γ -GTP	90 IU/L
Platelets	$32.0 \times 10^4/\mu\text{L}$	NH3	163 $\mu\text{g/dL}$
PT	88.3%	HBsAg	(-)
TP	7.3 g/dL	HCVAb	(+)
ALB	3.6 g/dL	HCV RNA	(-)
T-bil	1.3 mg/dL	AFP	30332.7 ng/mL
AST	92 IU/L	PIVKA-II	1395 mAU/mL
ALT	172 IU/L		

WBC: White blood cell; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALB: Albumin; TP: Total protein; PT: Pertussis toxin; AFP: Alpha-fetoprotein; HCV: Hepatitis C virus; HBsAg: Hepatitis B surface antigen; ALP: Alkaline phosphatase; γ -GTP: Gamma-glutamyl transpeptidase.

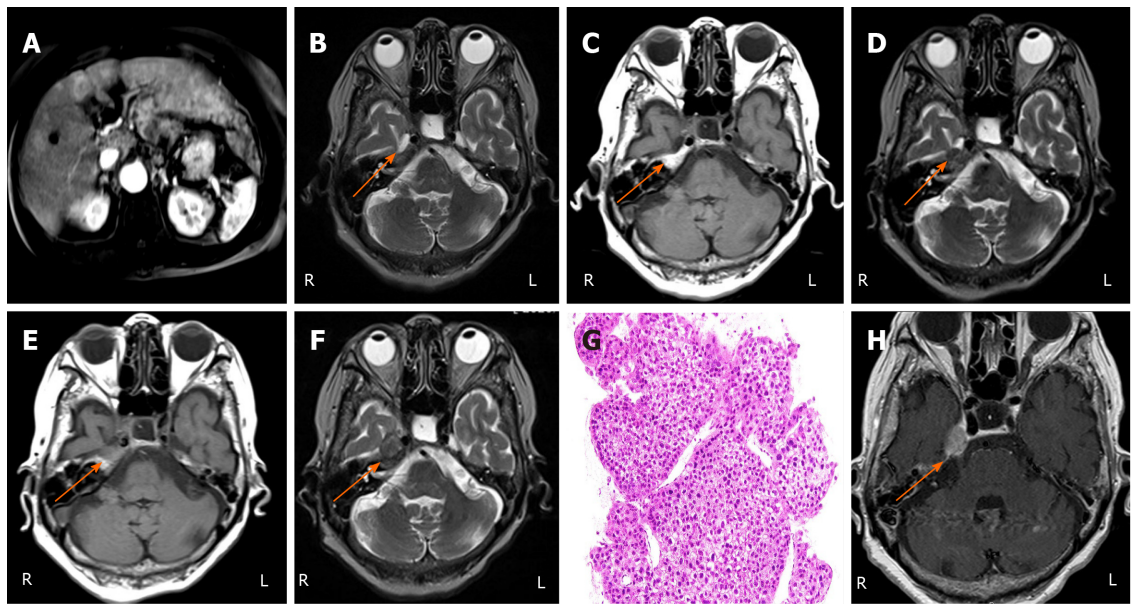


Figure 1 Imaging findings and histopathological findings. A: Ethoxybenzyl magnetic resonance imaging (MRI), hypervascular hepatocellular carcinoma (HCC) in the right and left lobes; B: Brain MRI [T2-weighted image (T2WI)], no findings in the cavernous sinus or Meckel's cave; C: Brain MRI [T1-weighted image (T1WI)], intact findings of bone marrow in the petrous bone; D: Brain MRI (T2WI), low intensity mass in the right Meckel's cave (arrow); E: Brain MRI (T1WI), loss of normal fatty bone marrow signal intensity in the right petrous bone (or apex); F: Brain MRI (T2WI), low intensity mass around the right cavernous node, the right Meckel's cave, and the right petrous bone on T2WI; G: Histopathological finding (hematoxylin and eosin staining), moderately differentiated HCC; H: Contrast enhanced MRI, well-defined mass with abnormal enhancement in the right cavernous sinus, and the right Meckel's cave (arrow). L: left; R: Right.

antigen 3.7 ng/mL (< 5.0), CA19-9 126.8 U/mL (< 37.0), interleukin-2R 824 U/mL (122-496).

Five months after the start of molecular targeted therapy, tumor markers were as follows: AFP 26795 mg/dL, PIVKA-II 258061 mAU/mL.

OUTCOME AND FOLLOW-UP

Based on the diagnosis, γ knife treatment was performed resulting in relief of the right side of the hyperalgesia. Fourteen days after γ knife treatment, the patient died due to the worsening of general condition.

DISCUSSION

Metastasis occurs as an advanced incident in the clinical course of liver cancer, and most patients expire because of hepatic insufficiency due to the cancer supplanting the liver. Distant metastases are routinely discovered at autopsy in over 50% of the cases [1-3]. On the contrary, the brain is an uncommon metastatic location. Accidental distant lesions at such more unusual locations are less a considered as possible metastases when metastatic HCC is not discovered at the more usual locations (the lungs, lymph nodes, and bone)[1-3].

The central nervous system is an uncommon location of metastatic HCC[4-8]. Before 1990, the diagnosis of HCC metastasizing to the craniospinal place was evidenced by histopathological findings of biopsy, operative and post-mortem tissues. Lately diagnosis is confirmed by neurologic tests and radiological findings, including computed tomography (CT) and MRI due to advances in such examinations[9-13]. In the 20th century, seven cases of HCC presenting as brain metastasis with no overt liver connection have been reported: Distant metastasis of liver cancer to the cerebrum in one case, and to the cranium in 6 cases[8]. Each showing slightly unusual hepatic examination early assessed, led to the diagnosis that in brain metastasis of obscure origin in a place where it is a usual illness, liver cancer should be viewed in differential diagnoses[8]. In Japan as in Taiwan, the place where liver cancer is a usual illness, HCC metastasizing to the cranium base relating to plural cranial nerves has not been described until now, but one case of cranium metastasis related to emergent epidural HCC[9].

After the 20th century, several cases of metastatic HCC to the cranial nerves have been reported: A 50-year-old female with HCV-associated recurrent multiple HCC metastasizing to the skull base involving multiple cranial nerves shows with conditions drop of eyelid, settlement of the right eyeball, and left abducens paralysis, suggesting disabilities of the right oculomotor and trochlear nerves, and both side abducens nerves. Contrast-enhanced CT of the brain shows an indistinct tumor with unusual increase surrounding the sella turcica. Brain MRI reveals that the tumor involves the clivus, the cavernous sinus, and the petrous apex. On contrast-enhanced MRI with gadolinium-chelated contrast medium, the tumor shows imbalanced middle increase. The diagnosis of metastatic liver cancer to the skull base is done based on of neurologic studies and radiological findings such as CT and MRI, but not on histopathological findings[13].

Two patients with HCC metastasizing to the skull base, the pituitary gland, the sphenoid sinus, and the cavernous sinus present with diplopia, retro-orbital headache, and multiple cranial nerve palsies. One is diagnosed with HCC prior to trans-sphenoidal operation of the pituitary metastasis. The second patient is, with histopathological examination, diagnosed to have HCC signs and symptoms associating with the primary tumor[14].

Two cases of HCC metastasizing to the cavernous cavity and the sphenoid cavity presenting with double vision and back eye socket headache, are performed operation for primary pituitary gland tumors. After operation, both cases are diagnosed as metastases from HCC[15].

A 73-year-old woman with HCV-related HCC shows a slightly limited abduction, more focused on the left eye with horizontal double vision. MRI of the face and paranasal cavity reveals a tumor in the left sphenoid cavity (22 mm × 16 mm × 16 mm) that invades the cavernous cavity and the forward slope of Meckel's cave[16,17]. HCC cases of metastasis to the brain from literature were summarized in Table 2 [Age: 56 (25-82), male: 16, female: 7]. Meckel's cave, a natural mouth-shaped aperture measuring 4 mm × 9mm wide at its opening and 15 mm in length within petrous apex's meningeal dura propria and periosteal layers, is the central part of the mid cranial fossa; it plays as a main route for the biggest cranial nerve (the fifth)[18,19]. The cavernous sinus is an important element of the cranial vascular organization, having immediate or indirect relations with the cerebrum, cerebellum, brainstem, face, eye, eye socket, nasopharynx, mastoid, and middle ear[20,21].

The neural components inside the cavernous sinus contain the sympathetic carotid plexus and 4 cranial nerves. The sites of these nerves, in superior to inferior turn, are the oculomotor (the third), trochlear (the fourth), abducens (the sixth), and ophthalmic divisions of the trigeminal (the fifth)[20].

Differential diagnosis of Meckel's cave lesions includes neoplastic and non-neoplastic ones.

Meckel's cave tumors account for only 0.5% of all intracranial tumors. Neoplastic lesions are trigeminal schwannoma (the most common with -33% of cases)[22], meningioma[22,23], pituitary macroadenoma, metastases: Including retrograde spread

Table 2 Hepatocellular carcinoma cases of metastasis to the brain from literature

No	Age	Sex	Presenting symptoms	Site of metastasis	Survival (from the onset of symptoms)	Ref.
1	25	M	Headache and left weakness	Right temporoparietal brain	1 d	Chang and Chen[5], 1979
2	50	M	Weakness of right leg, focal seizure of right leg	Calvarium of the skull, dura, brain	3 mo	Chang and Chen[5], 1979
3	51	F	Epistaxis, ptosis, diplopia, facial weakness in the left side	Skull base	6 mo	Chang and Chen[5], 1979
4	64	M	Loss of vision in the left eye, anorexia, weight loss	Lateral aspect of the temporal fossa and in the anterior portion of the middle cranial cavity	3 mo	Zubler <i>et al</i> [7], 1981
5	59	M	Left arm weakness and numbness, headache with left weakness, disturbed consciousness	Brain parenchyma (right frontotemporal parietal) with intracranial haemorrhage	2 mo	Lee[8], 1992
6	58	F	Progressive enlarging scalp mass over vertex for 4 mo	Calvarium, dura, brain parenchyma	10 mo	Lee[8], 1992
7	48	F	Progressive enlarging scalp mass over the left parietal and right frontal region for 6 mo	Calvarium	8 mo	Lee[8], 1992
8	36	M	Progressive enlarging scalp mass in right occipital region for 2 mo	Calvarium	3 mo	Lee[8], 1992
9	60	M	Diplopia and proptosis for 2 mo. Ophthalmoplegia for 1 mo	Skull base (retrobulbar)	7 mo	Lee[8], 1992
10	54	M	Progressive dysarthria and atrophy of left tongue for 2 mo	Skull base (jugular fossa hypoglossal canal)	4 mo	Lee[8], 1992
11	47	M	Right hemiparesis for 3 mo blurred vision with ptosis and limitation of eye movement (OD) numbness on the right forehead for one month	Skull base (parasellar)	6 mo	Lee[8], 1992
12	70	M	Left-sided weakness	Acute epidural hematoma adjacent to the right parietal bone	2 mo	Hayashi <i>et al</i> [9], 2000
13	58	F	Progressive weakness of her right leg, right hemianesthesia and weakness	Left parietal region, left high parietal area	6 mo	Lee and Lee [11], 1988
14	50	M	Hemiparesis and numbness of left upper arm, explosive headache and vomiting, disturbance of consciousness	Right frontotemporoparietal area	2 mo	Lee and Lee [11], 1988
15	65	M	Progressive painful right sided proptosis and ptosis, intermittent right temporal and facial pain, loss of sensation on the right side of the face	Right orbital apex	9 d	Phadke and Hughes[12], 1981
16	55	M	Mild right weakness	Left fronto-parietal cerebral hemisphere	11 d	Phadke and Hughes[12], 1981
17	50	F	Ptosis, diplopia, left abducens palsy	Clivus, cavernous sinus, petrous apex	Not described	Kim <i>et al</i> [13], 2006
18	40	M	Diplopia, retro-orbital headache, and occasional vomiting	Pituitary fossa, clivus, sphenoid sinus, and right petrous apex	3 mo	Aung <i>et al</i> [14], 2002
19	71	M	Headache, diplopia, ptosis of the right eye	Pituitary gland, optic chiasma, cavernous sinus	1 yr	Aung <i>et al</i> [14], 2002
20	67	M	Diplopia, left retro-orbital headache	Sphenoid sinus, pituitary gland, clivus	15 mo	Tamura <i>et al</i> [15], 2013
21	58	M	Headache, visual disturbance, general fatigue, diplopia, oculomotor nerve palsy	Pituitary fossa, cavernous sinus	3 wk	Tamura <i>et al</i> [15], 2013
22	73	F	Frontotemporal and left periorbital headache with associated photophobia	Left sphenoid sinus, cavernous sinus	Not described	Morais <i>et al</i> [16], 2018
23	82	F	Hyperalgesia of the right face and limited abduction of the right eye	Cavernous sinus, Meckel's cave, petrous bone	5.5 mo	Our case

of head and neck tumors[24-27], epidermoid cysts[28], lipoma, base of skull tumors. All these tumors should be differentiated from Meckel's cave tumors.

Non-neoplastic lesions include internal carotid artery aneurysms/vascular malformation[29,30], and petrous apex cephalocele.

In our case, benign neoplasms such as schwannoma, meningioma, pituitary macroadenoma, epidermoid cyst, lipoma, base of skull tumors, as well as internal carotid artery aneurysms, vascular malformation and petrous apex cephalocele were ruled out in differential diagnosis.

In our case, brain MRI (T1WI and T2WI) disclosed a mass involving the right cavernous sinus, the right Meckel's cave and the right petrous bone; MRI with contrast medium revealed abnormal enhancement around the right cavernous sinus, and the right Meckel's cave.

Moreover, no other malignancies, or lymphoma, have been observed clinically; metastasis from HCC is most likely, irrespective of the absence of histological findings.

CONCLUSION

Taken together with neurological and imaging findings, our case was diagnosed as metastatic HCC to the right cavernous sinus, the right Meckel's cave and the right petrous bone involving multiple cranial nerves including the right fifth, and sixth.

The diagnosis of HCC metastasizing to this area is difficult to confirm by histopathological examination because of the deep-seated location and the neurovascular structures; nevertheless, histopathological diagnosis of HCC metastases to the pituitary gland bone has been reported[13,14].

In a previous study, the reason for HCC metastasis to the skull base was explained by the long survival of 15 years with various treatment regimens of chemotherapy and chemoembolization[13]. In our case, HCC metastasis may be due to the biological behavior of HCC such as being moderately differentiated and the failure of molecular targeted therapy, resulting in disease progression.

To our knowledge, our case is the second case of HCC metastasizing to the cavernous sinus, and Meckel's cave.

Although HCC metastasizing to the cavernous sinus, Meckel's cave and the petrous bone complicating multiple cranial nerves is very exceptional, medical professionals should be careful and good at managing radiological examinations including CT and MRI, when the patients show neurologic dysfunction, especially cranial nerve connection.

ACKNOWLEDGEMENTS

The authors thank Ms. Mika Matsui for excellent technical assistance.

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