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World Journal of Hepatology (*World J Hepatol*, *WJH*, online ISSN 1948-5182, DOI: 10.4254), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJH covers topics concerning liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, biliary tract disease, autoimmune disease, cholestatic and biliary disease, transplantation, genetics, epidemiology, microbiology, molecular and cell biology, nutrition, geriatric and pediatric hepatology, diagnosis and screening, endoscopy, imaging, and advanced technology. Priority publication will be given to articles concerning diagnosis and treatment of hepatology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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Xiu-Xia Song, Director
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Baishideng Publishing Group Inc
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Telephone: +1-925-2238242
Fax: +1-925-2238243
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EDITORIAL

Is laparoscopic hepatectomy superior to open hepatectomy for hepatocellular carcinoma?

Jian-Hong Zhong, Ning-Fu Peng, Jian-Hong Gu, Ming-Hua Zheng, Le-Qun Li

Jian-Hong Zhong, Ning-Fu Peng, Le-Qun Li, Department of Hepatobiliary Surgery, Affiliated Tumor Hospital of Guangxi Medical University, Nanning 530021, Guangxi Zhuang Autonomous Region, China

Jian-Hong Gu, Yangzhou University and Jiangsu Co-Innovation Center, Yangzhou 225009, Jiangsu Province, China

Ming-Hua Zheng, Department of Hepatology, Liver Research Center, the First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325000, Zhejiang Province, China

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Correspondence to: Le-Qun Li, MD, PhD, Department of Hepatobiliary Surgery, Affiliated Tumor Hospital of Guangxi Medical University, #71, He Di Rd, Nanning 530021, Guangxi Zhuang Autonomous Region, China. zhongjianhong66@163.com
Telephone: +86-771-5330855
Fax: +86-771-5312000

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Abstract

The low perioperative morbidity and shorter hospital stay associated with laparoscopic hepatectomy have made it an often-used option at many liver centers, despite the fact that many patients with hepatocellular carcinoma have cirrhosis, which makes the procedure more difficult and dangerous. Type of surgical procedure proves not to be a primary risk factor for poor outcomes after hepatic resection for hepatocellular carcinoma, the available evidence clearly shows that laparoscopic hepatectomy is an effective alternative to the open procedure for patients with early-stage hepatocellular carcinoma, even in the presence of cirrhosis. Whether the same is true for patients with intermediate or advanced disease is less clear, since laparoscopic major hepatectomy remains a technically demanding procedure.

Key words: Hepatocellular carcinoma; Laparoscopic hepatectomy; Open hepatectomy

Core tip: Type of surgical procedure proves not to be a primary risk factor for poor outcomes after hepatic resection for hepatocellular carcinoma, the available evidence clearly shows that laparoscopic hepatectomy is an effective alternative to the open procedure for patients with early-stage hepatocellular carcinoma, even in the presence of cirrhosis.

Zhong JH, Peng NF, Gu JH, Zheng MH, Li LQ. Is laparoscopic hepatectomy superior to open hepatectomy for hepatocellular carcinoma? *World J Hepatol* 2017; 9(4): 167-170 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i4/167.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i4.167

Table 1 Propensity score studies comparing open and laparoscopic liver resection for hepatocellular carcinoma

Ref.	Country	Included period	Open/laparoscopic					*P* value	
			Sample size, *n*	Minor hepatectomy, %	Single tumor, %	Perioperative morbidity, %, *P* value	Perioperative mortality, %, *P* value	Overall survival	Disease free survival
Ahn *et al*[12]	South Korea	2005-2013	51/51	94/96	100/100	9.8/5.9, 0.470	0/0, 1.000	0.173	0.519
Cheung *et al*[1]	China	2002-2015	330/110	88/90	89/91	4.8/1.8, 0.266[1]	1.8/0, 0.342	0.033	0.141
Han *et al*[13]	South Korea	2004-2013	88/88	68/65	80/76	20.4/12.5, 0.042	1.1/1.1, 1.000	0.944	0.944
Han *et al*[14]	South Korea	2002-2012	198/99	85/84	87/93	24.7/13.1, 0.020	-	0.086	0.701
Kim *et al*[15]	South Korea	2000-2012	29/29	100/100	83/97	13.8/37.9, 0.018	-	0.267	0.929
Meguro *et al*[17]	Japan	2003-2011	35/35	-	83/80	25.7/25.7, 1.000	-	0.672	0.954
Sposito *et al*[20]	Italy	2006-2013	43/43	100/100	81/86	48.8/18.6, 0.004	0/0, 1.000	0.802	0.990
Takahara *et al*[18]	Japan	2000-2010	387/387	79/77	-	13.0/6.7, 0.003	1.0/0.3, 0.178	0.358	0.422
Tanaka *et al*[19]	Japan	2007-2014	20/20	-	85/90	45.0/0, 0.001	0/0, 1.000	0.606	0.533
Yoon *et al*[16]	South Korea	2007-2011	174/58	88/93	100/100	22.4/6.9, 0.020	-	0.480	0.31

[1]With complication of Clavien-Dindo grade ⅢA or above.

Recently, a large propensity score study comparing laparoscopic and open hepatectomy for treating hepatocellular carcinoma (HCC) was published in *Ann Surg*[1]. This parallel comparison comes at an important time, because technical and procedural improvements have led to increasing use of laparoscopic hepatectomy, including for more extensive hepatectomy and particularly in cases of left lateral sectionectomy[2]. In fact, the low perioperative morbidity and shorter hospital stay associated with laparoscopic hepatectomy have made it an often-used option at many liver centers[3-8], despite the fact that many patients with HCC have cirrhosis, which makes the procedure more difficult and dangerous. The long-term benefits of laparoscopic hepatectomy remain controversial, and this study[1] provides the first evidence that it is associated with better long-term overall survival (OS) than open hepatectomy ($P = 0.033$).

Our own clinical experience and evidence in the literature suggest that mortality risk following liver resection depends primarily not on the type of surgical procedure but on tumor-related factors[9-11]. In order to examine this possibility in more detail, we reviewed all randomized controlled trials and other studies involving propensity score analysis comparing laparoscopic and open hepatectomy published in 2014-2016. We identified 10 studies involving 2275 patients, comprising one from China[1], five from South Korea[12-16], three from Japan[17-19], and one from Italy[20] (Table 1). Across these 10 studies, 90% of patients had single tumors and 84% underwent minor hepatectomy. This means that most patients had early-stage HCC and surgical procedures were relatively straight forward. In 7 of 10 studies (accounting for 73% of all patients), laparoscopic hepatectomy was associated with a significantly lower rate of perioperative morbidity. None of the studies found significant differences in perioperative mortality or disease-free survival (DFS) between the laparoscopic and open procedures. Eight of the 10 studies (accounting for 86% of all patients) reported 5-year OS and DFS[1,12-15,17-19]. Meta-analyses based on these eight studies revealed that patients in the laparoscopic group had significantly higher 5-year OS than those in the open group [risk ratio (RR) = 0.91, 95% confidence interval (95%CI): 0.86-0.95, $P < 0.001$; $I^2 = 39\%$; Figure 1A], but similar 5-year DFS (RR = 0.96, 95%CI: 0.87-1.06, $P = 0.440$; $I^2 = 0\%$; Figure 1B). Similar results were obtained when the study by Cheung *et al*[1] was excluded.

Thus, substantial evidence suggests that laparoscopic hepatectomy is associated with significantly better long-term OS than open hepatectomy. It is possible that this reflects less tissue manipulation - and therefore less hematogenous dissemination of malignant tumor cells- in "no-touch" anterior-approach laparoscopic hepatectomy[1]. However, the two techniques were associated with similar DFS, indicating similar rates of tumor recurrence, which is the main cause of death among HCC patients. In fact, patients in the two groups across all 10 studies showed similar tumor characteristics, including diameter, number, vascular invasion, and New Edmondson grade. Since these characteristics are the main risk factors of tumor recurrence, the available evidence appears to be consistent with the idea that mortality risk following liver resection depends on tumor-related factors and not on type of surgical procedure.

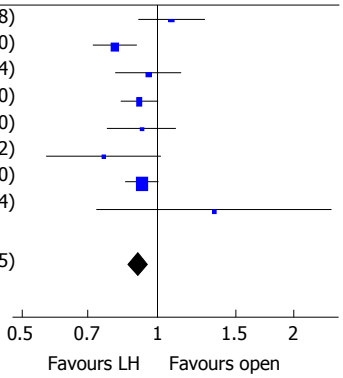
To examine this hypothesis rigorously, at least two questions must be answered. One is whether differences in blood loss and surgical complexity may help explain the difference in OS. Six of the 10 studies[1,13,16-19] reported significantly less blood loss in the laparoscopic group, yet the studies did not report whether tumors were close to the hepatic vein or portal hepatis, which would make the surgery more complex and increase risk of blood loss. Another question is whether economic differences may help explain the OS difference. Since laparoscopic hepatectomy costs substantially more than open hepatectomy, it stands to reason that patients opting for the laparoscopic procedure may be in a better financial position. This raises the possibility that such patients also receive better postoperative therapies, such as antiviral therapy, liver-protecting therapy, and/or psychological intervention. Such patients may also receive more extensive and/or more aggressive therapy after tumor recurrence. All these factors may explain the observed long-term OS advantage of laparoscopic hepatectomy

A

Study of subgroup	Open Events	Open Total	LH Events	LH Total	Weight (%)	Risk ratio M-H, fixed, 95%CI
Ahn	44	51	41	51	5.6	1.07 (0.90, 1.28)
Cheung	222	330	92	110	19.0	0.80 (0.72, 0.90)
Han	64	88	67	88	9.2	0.96 (0.80, 1.14)
Han-a	162	198	89	99	16.3	0.91 (0.83, 1.00)
Kim	25	29	27	29	3.7	0.93 (0.78, 1.10)
Meguro	22	35	29	35	4.0	0.76 (0.56, 1.02)
Takahara	274	387	297	387	40.9	0.92 (0.85, 1.00)
Tanaka	12	20	9	20	1.2	1.33 (0.73, 2.44)
Total (95%CI)		1138		819	100.0	0.91 (0.86, 0.95)
Total events	825		651			

Heterogeneity: χ^2 = 11.55, *df* = 7 (*P* = 0.12); I^2 = 39%

Test for overall effect: *Z* = 3.80 (*P* = 0.0001)



B

Study of subgroup	Open Events	Open Total	LH Events	LH Total	Weight (%)	Risk ratio M-H, fixed, 95%CI
Ahn	28	51	35	51	8.6	0.80 (0.59, 1.09)
Cheung	158	330	57	110	21.0	0.92 (0.75, 1.14)
Han	36	88	39	88	9.6	0.92 (0.65, 1.30)
Han-a	100	198	40	99	13.1	1.25 (0.95, 1.65)
Kim	12	29	16	29	3.9	0.75 (0.44, 1.29)
Meguro	13	35	15	35	3.7	0.87 (0.49, 1.54)
Takahara	152	387	157	387	38.6	0.97 (0.81, 1.15)
Tanaka	4	20	6	20	1.5	0.67 (0.22, 2.01)
Total (95%CI)		1138		819	100.0	0.96 (0.87, 1.06)
Total events	503		365			

Heterogeneity: χ^2 = 6.36, *df* = 7 (*P* = 0.50); I^2 = 0%

Test for overall effect: *Z* = 0.77 (*P* = 0.44)

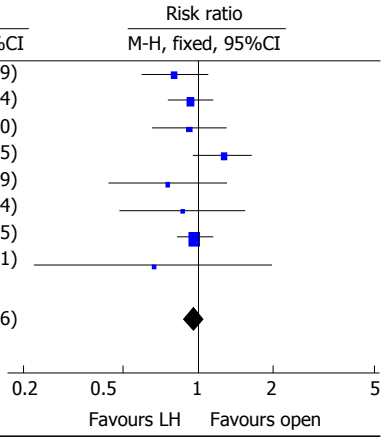


Figure 1 Forest plots of meta-analysis comparing the efficacy of laparoscopic with open hepatectomy. A: Rate of 5-year overall survival; B: Rate of 5-year disease-free survival. LH: Laparoscopic hepatectomy.

over open hepatectomy. Therefore, assessing the long-term impact of this procedure requires large randomized controlled trials that take surgical complexity and patient financial condition into account. At least, comparative studies with propensity score analysis should adjust surgical complexity and financial condition between groups.

Even if, as we suspect, type of surgical procedure proves not to be a primary risk factor for poor outcomes after resection, the available evidence clearly shows that laparoscopic hepatectomy is an effective alternative to the open procedure for patients with early-stage HCC, even in the presence of cirrhosis. Whether the same is true for patients with intermediate or advanced disease is less clear, since laparoscopic major hepatectomy remains a technically demanding procedure. Even so, we agree that laparoscopic hepatectomy is an alternative choice for treatment of HCC.

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MINIREVIEWS

Hepatic Kaposi sarcoma: A case report and review of the literature

Brett Van Leer-Greenberg, Abhisake Kole, Saurabh Chawla

Brett Van Leer-Greenberg, Abhisake Kole, Saurabh Chawla, Department of Medicine, Emory University School of Medicine, Atlanta, GA 30322, United States

Saurabh Chawla, Division of Digestive Diseases, Department of Medicine, Emory University School of Medicine, Atlanta, GA 30322, United States

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Correspondence to: Saurabh Chawla, MD, Assistant Professor of Medicine, Director of Endoscopy, Division of Digestive Diseases, Department of Medicine, Emory University School of Medicine, Faculty Office Building, 49 Jesse Hill Jr. Drive, Suite 431, Atlanta, GA 30322, United States. saurabh.chawla@emory.edu
Telephone: +1-140-47781684
Fax: +1-140-47781681

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Abstract

Kaposi sarcoma (KS) is an aggressive cancer caused by human herpesvirus-8, primarily seen in immunocompromised patients. As opposed to the well-described cutaneous manifestations and pulmonary complications of KS, hepatic KS is rarely reported before death as most patients with hepatic KS do not manifest symptoms or evidence of liver injury. In patients with acquired immune deficiency syndrome, hepatic involvement of KS is present in 12%-24% of the population on incidental imaging and in approximately 35% of patients with cutaneous KS if an autopsy was completed after their death. Patients with clinically significant hepatic injury due to hepatic KS usually have an aggressive course of disease with hepatic failure often progressing to multi-organ failure and death. Here we report an unusual presentation of acute liver injury due to hepatic KS and briefly review the published literature on hepatic KS.

Key words: Herpesvirus 8; Acquired immune deficiency syndrome-related Kaposi sarcoma; Acquired immune deficiency syndrome hepatopathy; Human; Kaposi sarcoma

Core tip: Hepatic Kaposi sarcoma (KS) is a clinical presentation that disproportionately affects the human immunodeficiency virus/acquired immune deficiency syndrome (AIDS) population. Up to 34% of patients with AIDS and KS have hepatic involvement. Usually hepatic KS is clinically indolent and diagnosed during autopsy. When clinically significant, hepatic KS presents with evidence of liver injury with elevation in bilirubin and liver enzymes, has characteristic findings on imaging and may progress to liver failure and death. Treatment is indicated in patients with progressive and symptomatic hepatic disease in the absence of other etiologies.

Van Leer-Greenberg B, Kole A, Chawla S. Hepatic Kaposi sarcoma: A case report and review of the literature. *World J Hepatol* 2017; 9(4): 171-179 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i4/171.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i4.171

CASE PRESENTATION

A forty-eight years old African American male with HIV and CD4 count of 8/μL presented with conjunctival icterus. Physical exam showed cachexia, icterus, a violaceous 1 cm plaque on the soft palate and similar lesion on the chest wall, and a soft, non-distended, non-tender abdomen. He denied prior treatment with antiretroviral medications. Laboratory studies were significant for AST (SPGT) 172 U/L, ALT (SGOT) 201 U/L, total bilirubin of 20.0 mg/dL, direct bilirubin 14.9 mg/dL, alkaline phosphatase 947 U/L, INR 2.5, and platelet count 52000/μL. Acute and chronic serologies for hepatitis A, B and C, histoplasma, and cytomegalovirus as well as a toxicology screen were negative. Patient did report taking cotrimoxazole for five days which he completed a month prior to presentation. Magnetic resonance imaging (MRI) of the Abdomen showed innumerable 10 mm T2 intense hepatic nodules without enhancement (Figure 1). Liver biopsy was positive for Cytokeratin 7 and human herpes virus-8, consistent with infiltrative Kaposi sarcoma (KS) (Figures 2 and 3). There was no evidence of drug induced liver injury on histopathology. There was no lymphadenopathy indicative of hemophagocytic lymphohistiocytosis or Castleman's disease. The diagnosis was most consistent with acute liver injury (ALI) secondary to infiltrative hepatic KS, stage T1, I1, S1. The patient was not a candidate for cytotoxic therapy given progressive liver injury and was started on rituximab and ganciclovir. Liver injury progressed and was further complicated by acute kidney injury, hypoxic respiratory failure, consumptive coagulopathy and septic shock. The patient received broad-spectrum antibiotics, blood products, vasopressors and ventilator support but unfortunately expired.

BACKGROUND

KS is an angioproliferative low-grade neoplasm that is associated with human herpesvirus-8 (HHV-8). KS can be codified into different clinical variants depending on the patient cohort and the presentation of the disease[1]. The "classical" form primarily affects men of Ashkenazic Jewish or Mediterranean background and follows an indolent cutaneous course. The "African endemic" form of the disease commonly affects Africans as the name implies, presents with lymphadenopathy and is usually fatal within 1-3 years. The "iatrogenic" form is due to HHV-8 activation caused by medical immunosuppression from treatment of autoimmune disorders or post-organ transplantation. The fourth and most common variant, acquired immune deficiency syndrome (AIDS)-related KS, is rapidly progressive and holds the highest rate of hepatic involvement[2].

The most common presentation of KS is a cutaneous papular disease with lesions on the legs, oral cavity, and genitalia. However, the most common site of visceral organ involvement is the gastrointestinal tract[3]. First described by Moritz Kaposi in 1872, hepatic KS was an autopsy diagnosis that rarely resulted in clinically significant disease or ALI[4]. To further understand hepatic KS, a systematic search of the literature was conducted on PubMed (1954 to 2015), EBSCO HOST (1956 to 2015), the Cochrane Database of Systematic Review, and the OVID interface (1946 to 2015) with comprehensive search terms as documented in Table 1.

EPIDEMIOLOGY

While most herpesviruses are widespread in the adult population, the prevalence of HHV-8 varies with human immunodeficiency virus (HIV) status and exposure risk factors. In the United States, only 5% of HIV uninfected men are seropositive for HHV-8 compared to 25%-60% of HIV-positive men who have sex with men (MSM)[5]. These rates are reflective of HHV-8 and HIV co-infection in MSM in other countries as well[6].

AIDS-patients have 20000 times greater risk of developing KS than the general population. Patients on HAART with a CD4 count of < 200 cells/μL are 18.9 times more likely to have KS than those with CD4 ⩾ 500 cells/μL[7]. In the era of ARV therapy, improved control of HIV viremia and preserved CD4 T-cell function has lead to an 80% decreased incidence of AIDS-associated KS[8], AIDS-related KS currently affects < 1% of AIDS patients, compared to 15% in the pre-HAART era[9].

MODES OF TRANSMISSION

Behavioral risk factors for HHV-8 transmission are incompletely understood. Saliva exchange appears to an important factor with HHV-8 DNA detected in the saliva of 61% of HHV-8-infected MSM[10,11]. With HHV-8 seropositivity higher in the MSM population, commercial sex workers and those with other sexually transmitted infections, a sexual route of transmission has also been proposed. HHV-8 DNA can be isolated from semen and vaginal secretions, but viral load is lower than that found in saliva, calling into question the clinical significance of sexual transmission[12]. HHV-8 seroprevalence in HIV-infected injection drug users is substantially lower than hepatitis B and C rates in HIV-infected MSM[13]. This finding is suggestive that blood exchange through contaminated needle sharing is a less significant route of HHV-8 transmission compared to salivary or sexual contact[14].

HEPATIC INVOLVEMENT

Hepatic KS is typically asymptomatic and rarely diagnosed in life. Therefore the true incidence of hepatic KS is not

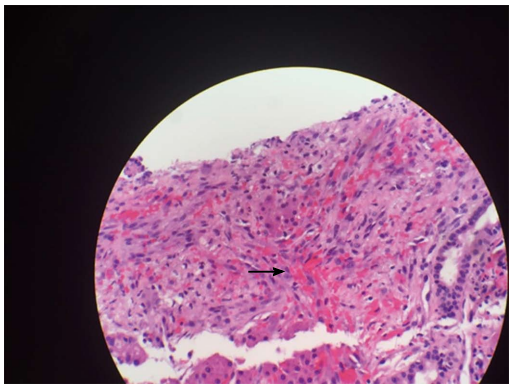

Figure 1 Spindle cells with cytokeratin 7 staining positive.

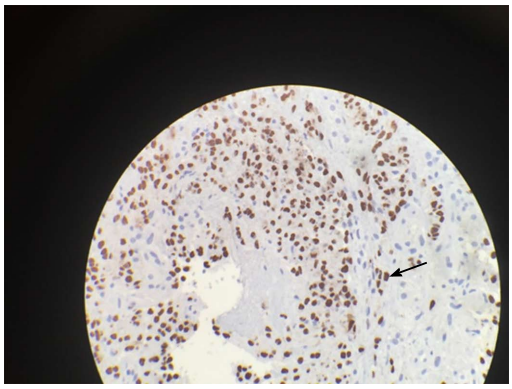

Figure 2 Positive human herpersvirus-8 immunohistochemical staining.

well-documented and is limited to small case series and reports. Prevalence of hepatic KS has primarily been determined from autopsy series with small sample sizes, which accounts for the wide variation in prevalence reported. In one autopsy series, approximately 34% of AIDS-related KS cases involved the liver while in another report, 8.3% had liver involvement[15,16]. In another retrospective review, hepatic involvement was present in 9 of 41 patients or 22% of cases of AIDS-related KS in post mortem dissection[17]. In this study ante-mortem hepatic KS was suspected in only one patient, in whom a computerized tomography (CT) scan demonstrated hepatosplenomegaly with a confluence of hypodense lesions in the left hepatic lobe. Autopsy confirmed disseminated KS. Schneiderman *et al*[18] found KS on liver biopsy in 18.6% of AIDS patients making KS the most common hepatic pathological diagnosis caused by AIDS. All of these patients already had a diagnosis of extrahepatic KS at time of biopsy. In contrast, 66% of patients with extrahepatic KS did not manifest hepatic involvement.

As mentioned above, most cases of hepatic KS were not clinically significant. In the study by Schneiderman *et al*[18], there were no statistically significant differences in transaminases, lactic dehydrogenase, alkaline phosphatase or bilirubin based on liver involvement with KS. However, in the few reported patients with clinically significant disease, a rapid progression to liver and multi-organ failure has been reported, usually with fatal outcomes (Table 2).

Table 1 MeSH search terms

Liver
Hepatopathy
Hepatitis
Hepatology
Cholestatic injury
Hepatocellular injury
Kaposi sarcoma
Herpesvirus 8, human
AIDS-related Kaposi sarcoma
Non-AIDS-related Kaposi sarcoma
Liver neoplasms

AIDS: Acquired immune deficiency syndrome.

In the non-HIV population, the incidence of KS is 0.2% in liver transplant patients from the United States, but the prevalence is higher in patients from Africa, the Middle East or the Mediterranean[19]. KS affected 4.7% of renal transplant patients in Saudi Arabia, 2.4% of recipients in Israel and 0.52% of recipients in France. While there is a well described clinical burden of post-transplant lymphoproliferative disorder including cutaneous and visceral manifestations of KS, there is no described literature of post-transplant hepatic KS.

PATHOGENESIS OF HHV-8 ASSOCIATED TUMORS

HHV-8 consists of a large double stranded DNA genome that includes approximately 145 kilobases (kbases) long region encodinging all the expressed viral genes, flanked by approximately 20-30 kbases that encode a number of mimicked human genes, several of which have immunologic or angiogenic properties[20,21]. HHV-8 has a tropism for both hematopoietic and non-hematopoietic cells including monocytes, B cells, endothelial cells and also hepatocytes[22]. Endothelial cells appear to be the most important host cells for oncogenic transformation as HHV-8 infection of these cells leads to their long-term proliferation and survival[23]. Similar to other herpesviruses, HHV-8 alternates between two metabolic cycles: Latent infection, where few genes are expressed, and the active lytic infection, where viral replication and multiple gene expression occurs. Lytic replication can be induced by oxidative stress, hypoxia, inflammatory cytokines, chemical exposure or concomitant infections, including HIV[24-27].

In hepatocytes infected with HHV8 genome by DNA polymerase chain reaction amplification, immunohistochemistry demonstrates expression of the transcriptional regulator, latency-associated nuclear antigen-1 (LANA-1)[28]. It has also been directly implicated in oncogenesis because of its ability to bind to the tumour-suppressing protein p53[29]. Furthermore, hepatocyte growth factor/scatter factor, a kinase that mediates epithelial cell proliferation and angiogenesis[30] has been demonstrated to induce HHV-8 lytic replication, providing a means of KS pro-

Table 2 Outcomes in patients with clinically symptomatic hepatic Kaposi sarcoma

Age (yr)	Sex	HIV status	CD4 count (cells/mm^3)	Liver chemistry profile	Pathology	Treatment	Hospital course + complications
45[63]	M	(+)	192	T Bil 19.35 ALP 1309 AST 204 ALT 188 GGT 827	HHV-8 PCR VL (+) 24000 copies/mL. Liver biopsy revealed features of KS with spindle cells, extravasation of red blood cells and haemosiderin deposition. IHC staining HHV8 (+)	Paclitaxel, Montelukast	Continued on chemotherapy. Subsequently developed respiratory and renal failure, anemia and thrombocytopenia from aggressive metastatic KS
36[64]	M	(+)	17	PTT 70 (s) ALT 185 T Bil 23	Necroscopy showed bile duct proliferation with diffuse fibrosis with lymphohistiocytic infiltration	Liposomal doxorubicin	Jaundice, renal failure, fulminant liver failure
28[65]	M	(+)	NR	NR	Biopsy residues of spindle cells lining portal tracts. Immunoperoxidase staining factor Ⅷ (+)	Palliative care	Liver function continued to decline and patient died from respiratory failure two weeks later
38[66]	M	(+)	< 200[1]	AST 147 ALT 180 ALP 573	Gross specimen with fibrous thickening of portal tracts and dark red nodules in periportal areas and diffusely infiltrating liver parenchyma	Chemotherapy, NOS	Partial cutaneous response, died several weeks later
40[4]	M	(+)	NR	Reportedly, "normal"	KS present on biopsy of lymph nodes. US with three 7-12 mm hyperechoic nodules. Periportal groups of dilated blood filled cavernous spaces lined by flat endothelial cells and interspersed of spindle cells. Extravasated erythrocytes and minimal hemosiderin deposits	Combination Chemotherapy, NOS	Complete remission of cutaneous lesions and reduction in size of two of the lesions with the third not visible. Readmitted six months later for severe relapse of cutaneous KS. Reinitiated chemotherapy with rapid deterioration and death within one month
48[67]	M	(+)	8	TBili 20.0 ALP 947 AST 186 ALT 155 INR 1.9	Liver biopsy was Cytokeratin-7 and HHV-8 staining positive	Ganciclovir and Rituximab	Presented with jaundice and acute liver injury with a cholestatic pattern, progressed to fulminant hepatic failure and ultimately death
44[68]	M	(+)	CD4/CD8 ratio 0.08	AST 153 ALT 124 ALP 1228	Laproscopy demonstrated enlarged liver with multiple purple 2-3 mm nodules Biopsy demonstrated spindle cells, vascular slits, extravasated red cells and lymphocytic infiltration	Platinum based chemotherapy, NOS	Primary hepatic manifestations without cutaneous lesions. Persistent abdominal pain after treatment. Progressed to cutaneous lesions six weeks after treatment. Lost to follow-up

[1]Less than 200, not otherwise reported. M: Male; F: Female; TBili: Total Bilirubin (units, mg/dL); ALP: Alkaline phosphatase (units, IU/L); AST: Aspartate transaminase (IU/L); ALT: Alanine transaminase (IU/L); GGT: Gamma glutamyl transpeptidase (units, IU/L); HHV-8: Human herpes virus-8; PCR: Polymerase chain reaction; VL: Viral load; IHC: Immunohistochemistry; NR: Not reported; NOS: Not otherwise specified; US: Ultrasound; KS: Kaposi sarcoma.

gression in the liver[31].

DIAGNOSIS OF KS

A full integumentary survey including oral and rectal examination quantifying extent of disease should be completed in patients with suspected KS. Cutaneous or visceral biopsy is required for diagnosis. On gross pathology, there are usually multiple, grossly irregular, variable sized red-brown spongiform nodules seen in the periportal connective tissue[32]. Histopathologic features of disease include thin walled vascular formations and inflammatory infiltration. Spindle cell formation is also characteristic of angioproliferative HHV-8-infected cells that have undergone reprogramming of vascular endothelium and tumorigenesis[33]. Immunohistochemical staining is characteristic for HHV-8 LANA expression within the spindle cell formation[34]. Immunohistochemistry staining for endothelial cell markers factor Ⅷ, CD31, CD34 and lymphatic vessel endothelial receptor 1 further corroborate a diagnosis of KS[35].

Further investigation of visceral KS is warranted in the presence of adenopathy or occult bleeding. Patients with cutaneous KS and iron deficiency anemia, fecal occult blood or gastrointestinal symptoms warrant GI endoscopic evaluation. Patients with cutaneous KS and concurrent adenopathy should receive CT of the chest, abdomen, and pelvis to evaluate for visceral KS and HHV-8-related lymphoproliferative disorders including primary effusion lymphoma, Castleman disease, and plasmablastic lymphoma[36].

KS STAGING AND PROGNOSIS

As KS is a disseminated angioproliferative virally mediated malignancy, classic tumor, node, metastasis staging as used in other cancers does not accurately prognosticate disease or dictate treatment. AIDS Clinical Trials Group (ACTG) Oncology Committee has codified staging of AIDS-associated KS[37]. The ACTG staging system risk stratifies patients low risk (0) or high risk (1) based on three criteria: Tumor burden (T), immune status (I), and systemic illness (S). For tumor burden, poor risk (T1) is defined by presence of extensive cutaneous, oral disease or visceral disease. For immune status, poor risk (I1) is defined by CD4 count of less than 150 cells/μL. For

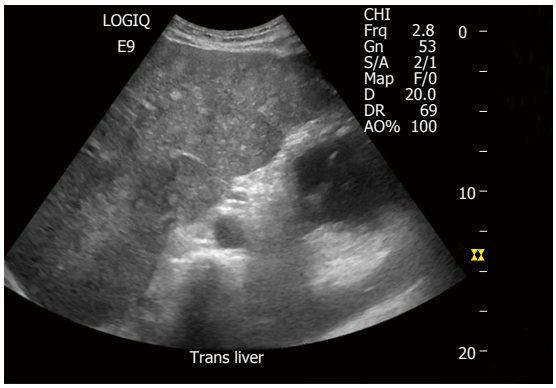

Figure 3 Ultrasound image with multiple small round hyperechoic nodules.

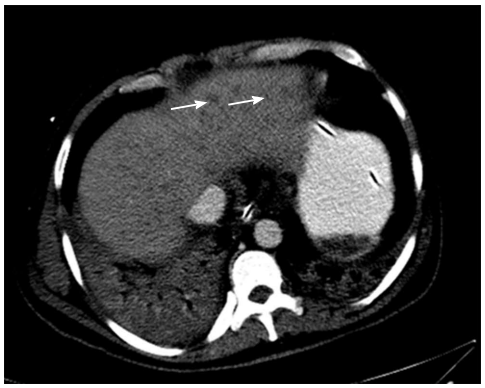

Figure 4 Computerized tomography scan enlarged inhomogeneous liver with multiple hypodense lesions.

systemic illness, poor risk (S1) is defined by the presence of constitutional symptoms, poor performance status, or other opportunistic infections. While these criteria were validated in the pre-ART era, post-ART therapy, a CD4 cutoff of 100 cells/mm^3 has an unclear role in predicting mortality[38,39].

IMAGING IN HEPATIC KS

Hepatic KS has characteristic findings on individual imaging modalities that can help delineate clinically significant disease. Abdominal ultrasound imaging of the liver can demonstrate inhomogeneous cystic lesions with hyperechoic bands and nodules along the peripheral branches of portal veins. Likewise, computed tomography of the abdomen is characteristic for inhomogeneous hepatomegaly with multiple small hypodense nodules, often in the periportal area[40] (Figure 4). Mild hepatomegaly is a non-specific finding in 19% of patients with AIDS-related KS[41]. MRI shows hyperintense nodules on T1-weighted in-phase imaging and hypointense nodules on T1- weighted out-of-phase imaging (Figure 5). Neither T2-weighted imaging nor late hepatobiliary volumetric interpolated breath hold examination have any specific findings in hepatic KS[42].

Image guided biopsy of hepatic nodules in patients suspected to have liver involvement demonstrate hyaline globules, hemosiderin accumulation, macrovaculoar steatosis, large fibrotic portal spaces, bile duct ectasia, neoductogenesis and spindle cells with large, irregular nuclei. Staining of the perinodular tissues is positive for CD31, CD34 and factor Ⅷ as can be seen in extrahepatic KS as well.

TREATMENT

As shown in Table 2, hepatic KS is predominantly manifested in patients with HIV/AIDS. While overall HIV mortality is improving in the era of ARV therapy, patients with AIDS-associated KS have an increased risk of death, compared to HIV controls, irrespective of CD4 count[43]. HIV-infected patients initiating ARV commonly have progression of their KS lesions[44]. However, long term ARV therapy is associated with a reduced incidence of KS. Guidelines currently recommend correcting underlying immunodeficiency by treating AIDS with ARV therapy. Studies indicate that control of KS progression is related to the degree of control of HIV, rather than the specific cART regimen utilized[45]. Beyond ARVs, a variety of systemic therapies may be used in KS. Usually systemic therapy is indicated in progressive disease, with symptomatic visceral involvement, in the presence of immune reconstitution inflammatory syndrome (IRIS) or with extensive cutaneous involvement. These strategies are not specific to hepatic dysfunction in the setting of KS.

Radiotherapy is a well-established treatment and has a robust clinical response for classic nodular KS but tends to be a palliative approach. While it may be a good modality for superficial lesions, electron beam radiation therapy (EBRT) has limited penetration below the dermis; deeper or unresponsive KS may be treated with standard non-EBRT approaches[46].

Retinoid products appear to inhibit IL-6, a cytokine implicated in KS pathogenesis, and have an antiproliferative effect on KS lesions[47]. Application of alitretinoin can reduce cutaneous lesions of both classic and HIV-KS but has no role in systemic disease[48].

The role of chemotherapy in addition to standard antiretroviral therapy has been explored. A meta-analysis of studies demonstrated that although chemotherapy in addition to ARVs did not have a mortality benefit, it did reduce disease progression[49]. Current first-line therapy for advanced AIDS-KS is liposomal anthracyclines, including pegylated liposomal doxorubicin (PLD). In a randomized control trial (RCT), PLD demonstrated superiority to previous conventional chemotherapy, bleomycin and vincristine with 58.7% *vs* 23.3% ($P < 0.001$) response rate and a decreased adverse event rate (10.7% *vs* 26.7%)[50]. Another RCT of liposomal daunorubicin verses doxorubicin, bleomycin and vincristine showed no statistical difference in response rate or disease progression (25% *vs* 28%)[51]. When the analysis was restricted to patients receiving prior zidovudine, however, survival was improved in the liposomal daunorubicin group. Another non-randomized study showed a trend

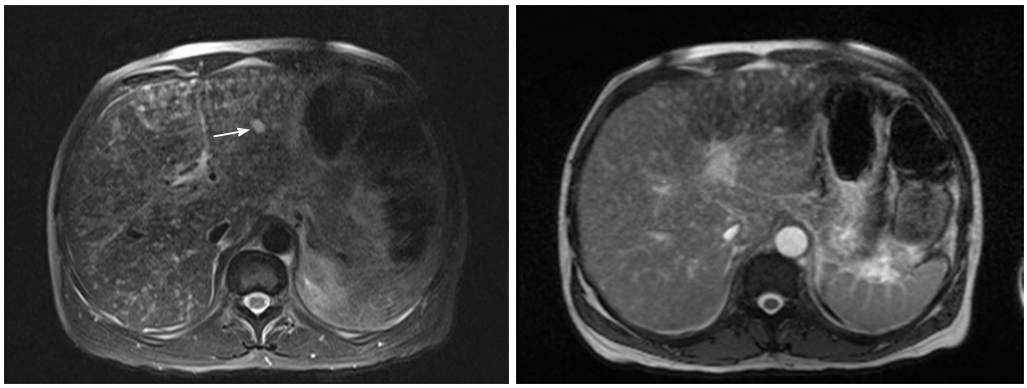

Figure 5 SPAIR and T2 images Kaposi sarcoma on magnetic resonance imaging.

toward mortality benefit with liposomal doxorubicin as compared to bleomycin plus vinblastine, vincristine or ARV monotherapy alone, although this did not reach statistical significance[52].

Interferon-alpha, has an array of antiviral and antiangiogenic properties with efficacy in AIDS-KS, but its use is limited due to hepatotoxicity[48].

Paclitaxel has systemic response rates from 59%-71% and is approved as second-line treatment for KS. In randomized controls, paclitaxel does not demonstrate benefit over PLD in complete or partial remission and no mortality data were available according to KS staging[53]. Less well tolerated than doxorubicin, adverse events include peripheral neuropathies, cytopenias, and gastrointestinal upset. Third line agents for AIDS related visceral KS include etoposide, bleomycin, vinblastine, and vincristine with overall response rates ranging from 23% to 36%. The median survival times are 11 (6 to 20) mo in the bleomycin only group and 13 (7 to 36) mo in the ABV group. With extensive side effect profiles, including secondary malignancies, these treatment modalities are maintained in resource-limited settings[54,55].

Although HAART with or without chemotherapy is the current recommended treatment, novel targets are being explored including inhibitors of angiogenesis and matrix metalloproteinases. These drugs are currently in various phases of clinical trials[56]. Inhibition of HHV-8 replication with agents such as foscarnet and ganciclovir have also been explored[57].

Finally, it bears mentioning that treatment for HIV/AIDS in patients co-infected with HHV-8 can cause a paradoxical worsening of disease. In the KS AIDS AntiRetroviral Therapy Trial, 23/112 (21%) of co-infected patients receiving ARV therapy developed KS-IRIS, which was defined as a rapid worsening of KS beyond its natural course within 12 wk of initiating ARV therapy. Of those 23 patients, 10 died, 9 of which had visceral KS. Eighteen patients in the study overall (16%) had worsening elevation in their liver enzymes and two patients (1.8%) died of liver failure. In this study, exclusion criteria included HIV-KS patients with direct serum bilirubin > 85 μmol/L or aspartate aminotransferase or alanine aminotransferase > 2.5 times the normal range[39].

Biologic and targeted molecular therapies may have a supplementary or alternative role in AIDS-KS, but are currently in early stages of clinical trials. In the AIDS Malignancy Consortium, a phase Ⅱ trial of imantinib with a small sample size showed a partial response in approximately one third of patients[58]. In another study focusing on patients who did not respond to chemotherapy and chimeric antigen receptor T cell therapy, bevacizumab, an anti-vascular endothelial growth-factor monoclonal antibody, had a response rate in again approximately one third of patients[59]. The cytokine, interleukin-12 had a response rate of 71% in small phase Ⅰ and phase Ⅱ trials. However, patients were ineligible if they had transaminitis or a history of hepatic disease[60]. Ongoing studies include a phase Ⅱ trial for the utility of combined PLD and bevacizumab in the treatment of advanced AIDS-KS[61] and a phase I study for dosing and side effect profile of combination therapy with ipilimumab, a cytotoxic T-lymphocyte antigen 4 antibody and nivolumab, an antibody against programmed cell death 1 for the treatment of advanced KS solid tumors[62]. These trials show that biologic and molecular therapies may have a role in the future as alternative treatment therapy for some patients with AIDS-KS.

Currently, HHV-8 infection cannot be eradicated but long-term remission is feasible. Treatment is indicated in patients with progressive hepatic disease in the absence of other etiologies.

CONCLUSION

Hepatic KS is a clinical presentation that disproportionately effects the HIV/AIDS population. Up to 34% of patients with AIDS and KS have hepatic involvement. It is rarely clinically significant and often diagnosed during autopsy, but can cause liver injury or even fatal liver failure, as demonstrated in the case presentation above and case series in Table 2. In an immune compromised patient with ALI or failure, a thorough skin exam in addition to abdominal imaging and biochemical testing should be pursued and a diagnosis of hepatic KS should be considered. Treatment of hepatic KS does not differ

from systemic treatment of other KS manifestations with HAART and chemotherapy, and should be considered within the context of medical comorbidities and severity of disease. Due to wide population prevalence, a lack of clinically significant disease and variable presentations, there is little clinical data or dedicated clinical trials for liver specific disease, and further investigation is warranted.

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MINIREVIEWS

Interferon-free regimens in patients with hepatitis C infection and renal dysfunction or kidney transplantation

Evangelos Cholongitas, Chrysoula Pipili, George V Papatheodoridis

Evangelos Cholongitas, 4th Department of Internal Medicine, Medical School of Aristotle University, Hippokration General Hospital of Thessaloniki, 54642 Thessaloniki, Greece

Chrysoula Pipili, Division of Nephrology, Queen Elizabeth University Hospital, Glasgow G51 4TF, United Kingdom

George V Papatheodoridis, Department of Gastroenterology, Medical School of National and Kapodistrian University of Athens, Laiko General Hospital, 11527 Athens, Greece

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Correspondence to: Evangelos Cholongitas, Assistant Professor of Internal Medicine, 4th Department of Internal Medicine, Medical School of Aristotle University, Hippokration General Hospital of Thessaloniki, 49, Konstantinopoleos Street, 54642 Thessaloniki, Greece. cholongitas@yahoo.gr
Telephone: +30-23-10892110
Fax: +30-23-10855566

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Abstract

Treatment of patients with chronic kidney disease (CKD) and chronic hepatitis C (CHC) differs from that used in the general CHC population mostly when glomerular filtration rate (GFR) is below 30 mL/min, as sofosbuvir, the backbone of several current regimens, is officially contraindicated. Given that ribavirin free regimens are preferable in CKD, elbasvir/grazoprevir is offered in CHC patients with genotype 1 or 4 and ombitasvir/paritaprevir and dasabuvir in genotype 1b for 12 wk. Although regimens containing peginterferon with or without ribavirin are officially recommended for patients with CKD and genotype 2, 3, 5, 6, such regimens are rarely used because of their low efficacy and the poor safety and tolerance profile. In this setting, especially in the presence of advanced liver disease, sofosbuvir-based regimens are often used, despite sofosbuvir contraindication. It seems to have good overall safety with only 6% or 3.4% of CKD patients to discontinue therapy or develop serious adverse events without drug discontinuation. In addition, sustained virological response (SVR) rates with sofosbuvir based regimens in CKD patients appear to be comparable with SVR rates in patients with normal renal function. Treatment recommendations for kidney transplant recipients are the same with those for patients with CHC, taking into consideration potential drug-drug interactions and baseline GFR before treatment initiation. This review summarizes recent data on the current management

of CHC in CKD patients highlighting their strengths and weaknesses and determining their usefulness in clinical practice.

Key words: Chronic hepatitis C virus infection; Kidney; Renal; Kidney transplantation; Direct acting antiviral agents; Glomerular filtration rate; Hepatitis C

Core tip: Recent evidence showed very good safety and efficacy of both interferon and ribavirin-free direct acting antivirals (DAAs) regimens in patients with severe kidney disease (CKD) or kidney transplantation. Nevertheless, sofosbuvir, the backbone of most antiviral schemes is officially contraindicated in patients with CKD (creatinine clearance < 30 mL/min). Accordingly, CKD patients with genotype 1 or 4 can be currently treated with available ribavirin free DAAs regimens without sofosbuvir, while those with non-1, non-4 genotype can officially be treated with peginterferon with or without ribavirin, but they are actually treated with sofosbuvir-based regimens mostly if they have advanced liver disease.

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INTRODUCTION

The prevalence of hepatitis C virus (HCV) infection among hemodialysis (HD) patients has been reported to range from 10% to 25%[1]. Chronic hepatitis C (CHC) has been related with high morbidity and reduced survival in both patients with renal dysfunction and kidney transplant (KT) recipients[2]. HCV treatment in patients with renal dysfunction has been a complex and challenging issue in the pre-direct acting antiviral (DAAs) era. Interferon-alpha (IFN) or pegylated IFN (PEG-IFN) with or without low doses of ribavirin (RBV) (200-400 mg three times weekly) was associated with low rates of sustained virological response (SVR) and several potentially dangerous side effects[3] such as steroid resistant acute allograft rejection in KT recipients[4].

In general, the introduction of first generation DAAs (*i.e.*, telaprevir and boceprevir) improved the SVR rates in CHC patients infected with genotype 1 but did not substantially improve the treatment of such patients with renal dysfunction or KT[5]. Initially, both telaprevir and boceprevir had to be used in combination with PEG-IFN and RBV resulting in the potential appearance of limitations, worse tolerability and safety profile of both PEG-IFN and RBV. These could account for severe anemia with both drugs, rash and pruritus with telaprevir and dysgeusia with boceprevir[5]. Moreover, glomerular filtration rate (GFR) deterioration was reported to develop in about 5% of CHC patients who received telaprevir- or boceprevir-based therapy, particularly if they had additional risk factors for renal impairment (*e.g.*, arterial hypertension)[6,7].

After 2014, newer DAAs have been licensed for the treatment of CHC by EMA and FDA. They include a nucleotide analogue NS5B polymerase inhibitor, sofosbuvir (tablet of 400 mg, Sovaldi®, Gilead)[8], the NS3/4 protease inhibitor, simeprevir (tablet of 150 mg, Olysio®, Janssen)[9], the NS5A inhibitor, daclatasvir (tablet of 60 mg, Dankliza®, Bristol-Myers Squibb)[10], the co-formulation of a NS5A inhibitor, ledipasvir, with sofosbuvir (tablet of 90/400 mg, Harvoni®, Gilead)[11], the co-formulation of a NS5A inhibitor, ombitasvir, with a NS3/4 protease inhibitor, paritaprevir, boosted by ritonavir (r) (tablet of 12.5/75 per 50 mg, Viekirax®, Abbvie), with a non-nucleos(t)ide analogue NS5B polymerase inhibitor, dasabuvir (tablet of 250 mg, Exviera®, Abbvie)[12], the co-formulation of a NS5A inhibitor, elbasvir, with a NS3/4 protease inhibitor, grazoprevir (tablet of 50/100 mg, Zepatier®, Merck)[13] and the co-formulation of a NS5A inhibitor, velpatasvir, with sofosbuvir (tablet of 100/400 mg, Epclusa®, Gilead)[14] (Table 1). IFN-free and often RBV-free combinations of the newer DAAs given for 8-24 wk have been associated with very high (> 95%) SVR rates in most subgroups of CHC patients. Such combinations seem to represent the optimal choice against HCV infection in patients with chronic kidney diseases (CKD) or KT recipients, although its potential effects on renal function in all HCV patients and in HCV patients with renal impairment have just started to be evaluated. All newer DAAs are mainly eliminated through the liver, except for sofosbuvir which is eliminated through the kidney[15]. According to licensed summaries of product characteristics, daclatasvir, dasabuvir, ombitasvir/paritaprevir/r and elbasvir/grazoprevir could be administered to patients with any severity of renal impairment. However, sofosbuvir and consequently its co-formulations, ledipasvir/sofosbuvir and velpatasvir/sofosbuvir, should not be used in patients with severe renal impairment [estimated GFR (eGFR) < 30 mL/min per 1.73 m^2] and/or patients requiring HD. Furthermore, caution is required when simeprevir is offered in patients with severe renal impairment and/or on HD because the knowledge of how it affects kidney function is limited[15].

The purpose of this review is to summarize the most recent data on the impact of the recent IFN-free anti-HCV regimes on kidney function in CHC patients as well as the safety and efficacy of these regimens in CHC patients with CKD and KT recipients.

IMPACT OF NEW DAAS ON RENAL FUNCTION

Non transplant setting

Given that sofosbuvir represents the back-bone of many current IFN-free regimens and at the same time it is the

Table 1 Main characteristics of the approved direct acting antivirals that are currently used for the treatment of hepatitis C

DAA (commercial name), dose	Category	Dose adjustment in renal impairment	Antiviral activity	CNIs co-administration
Sofosbuvir (Sovaldi®), tablet 400 mg, once daily	Nucleotide analogue NS5B polymerase inhibitor	Contraindicated in patients with GFR < 30 mL/min	Genotypes 1-6 High genetic barrier	No change
Simeprevir (Olysio®), tablet 150 mg, once daily with food	NS3/4A protease inhibitor	No change in renal impairment	Genotypes 1,4 Low genetic barrier	Contraindicated with cyclosporine
Daclatasvir (Daklinza®), tablet 60 mg, once daily	NS5A inhibitor	No change in renal impairment	Genotypes 1, 2, 3, 4 Low genetic barrier	No change
Ledipasvir/sofosbuvir/(Harvoni®), tablet 90/400 mg, once daily	NS5A inhibitor + nucleotide analogue NS5B polymerase inhibitor	Contraindicated in patients with GFR < 30 mL/min	Genotypes 1, 4, 5, 6 High genetic barrier	No change
Ombitasvir/paritaprevir/ritonavir (Viekirax®), tablet 12.5/75/50 mg, two once daily with food	NS5A inhibitor + NS3/4A protease inhibitor boosted by ritonavir boosted	No change in renal dysfunction	Genotypes 1, 4 Genetic barrier depending on HCV genotype	Cyclosporine: 20% of pretreatment total daily dose; tacrolimus: 0.2 mg/72 h or 0.5 mg once weekly
Dasabuvir (Exviera®), tablet 250 mg, every 12 h	Non-nucleos(t)ide analogue NS5B polymerase inhibitor	No change in renal dysfunction	Genotype 1 Low genetic barrier	
Elbasvir/Grazoprevir (Zepatier®), tablet 100/50 mg, once daily	NS5A inhibitor + NS3/4A inhibitor	No change in renal dysfunction	Genotypes 1,4	Co-administration increases tacrolimus concentrations
Velpatasvir/sofosbuvir/(Epclusa®), tablet 100/400 mg, once daily	NS5A inhibitor + nucleotide analogue NS5B polymerase inhibitor	Contraindicated in patients with GFR < 30 mL/min	Genotypes 1-6 High genetic barrier	No change

CNI: Calcineurin inhibitor; DAA: Direct acting antiviral; GFR: Glomerular filtration rate.

only agent with renal elimination, only sofosbuvir based regimens have been evaluated for potential effects on renal function. One study[16] assessed the rate of renal impairment in patients treated with sofosbuvir-based regimens comparing it to that of telaprevir or boceprevir based regimens, which have been previously shown to cause renal impairment in 5%-7% of treated CHC patients[7]. In total, 442 patients (50% with cirrhosis, > 95% with baseline GFR ⩾ 60 mL/min)[16]. Renal impairment (defined as increase in serum creatinine ⩾ 50% from baseline) was observed at similar rates in all groups: 7% of 228 patients under boceprevir/telaprevir-based regimens, 5% of 76 patients under sofosbuvir plus PEG-IFN/RBV and 4% of 152 patients under IFN-free sofosbuvir-based regimens (P = 0.40), but the on-treatment median creatinine peak was lower in the boceprevir/telaprevir group compared to sofosbuvir containing groups (1.4 mg/dL *vs* 2.0 mg/dL, P = 0.04). In multivariable analysis, only ascites [odds ratio (OR) = 3.16] and preexisting proteinuria (OR = 5.74) were significantly associated with development of renal impairment and SVR did not differ between patients who did or did not develop renal impairment (88% *vs* 86%, P = 0.90). According to the authors, monitoring of renal function and standard nephroprotective measures may be useful when sofosbuvir-based regimens are applied, particularly in patients with ascites or pre-existing kidney disease. This finding was confirmed in a recent study[17], in which 90 patients with HCV infection were treated with sofosbuvir plus ledipasvir: 17 patients had abnormal baseline renal function (GFR < 60 mL/min), while 42% had worsening GFR while on treatment. In multivariate analysis, baseline GFR < 60 mL/min was independently associated with worsening renal function on treatment (P = 0.04).

On the other hand, HCV infection may have a negative impact on renal function, and thus, HCV eradication could be associated with improvement of GFR. This was shown in a recent study[18] including 124 patients treated with DAAs (mean age 53.8 years, 67.7% treatment experienced, 83% had genotype 1 and 41% had cirrhosis). The achievement of SVR was associated with GFR improvement (baseline: 78.55 ± 8.96 *vs* SVR at week 12: 81.85 ± 12.87 mL/min, P = 0.037). Thus, renal function may be improved after effective treatment of HCV infection with DAAs-based regimens. However, caution is still advised if sofosbuvir is administered in patients with renal impairment, as renal function may get worse in addition to more adverse events particularly if RBV is also used in combination.

Another study assessed the potential effect of sofosbuvir-based regimens on renal function in patients with HCV decompensated cirrhosis, who represent a group at high risk for renal dysfunction[19]. The on-treatment changes of serum cystatin C, as a marker of glomerular function, and of neutrophil gelatinase-associated lipocalin (NGAL), as a marker of tubular function, were evaluated in 52 patients with Child-Pugh score ⩾ 7 treated with sofosbuvir and a NS5A inhibitor (ledipasvir or daclatasvir) and RBV for 12 wk. Half of the patients had at least one renal risk factor (*e.g.*, hypertension, diabetes, therapy with diuretics), while 14% of the patients had eGFR < 60 mL/min. The eGFR did not change significantly during antiviral therapy, but cystatin C and NGAL levels increased from baseline to week 4 of therapy (cystatin C: 1.46 mg/L *vs* 1.55 mg/L, P < 0.01; NGAL: 28.1 ng/mL *vs* 32.8 ng/mL, P < 0.01) indicating transient renal dysfunction. Unfortunately, the evolution of these renal markers at

longer follow-up was not provided.

Transplant setting

The impact of sofosbuvir-based regimens on renal function was assessed in liver transplant (LT) recipients who are at high risk for renal dysfunction for several reasons including the long-term use of calcineurin inhibitors. A recent multicenter study[20] evaluated 193 LT recipients with HCV recurrence treated with sofosbuvir-based regimens (mean age 58.7 ± 9.0 years, 30.6% cirrhotics). Renal dysfunction developed in 38% of patients. The presence of a preexisting renal disease (OR = 3.49), the baseline GFR (OR = 1.02) and tacrolimus-based immunosuppressive therapy (OR = 0.43) were all three predictive factors of renal dysfunction development. The same study group[21] focused on 20 patients with combined liver-kidney transplantation (cirrhosis 25%, genotype 1 in 70%) who received sofosbuvir-based therapy for HCV recurrence. The authors reported that GFR decreased significantly from baseline value 50.9 mL/min to 41.8 mL/min at week 12 and to 42.7 mL/min at 12 wk after the end of antiviral therapy (*P* values always $\leqslant$ 0.0001).

Finally, 165 LT patients with HCV recurrence[22] received sofosbuvir-based regimens. A decline in renal function was observed in 22% of patients, particularly in those with baseline eGFR < 30 mL/min (*P* = 0.01), cirrhosis (*P* = 0.01) and prior treatment failure (*P* = 0.03). Similarly to the non-LT setting[18], renal function improvement after treatment was observed in 58% of patients and more commonly in those who achieved SVR, compared to those who did not (81% *vs* 19%, *P* < 0.05).

INTERFERON-FREE REGIMENS IN PATIENTS WITH CHC AND CKD

Interferon-free antiviral schemes approved for CHC and CKD

Ombitasvir/paritaprevir/dasabuvir based regimens: The combination of ombitasvir/paritaprevir/r and dasabuvir, which has been abbreviated as 3D regimen, is used with or without the addition of RBV for the treatment of genotype 1a or 1b CHC patients. Moreover, the combination of ombitasvir/paritaprevir/r (2D) with RBV is administered for the treatment of genotype 4 CHC patients. The potential effect of renal impairment on the pharmacokinetics of 3D combination was evaluated in more than 2000 patients from seven phase 2/3 studies[23]. The severity of renal dysfunction was not found to affect the area under the plasma concentration curve (AUC) of 3D in 22 patients with GFR between 30 and 59 mL/min and therefore no dose-adjustments are required. However, no patients with end stage renal disease (GFR < 30 mL/min) were included in that initial evaluation. In a smaller study[24], HCV patients with normal or mild renal impairment (*n* = 38), were compared to those with stage 4 or 5 CKD patients (with or without HD) (*n* = 19). During a 12-wk course with the 3D regimen, renal dysfunction did not affect significantly the pharmacokinetics of the 3D regimen. Ombitasvir and paritaprevir exposures were comparable (< 20% difference) in both groups and ritonavir and dasabuvir exposures were 33% and 37% lower, respectively. Thus, the authors concluded that no dose adjustment for the 3D regimen is required in HCV patients with severe renal impairment.

In the RUBY-I study[25], the safety and efficacy of 3D given for 12 wk was evaluated in 20 genotype 1 treatment-naïve non-cirrhotics patients with CHC and CKD stage 4 or 5 (RBV was given at 200 mg/d in genotype 1a patients). Thirteen patients were under HD. The efficacy was high since SVR was achieved in 18 (90%) of 20 patients in the intention to treat analysis: One F3 genotype 1a patient relapsed 4 wk post-treatment, while a second patient died 14 d after the end of therapy due to left ventricular systolic dysfunction. Regarding safety profile, most adverse events were of mild to moderate severity. There were nine serious adverse events in 4 patients (including the patient who died), but none of them was considered to be related with antiviral therapy (including RBV). Four patients received erythropoietin for anemia but none required blood transfusion. No deterioration of liver or kidney function was observed during the study period.

More recently, real life data have been reported from two studies[26,27] which evaluated the safety and effectiveness of 3D with or without RBV in 69 CHC patients with stage 4 or 5 CKD (*i.e.*, GFR < 30 mL/min) or under HD. Sixty-five (94.2%) patients had genotype 1 including 29 (44.6%) cases with genotype 1a. Twenty five (75.7%) of 33 patients were treatment naïve[26] and 31 (45%) of 69 patients had cirrhosis[26,27]. 3D was given for 12 wk in all 69 patients, combined with RBV in 32 (46.3%) of them[26,27]. SVR rates at week 12 (SVR12) were 97% (65/67) [94.4% (17/18) for 3D and 94.4% (17/18) for 3D plus RBV, as provided by the study data]. In regards to safety profile, no patient discontinued 3D, two patients stopped RBV and five out of 69 patients (7.2%) developed serious adverse events requiring hospitalization (1 urinary tract infection, 2 heart failure, 1 arthritis and 1 atrial fibrillation) (Table 2).

Elbasvir/grazoprevir: Elbasvir/grazoprevir co-formulated in one tablet, with or without the addition of RBV, has been recently licensed by FDA and EMA for the treatment of HCV genotype 1 and 4[13]. Given that these agents are cleared by the liver, they can be a good option for patients with CKD stages 4 and 5. In the C-SURFER phase Ⅲ study[28], 224 patients with eGFR < 30 mL/min were randomized to receive elbasvir/grazoprevir (*n* = 111) or placebo (*n* = 113) for 12 wk. At week 16, unmasking occurred and all patients in the placebo arm received elbasvir/grazoprevir as well. Almost half (52%) of the patients had genotype 1a, 83% were HCV treatment-naïve, 6% had cirrhosis, 19% had CKD stage 4 and 81% CKD stage 5 (76% of them under HD). In the intention to treat analysis, SVR was achieved in 94%

Table 2 Studies of interferon free regimens for treatment of hepatitis C virus patients with severe renal disease or under hemodialysis

Ref.	Patients, *n*	Patient characteristics	Regimen: Patients number (dose of sofosbuvir)	Sustained virological response at 12 wk, *n/N*	Adverse events, *n*
Pockros *et al*[25]	20	GT1: 20 patients (1a: 13)	3D ± RBV: 20	18/20 (EOT-VR: 20/20)	Death from drug unrelated cause (cardiac arrest at 14 d after the end of therapy): 1
Gomez *et al*[26]	33	GT1: 29 (1a: 6) Age: 57 yr	3D ± RBV: 33	31/31	Serious adverse events: 5 (all unrelated to study drugs)
Basu *et al*[27]	36	GT1: 36 (1a: 23)	3D ± RBV: 36	34/36	No serious adverse event
Roth *et al*[28]	122	GT1: 122 patients	Elbasvir/grazoprevir: 122	115/122	Serious adverse events: 16
Czul *et al*[29]	28	GT1: 26 (1a: 16) Age: 58 yr	SOF + SMV: 26 SOF + RBV: 2 (200 mg/eod-400 mg/d)	21/25	Encephalopathy: 1 Uncontrolled diarrhea: 1
Beinhardt *et al*[30]	15	GT1: 11 patients Age: 52 yr	SOF + DCV: 9 SOF + SMV: 5 SMV + DCV: 1 (400 mg/d)	1/1 (EOT-VR: 5/5)	Pancytopenia at week 7: 1 (change SOF from every 24 h to every 48 h)
Dumortier *et al*[31]	50	GT1: 28 patients Age: 60 yr	SOF + RBV: 7 SOF + RBV + PEG-IFN: 2 SOF + DCV ± RBV: 30 SOF + SMV ± RBV: 11	24/26 (EOT-VR: 50/50)	No serious adverse event
Gane *et al*[32]	10	GT1: 9 (1a: 7) Age: 62 yr	SOF + RBV: 10 (200 mg/d)	4/10	Serious adverse events: 2 (diabetic acidosis, angina)
Nazario *et al*[33]	40	GT1: 26 (1a: 26) Age: 57 yr	SOF + LDV: 9 SOF + DCV: 2 SOF + SMV: 29 (400 mg/d)	29/29	Drug discontinuation: 1 (unknown reason)
Baliellas *et al*[34]	21 (10 on hemodialysis)	GT1: 20 patients (1a: 2) Age: 57 yr	SMV + DCV: 12 SMV + DCV + RBV: 9	17/19	No serious adverse event
Moreno *et al*[35]	42	GT1: 25 (1a: 8) Age: 54 yr	SOF + RBV: 5 LDV/SOF: 8 SOF + DCV: 14 SOF + SMV: 3 SMV + DCV: 12	32/42	Drug discontinuation: 11
Saxena *et al*[36]	19	GT1: 16 (1a: 8)	SOF + SMV + RBV: 2 SOF + SMV: 11 SOF + RBV: 5 SOF + RBV + PEG-IFN: 1 (400 mg/d)	SOF + SMV + RBV: 2/2 SOF + SMV: 8/10 SOF + RBV: 4/4 SOF + RBV + PEG: 1/1	Therapy discontinuation: 1 Serious adverse events: 3
Martin *et al*[37]	10	GT1: 8 patients Age: 58 yr	SOF + RBV: 10 (400 mg/d)	6/10	Acute respiratory failure - drug discontinuation: 1, hematemesis: 1

DCV: Daclatasvir; EOT-VR: End of treatment virological response; GT: Genotype; RBV: Ribavirin; LDV: Ledipasvir; PEG-IFN: Pegylated interferon-alfa; SMV: Simeprevir; SOF: Sofosbuvir; 3D: Ombitasvir/paritaprevir/ritonavir plus dasabuvir; eod: Every other day; HCV: Hepatitis C virus.

(115/122) of patients in the active arm: 1 noncirrhotic patient relapsed during the first 12 wk after the end of treatment, while 6 patients discontinued treatment for reasons unrelated to antiviral therapy. Serious adverse events occurred in 16 (14%) and 17 (15%) patients in the elbasvir/grazoprevir and placebo arms, respectively. None and 4% of the patients in the active and placebo groups, respectively, discontinued therapy due to an adverse event. The most common adverse events in the active arm were headache, nausea and fatigue (Table 2).

Interferon-free antiviral schemes not approved for CHC and CKD

In total, nine studies[29-37] evaluated the safety and efficacy of various antiviral schemes in 235 patients with CHC and CKD. All patients had stage 4 or 5 CKD (*i.e.*, GFR < 30 mL/min) or were under HD. The mean age was provided in 7 studies and ranged between 52.4 and 62 years[29-35]. Based on the available data, 169 (71.9%) of 235 patients had genotype 1 [67/122 (54.9%) genotype 1a][29,32-36]. One hundred (47.6%) of 210 patients were treatment naïve[29,31,33-36] and 121 (51.4%) of 235 patients had cirrhosis[29-37].

Sofosbuvir was given for 12-24 wk in combination with RBV in 42 (and PEG-IFN in 3)[29,31,32,35-37], simeprevir in 87[29-31,33,35,36] (and RBV in 2, unclarified in 11)[31,36], daclatasvir in 55 patients[30,31,33,35] and ledipasvir in 17[33,35]. The dosage of sofosbuvir was 400 mg per day in 84[29,30,33,36,37], 200 mg per day in 33[29,32,36], 200 mg every other day in 2[29] and unclarified in 82 patients. The dosage of PEG-IFN was not provided in the few studies including PEG-IFN containing regimens, while the dosage of RBV was 200 mg per day in 20[32,37], variable (200 mg three times per week to 600 mg per day) in 35[31] and unknown in the remaining patients receiving RBV. The daily dosage of simeprevir was 150 mg and of daclatasvir 60 mg in all patients. The dose of ledipasvir was dependent on the dose of sofosbuvir.

The efficacy of sofosbuvir-based antiviral therapy was provided in all studies. Based on the available data, the rates of end of treatment virological response and SVR at week 12 were 100% (91/91) and 87.1% (129/148), respectively [SVR: 55.2% (16/29) for sofosbuvir plus RBV, 92.1% (35/38) for sofosbuvir plus simeprevir (with or without RBV), 100% (14/14) for ledipasvir/sofosbuvir and 85.7% (12/14) for sofosbuvir plus daclatasvir]. The SVR rates were 80.6% (25/31) for simeprevir plus daclatasvir with or without RBV.

Regarding safety profile, only 14 (5.9%) of the 235 patients discontinued therapy due to adverse events (one under combination of sofosbuvir plus RBV due to acute respiratory failure and one under sofosbuvir plus simeprevir for unclarified cause, while no details were provided for 12 patients)[33,35-37]. In addition, one patient developed pancytopenia at week 7 under therapy (no further data were given regarding antiviral therapy, but sofosbuvir was reduced from 400 mg/d to 400 mg every other day)[30]. Finally, 8 (3.4%) of 235 patients developed serious adverse events requiring hospitalization without treatment discontinuation: Hematemesis[37], new onset encephalopathy[29], uncontrolled diarrhea[29], diabetic ketoacidosis or angina[32] (unclarified causes in 3 patients)[36]. Renal safety was evaluated in two studies[31,36] which reported no significant change of GFR from baseline to the end of treatment in non-haemodialysis patients under sofosbuvir-based regimens (Table 2).

Recently, the co-formulation of velpatasvir/sofosbuvir was approved for the treatment of all HCV genotypes. Its short-term safety and pharmacokinetics were evaluated in 10 subjects with eGFR < 30 mL/min[38]. A single dose of 100 mg velpatasvir was followed by a 120-h intensive blood monitoring. Records were compared to control subjects with normal renal function (eGFR $\geqslant$ 90 mL/min) matched for age, sex and body mass index. Velpatasvir was well tolerated and all adverse events were of mild severity. Only an approximately 50% increase in the velpatasvir AUC was observed in the group of patients with renal dysfunction, while the maximum velpatasvir concentrations (Cmax) were similar between the two groups. The authors concluded that velpatasvir could be administered without dose adjustment in patients with any GFR. However, since velpatasvir is available only in co-formulation with sofosbuvir, its use is driven by the limitations of sofosbuvir in patients with renal impairment.

INTERFERON-FREE REGIMENS IN KT RECIPIENTS WITH CHC

In total, 10 studies[39-48] evaluated the safety and efficacy of current DAAs based regimens in 330 KT recipients with CHC for 12-24 wk. The mean age ranged from 53 to 65 years. Based on the available data, 247 out of 281 patients (87.9%) had genotype 1 CHC [54/143 (37.8%) genotype 1a][39-46]. One hundred and fifty one out of 238 patients (63.4%)[40,42-44,46,47] were treatment naïve and 64 out of 252 patients (25.4%) had cirrhosis[39,40,43,44,46,47]. Sofosbuvir was given in combination with RBV in 30 patients, simeprevir (± RBV) in 31, daclatasvir (± RBV) in 20 and ledipasvir (± RBV) in 230 for 12-24 wk. The 3D (or 2D) combination (± RBV) was given in 12[46,48] and the combination of simeprevir and daclatasvir (± RBV) in 7 patients[46]. The daily dosage of RBV was provided in only 2 studies[42,43] ranging from 200 mg to 1200 mg per day.

Based on the available data, the week-12 SVR rates of sofosbuvir based regimens were 94.2% (193/205): 66.7% (10/15) for sofosbuvir plus RBV [100% (4/4) for genotype 2], 88% (22/25) for sofosbuvir plus simeprevir (with or without RBV), 75% (3/4) for sofosbuvir plus daclatasvir, 98% (158/161) for sofosbuvir plus ledipasvir (with or without RBV). In addition, in one study the week-12 SVR rates were 97.8% (45/46) for various antiviral schemes[46]. No data have been available for the efficacy of 3D or simeprevir plus daclatasvir regimens[46,48].

Regarding safety profile, 7 (2.1%) of 330 KT recipients discontinued therapy (4 under combination sofosbuvir and RBV due to pruritus, myalgia, anemia and unclarified reason; 1 under sofosbuvir plus daclatasvir due to virological failure; 2 under ledipasvir/sofosbuvir plus RBV for unclarified reasons)[39,41,44,47], while one patient died 4 wk after the end of antiviral therapy due to bleeding from donor aorta graft[40]. In addition, 15 KT recipients developed anemia requiring RBV dose reduction and/or erythropoietin injection or blood transfusion, one patient had an episode of bradycardia requiring pacemaker placement despite on regular amiodarone treatment, 2 patients presented worsening proteinuria (> 3 g/d), 4 patients developed rejection of kidney graft, and 12 patients developed unclarified serious adverse events[47]. No dose adjustment of calcineurin inhibitors was required. Renal and liver function tests remained stable during antiviral treatment (Table 3).

DISCUSSION

Current DAAs against HCV have very good safety profiles. However, baseline GFR and potential drug-drug interactions should be always considered before treatment initiation. Since sofosbuvir is the only DAA with renal elimination, concerns for potential nephrotoxicity have been raised mainly for this agent. There have been reports suggesting that sofosbuvir might have a negative impact on renal function in patients at high renal risk (*e.g.*, decompensated cirrhosis, LT, proteinuria), particularly if more sensitive renal function markers are used (*e.g.*, cystatin C or serum or urine NGAL). However, renal function decline in such high renal risk patients does not necessarily reflect drug related toxicity, as shown in uncontrolled reports. In addition, improvement in renal function after treatment has also been reported in patients who achieved SVR despite the scarcity of long follow-up data after the end of therapy. Only nephrotoxicity related to sofosbuvir has been observed but seems to be minimal given the short duration of therapy. Therefore, no definite conclusion can be drawn,

Table 3 Studies of interferon-free regimens for treatment of hepatitis C virus positive kidney transplant recipients

Ref.	Patients, *n*	Patient characteristics	Regimen: Patients number	Sustained virological response at 12 wk, *n*/*N*	Adverse events, *n*
Huard *et al*[39]	17	GT1: 16 patients (1a: 5) Age: 65 yr	SOF + RBV: 17 (400 mg/d)	1/6	Therapy discontinuation: 4 (3 due to pruritus, myalgia, anemia, 1 unclarified) Anemia: 8
Lin *et al*[40]	15	GT1: 14 (1a: 10) Age: 55.8 yr	SOF + SMV ± RBV: 12 (SOF + SMV: 9) SOF + RBV: 2 SOF + LDV: 1	13/15	No serious adverse events under therapy (1 died by massive hemorrhage 4 wk after therapy) Proteinuria: 2 Bradycardia under amiodarone (pacemaker placement): 1
Bhamidimarri *et al*[41]	14	GT1: 14 (1a: 12) Age: 54 yr	SOF + LDV: 13 (in 9 plus RBV) SOF + SMV: 1	13/14	No serious adverse events Therapy discontinuation: 1 Anemia: 7
Hussein *et al*[42]	3	GT4: 3	SOF + RBV (400 mg/d)	3/3	No serious adverse events
Sawinski *et al*[43]	20	GT1: 17 (1a: 7) Age: 57 yr	SOF + SMV: 9 SOF/LDV: 7 SOF + RBV: 3 SOF + DCV: 1 (400 mg/d)	20/20	No serious adverse events
Moreno *et al*[44]	12	GT1: 11 (1a: 4) Age: 53 yr	SOF + SMV: 1 SOF/LDV: 8 SOF + DCV: 3 (400 mg/d)	11/12	Therapy discontinuation: 1
El-Halawany *et al*[45]	11	GT1: 10 (1a: 10) Age: 57.6 yr	SOF + SMV: 2 SOF/LDV: 8 SOF + RBV: 1	10/11	No serious adverse events
Londono *et al*[46]	74	GT1: 61 (1a: 6) Age: 54 yr	SOF/LDV ± RBV: 37 SOF + DCV ± RBV: 15 SOF + SMV ± RBV: 6 SMV + DCV ± RBV: 7 SOF + RBV: 4 3 "D" or 2 "D": 5	45/46	Rejection episodes: 3
Colombo *et al*[47]	114	GT1: 104	SOF/LDV	112/114	Therapy discontinuation: 1 Serious adverse events: 12
Reddy *et al*[48]	50		SOF/LDV ± RBV: 42 SOF + DCV ± RBV: 1 3 "D": 7	10/10	Rejection episode: 1

DCV: Daclatasvir; GT: Genotype; RBV: Ribavirin; LDV: Ledipasvir; PEG-IFN: Pegylated interferon-alfa; SMV: Simeprevir; SOF: Sofosbuvir; 3D: Ombitasvir/paritaprevir/ritonavir plus dasabuvir; 2 "D": Ombitasvir/paritaprevir/ritonavir.

while it seems reasonable to apply nephroprotective measures and careful renal monitoring during treatment with sofosbuvir-based regimens in patients at high renal risk. Anyway, eGFR monitoring is currently recommended at 4 wk of therapy and as clinically indicated for all patients receiving any regimen with DAAs[49].

All current DAAs can be given in CHC patients with mild to moderate renal impairment (*i.e.*, eGFR ⩾ 30 mL/min) without dose modification. Similarly, they could all be administered in severe renal impairment (*i.e.*, eGFR < 30 mL/min) or end-stage renal disease without dose modification as well, except for sofosbuvir. Of note, the currently recommended regimens for CHC patients with severe renal impairment or end-stage renal disease according to the AASLD and EASL are presented in Table 4[49,50]. To date, HCV therapy is only recommended for patients with high urgency for treatment of the liver disease and without KT as an immediate option. Furthermore, antiviral therapy can be given after KT or even simultaneous liver and kidney transplantation, when patients usually have eGFR > 30 mL/min and can receive any regimen. HCV therapy with an IFN free regimen is mandatory for CHC patients with cirrhosis and severe renal impairment usually due to hepatorenal syndrome, since HCV eradication may lead to liver function stabilization and such an improvement resulting in LT elimination. But more data are required in this subgroup before the optimal regimen can be decided. Regrettably, lack of adequate supporting evidence halts a widely disseminated recommendation.

The indication of elbasvir/grazoprevir as first line treatment for CHC patients with genotype 1 or 4 and severe renal impairment, always given without RBV for 12 wk, has been based on the results of the C-SURFER trial. In contrast to genotype 1a patients with eGFR > 30 mL/min who should be tested for NS5A resistance associated variants (RAVs) before therapy and require 16 instead of 12 wk treatment period - of elbasvir/grazoprevir combined with RBV in case of NS5A RAVs presence-, there is no recommendation for such pre-

Table 4 Recommended regimens from the American Association for the Study of Liver Diseases and European Association for the Study of the Liver for patients with chronic hepatitis C and severe renal impairment (glomerular filtration rate < 30 mL/min) who need urgent hepatitis C virus therapy and renal transplantation is not an immediate option

HCV genotype	AASLD recommended regimen	EASL recommended regimen[3]
1	Elbasvir/grazoprevir for 12 wk (for 1a or 1b) or ombitasvir/paritaprevir/ritonavir plus dasabuvir[1] (for 1b) for 12 wk	Elbasvir/grazoprevir or ombitasvir/paritaprevir plus dasabuvir (for 1a or 1b), for 12 wk (plus RBV 200 mg/d for 1a if the haemoglobin level is > 10 g/dL at baseline)
2, 3, 5 or 6	Pegylated interferon-alfa plus dose-adjusted ribavirin (200 mg daily)[2]	Sofosbuvir/velpatasvir or sofosbuvir plus daclatasvir (plus ribavirin if the haemoglobin level is > 10 g/dL at baseline for genotype 3) for 12 wk (or for 24 wk without ribavirin for genotype 3)[4]
4	Elbasvir/grazoprevir for 12 wk	Elbasvir/grazoprevir for 12 wk or ombitasvir/paritaprevir plus dasabuvir plus ribavirin (if the haemoglobin level is > 10 g/dL at baseline) for 12 wk

[1]For HCV genotype 1a: Ombitasvir/paritaprevir/ritonavir plus Dasabuvir plus ribavirin at reduced doses (200 mg thrice weekly to daily) may be also used; [2]Ribavirin should be discontinued when hemoglobin decreases by > 2 g/dL despite use of erythropoietin (or in case of severe anaemia (haemoglobin < 8.5 g/dL according to EASL guidelines); [3]According to EASL guidelines: (1) antiviral therapy is indicated in those without an indication for kidney transplantation otherwise after kidney transplantation may be preferred; and (2) sofosbuvir should be used with caution (no dose recommendation can currently be given for these patients) and with careful monitoring of renal function; [4]If treatment is urgently needed. HCV: Hepatitis C virus; AASLD: American Association for the Study of Liver Diseases; EASL: European Association for the Study of the Liver.

treatment testing in patients with genotype 1a and eGFR < 30 mL/min. The higher exposure to antiviral agents, the lower baseline HCV RNA levels in CHC patients and the severe renal impairment attribute for the previous difference. The 3D combination is considered an acceptable alternative for genotype 1 patients based on the results of the smaller RUBY Ⅰ study and few real life data. The 3D combination is more attractive for patients with at least severe renal impairment and genotype 1b given for 12 wk without RBV. In contrast, the need for the addition of RBV makes it less attractive for such patients with genotype 1a. The safety and efficacy of the 2D regimen in patients with genotype 4 and CKD is currently under evaluation in the RUBY Ⅱ trial.

The progress in HCV therapy seems to have been minimal for CHC patients with non-1, non-4 genotype and CKD, since current guidelines still recommend the PEG-IFN and RBV combination, which is associated with low efficacy, poor tolerance and potentially several adverse events. Therefore, several efforts have been focused on sofosbuvir based regimens despite its official contraindication in patients with stage 4 or 5 renal impairment (*i.e.*, with GFR < 30 mL/min or under HD)[51]. The package labels record that up to 20-fold accumulation of the sofosbuvir metabolite GS-331007 is expected in patients with severe renal dysfunction, but the clinical significance of GS-331007 accumulation remains unknown. Moreover, a recent prospective observational study[52] evaluated the pharmacokinetics of sofosbuvir in 2 dosing (400 mg per day or 3 times per week after HD), in HCV-infected patients under HD. No accumulation of sofosbuvir or GS-331007 was observed, while HD removed 53% of GS-331007.

Since sofosbuvir was chronologically the first licensed current DAA in most countries and is still required for the IFN-free treatment of patients with non-1, non-4 genotype, the safety and efficacy of sofosbuvir based regimens in patients with end stage renal disease (CKD stage 4 or 5) on or off HD have been reported in several "real life" studies (Table 2). Its overall safety profile has been very good even in this setting with only 6% of patients (14/235) discontinuing therapy and 3.4% of patients (8/235) developing serious adverse events but without drug discontinuation. The SVR rates seem to be comparable with SVR rates in patients with normal renal function, although no definite conclusion can be drawn due to the suboptimal design of the studies, the suboptimal regimens used in some studies according to chronological availability, the small patient numbers and the variable sofosbuvir dosage. Provided that reduced sofosbuvir dosage reduces not only the plasma concentrations of GS-331007, but also the liver concentrations of the active sofosbuvir metabolite, GS-461203[53] and no major safety issues have been raised with the use of any sofosbuvir dosage in patients with at least severe renal impairment, the standard dose of sofosbuvir (400 mg daily) seems to be optimal even for this setting but should be linked with close clinical, biological, cardiovascular, and therapeutic drug monitoring. Nevertheless, further studies including more patients are required to provide stronger answers to all unresolved issues with sofosbuvir use in patients with CKD. In addition, further studies are needed in children and adolescents with CHC. It is estimated that the prevalence of chronic HCV infection is low (*e.g.*, < 0.5 among European children)[54] and currently no data on the efficacy and adverse effects of DAA are available in children with CHC.

For KT recipients, IFN-free, sofosbuvir based regimens are highly recommended providing that there is no severe underlying renal dysfunction because they are very effective with good tolerance, safety and minimal drug-drug interactions. Alternatively, the 3D or 2D regimens and the fixed elbasvir/grazoprevir combination could be the additional treatment options for patients with genotypes 1 and 4, but their safety and efficacy in the KT setting has not been evaluated yet. In general, the concurrent use of immunosuppressive agents has not been shown to affect the efficacy of any DAA

regimen and the main concern in transplant patients has been the potential drug interactions. Of the currently licensed DAAs, sofosbuvir, daclatasvir and ledipasvir have no interaction with the usual immunosuppressive agents and require no dosage modifications in transplant patients. On the other hand, simeprevir should not be given in patients receiving cyclosporine and initiation of 3D or 2D regimens should be given with reduced daily dose of cyclosporine (start with 20% of previous dose) or tacrolimus (start with 0.2 mg every 72 h or 0.5 mg once per week) in parallel with close level monitoring and dosage adjustment as required. Similarly, close monitoring of tacrolimus levels should be performed in patients undertaken elbasvir/grazoprevir because their co-administration results in increased tacrolimus plasma concentrations (Table 1).

In conclusion, IFN-free recent DAAs regimens offer for the first time the opportunity to treat effectively and safely most CHC special populations including those with severe renal dysfunction or KT. In particular, excellent IFN and RBV free options are already available for patients with genotypes 1 and 4 and severe renal impairment (eGFR < 30 mL/min) on or off HD such as elbasvir/grazoprevir for genotypes 1 and 4 and 3D for genotype 1b. To date, the patients with severe renal impairment and genotype 2, 3, 5 or 6 can be treated officially with PEG-IFN with or without RBV. Nevertheless, sofosbuvir-based regimens are actually applied if urgent treatment for the liver disease is required. Otherwise, such patients can wait for HCV treatment after KT or for future options with safer kidney profile, anticipated within the next few years.

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ORIGINAL ARTICLE

Basic Study

Regulation of hepatic microRNA expression by hepatocyte nuclear factor 4 alpha

Hong Lu, Xiaohong Lei, Jerry Liu, Curtis Klaassen

Hong Lu, Xiaohong Lei, Department of Pharmacology, SUNY Upstate Medical University, Syracuse, NY 13210, United States

Jerry Liu, Curtis Klaassen, Department of Medicine, University of Kansas Medical Center, Kansas City, KS 66160, United States

Author contributions: Lu H wrote the paper; Lu H, Lei X and Liu J performed the experiments and analyzed the data; Lu H and Klaassen C conceived and designed the experiments.

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Institutional animal care and use committee statement: All animal procedures in the study were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center. The animal protocol was designed to minimize the pain or distress to the mice. Agematched young-adult HNF4α Liv-KO mice and their wild-type control littermates were fed rodent chow (#8064, Teklad; Harlan, Indianapolis, IN). Mice were housed at an ambient temperature of 22 ℃ with alternating 12-h light/dark cycles and allowed water and feed ad libitum.

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Correspondence to: Hong Lu, PhD, Assistant Professor, Department of Pharmacology, SUNY Upstate Medical University, 750 E Adams ST, Syracuse, NY 13210, United States. luh@upstate.edu
Telephone: +1-315-4647978
Fax: +1-315-4648008

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Abstract

AIM

To uncover the role of hepatocyte nuclear factor 4 alpha (HNF4α) in regulating hepatic expression of microRNAs.

METHODS

Microarray and real-time PCR were used to determine hepatic expression of microRNAs in young-adult mice lacking *Hnf4α* expression in liver (*Hnf4α*-LivKO). Integrative genomics viewer software was used to analyze the public chromatin immunoprecipitation-sequencing datasets for DNA-binding of HNF4α, RNA polymerase-Ⅱ, and histone modifications to loci of microRNAs in mouse liver and human hepatoma cells. Dual-luciferase reporter assay was conducted to determine effects of HNF4α on the promoters of mouse and human microRNAs as well as effects of microRNAs on the untranslated regions (3′ UTR) of two genes in human hepatoma cells.

RESULTS

Microarray data indicated that most microRNAs remained unaltered by *Hnf4α* deficiency in *Hnf4α*-LivKO mice. However, certain liver-predominant microRNAs were down-regulated similarly in young-adult male and female *Hnf4α*-LivKO mice. The down-regulation of miR-101, miR-192, miR-193a, miR-194, miR-215, miR-802, and miR-122 as well as induction of miR-34 and miR-29 in male *Hnf4α*-LivKO mice were confirmed by real-time

PCR. Analysis of public chromatin immunoprecipitation-sequencing data indicates that HNF4α directly binds to the promoters of miR-101, miR-122, miR-194-2/miR-192 and miR-193, which is associated with histone marks of active transcription. Luciferase reporter assay showed that HNF4α markedly activated the promoters of mouse and human miR-101b/miR-101-2 and the miR-194/miR-192 cluster. Additionally, miR-192 and miR-194 significantly decreased activities of luciferase reporters for the 3'UTR of histone H3F3 and chromodomain helicase DNA binding protein 1 (CHD1), respectively, suggesting that miR-192 and miR-194 might be important in chromosome remodeling through directly targeting H3F3 and CHD1.

CONCLUSION

HNF4α is essential for hepatic basal expression of a group of liver-enriched microRNAs, including miR-101, miR-192, miR-193a, miR-194 and miR-802, through which HNF4α may play a major role in the post-transcriptional regulation of gene expression and maintenance of the epigenome in liver.

Key words: Liver; Hepatocyte nuclear factor 4 alpha; Knockout; Mice; Human; miR-122; miR-192; miR-194; miR-101; miR-802

Core tip: Hepatocyte nuclear factor 4 alpha (HNF4α) is a liver-enriched master regulator of liver development and function. HNF4α plays a key role in regulating hepatic transcriptome and epigenome. However, little was known about the role of HNF4α in regulating hepatic expression of microRNAs, essential modulators of the transcriptome and epigenome. Results from this study uncover species differences and similarities between humans and mice in the role of HNF4α in regulating hepatic expression of certain important microRNAs. Such novel knowledge will help understand the role of HNF4α in post-transcriptional regulation of gene expression and maintenance of the normal epigenome and physiology in mouse and human liver.

Lu H, Lei X, Liu J, Klaassen C. Regulation of hepatic microRNA expression by hepatocyte nuclear factor 4 alpha. *World J Hepatol* 2017; 9(4): 191-208 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i4/191.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i4.191

INTRODUCTION

Hepatocyte nuclear factor 4 alpha (HNF4α) is a master regulator of liver development and function[1]. HNF4α is essential for hepatocyte differentiation in fetal liver[2-4], maintenance of liver function in adult[5,6], and protection against liver cirrhosis and liver cancer[7,8]. HNF4α is critical in regulating hepatic metabolism of fatty acids, bile acids, and ureagenesis[5,9-11]. Moreover, HNF4α is essential in regulating hepatic expression of drug processing genes, namely cytochrome P450s, phase-Ⅱ conjugation enzymes, and transporters[1,12,13].

There are very large individual variations in hepatic basal expression of HNF4α in humans[14], and mutation of HNF4α causes maturity onset diabetes of young humans[15]. The expression and/or transcriptional activity of HNF4α is decreased markedly in severe cirrhotic livers, alcoholic liver disease, tumor necrosis factor-α-induced hepatotoxicity, and hepatoma progression[16-19]. Thus, it is important to understand how HNF4α deficiency affects hepatic gene expression and its underlying mechanism.

Interestingly, overexpression of HNF4α in hepatocellular carcinoma (HCC) markedly decreases the stemness of gene expression and the percentage of cancer stem cells in HCC[7]; however, the underlying mechanism is unknown. Epigenetic modifications play key roles in regulating gene expression and stem cell differentiation. Our recent study demonstrates that *Hnf4α* deficiency in young-adult mouse livers causes marked alteration in histone methylation and acetylation, which is associated with induction of certain key epigenetic enzymes, including enhancer of zeste homolog 2 (EZH2), G9a and DNA methyltransferase (cytosine-5) 1 (Dnmt1)[20]. EZH2 plays a key role in maintaining the stemness of stem cells[21]. Therefore, establishment and maintenance of the epigenome of differentiated hepatocytes may be a key mechanism in the regulation of gene expression and cell differentiation by HNF4α.

The importance of HNF4α in regulating hepatic expression of mRNAs has been well established, however, the underlying mechanism remains less clear. HNF4α directly binds to a large number of gene promoters in human and mouse liver[22-24]. Hnf4α deficiency in young-adult mouse liver caused induction of certain key epigenetic modifiers[20]. However, our analysis of published data of chromatin immunoprecipitation-sequencing (ChIP-seq) of Hnf4α in adult mouse liver[25] revealed no binding of Hnf4α to these epigenetic modifiers, suggesting indirect regulation of these epigenetic modifiers by Hnf4α in liver. microRNAs are important post-transcriptional regulators of gene expression, and deregulation of microRNAs is common in human hepatocarcinogenesis[26]. Through binding to the untranslated regions (UTRs, usually the 3'UTR) of mRNAs, microRNAs affect the stability/translation of mRNAs and thus the mRNA and/or protein levels of their target genes. We hypothesized that HNF4α can indirectly regulate hepatic gene expression through directly regulating hepatic expression of certain microRNAs. Thus, the purpose of this study was to uncover the role of HNF4α in regulating hepatic expression of microRNAs. We used microarray and real-time PCR to determine hepatic expression of microRNAs in young-adult mice lacking *Hnf4α* expression in liver (*Hnf4α*-LivKO). We used integrative genomics viewer (IGV) software to analyze the public ChIP-seq datasets

for DNA-binding of HNF4α, RNA polymerase-Ⅱ, and histone modifications to loci of microRNAs in mouse liver and human hepatoma cells. Additionally, we conducted dual-luciferase reporter assay to determine effects of HNF4α on the promoters of mouse and human microRNAs as well as effects of microRNAs on the 3'UTR of two putative target genes in human hepatoma cells.

MATERIALS AND METHODS

Preparation of liver samples

The livers of male and female young-adult mice with liver-specific knockout of *Hnf4α* (*Hnf4α*-LivKO) (*Hnf4α* flox/flox, Alb-cre/+) and age-matched wild-type (*Hnf4α*flox/flox, Alb-cre/-) littermates at the age of 45 d were collected in the previous study[27] and stored at -80 ℃ until use. All animal procedures in the study were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center[27].

Microarray profiling of microRNA expression in Hnf4α-LivKO mice

Pooled total RNAs from livers of young-adult (42-45 d old) male and female *Hnf4α*-LivKO and their age-matched wild-type littermates (n = 5-6) were used for microarray analysis of microRNAs, utilizing miRCURY™ LNA array version 11.0 (Exiqon, Denmark), which contains probes targeting all mouse microRNAs registered in the miRBASE version 13.0. Background correction was conducted utilizing normexp plus offset method with offset value 10[28]. The non-linear regression method was used for data normalization to remove certain systematic biases from microarray data, such as dye effects or intensity dependence.

Heat map and unsupervised hierarchical clustering of microRNAs

The heat map diagram shows the result of the 2-way hierarchical clustering of microRNAs and samples[29]. Each row represents a microRNA and each column represents a pooled liver sample. The microRNA clustering tree is shown on the left, and the sample clustering tree appears at the top. The color scale shown at the bottom illustrates the relative expression level of a microRNA across all samples: Red color represents an expression level above mean, blue color represents expression lower than the mean. The clustering is performed on log2(Hy3/Hy5) ratios which passed the filtering criteria on variation across samples; LogMedianDRatios differencies > 0.58, corresponding to 50% differential expression.

Quantification of microRNAs using real-time PCR

miRCURY LNA™ Universal RT microRNA PCR (Exiqon) was used to quantify microRNAs in individual RNA samples from livers of male *Hnf4α*-LivKO mice. All PCR reagents and specific LNA-modified PCR primer sets were purchased from Exiqon. The PCR primer sets for mmu-miR-19b, 26a, 29b, 34a, 122, 192, 193a-3p, 194 and 195 target both human and mouse microRNA homologs, whereas PCR primer sets for mmu-miR-101b, 215, and 802 were specific for mouse microRNAs. The relative expression of each microRNA was normalized by 5s rRNA and U6 rRNA with values of wild-type mice set at 100.

Use of public database to analyze DNA-binding of HNF4α and the chromatin status of microRNAs in mouse liver, intestine, and human hepatoma HepG2 cells

Actively transcribed genes typically remain in loosely-packed euchromatin, where DNA is more accessible to the transcriptional machinery. DNAse-Ⅰhypersensitive sites (DHSs), determined by DNAse-sequencing (DNAse-seq), is a key determining factor of the chromatin accessibility of transcription factors. DNA-binding of RNA polymerase 2 (Pol2) is widely used as a marker of active transcription. Histone H3 trimethylation at lysine-4 (H3K4me3) is enriched around the transcription start sites (TSS) and correlates tightly with active gene transcription[30,31], whereas H3 trimethylation at lysine-36 (H3K36me3) along the gene coding regions after TSSs correlated highly with transcription elongation[32]. Our previous study shows that alterations of H3K4me3 correlate bi-directionally with mRNA expression in HNF4α-null livers[20]. Conversely, Histone H3 trimethylation at lysine-27 (H3K27me3) and at lysine-9 (H3K9me3) are well-established epigenetic signatures of gene silencing[31,33]. The public genome-wide datasets of DNAse-seq (GSM1003818) as well as ChIP-seq of H3K4me3 (GSM769014), H3K36me3 (GSM1000151), H3K9me3 (GSM1087075), H3K27me3 (GSM1087069), Pol2 (GSM722763) and HNF4α (GSM1390711) in wild-type mouse liver were retrieved from GEO DataSets and uploaded into the IGV software[34] to visualize the DNA-binding of HNF4α, Pol2 and these epigenetic signatures in each microRNA locus in mouse liver. Similarly, the public genome-wide datasets of DNAse-seq (GSM816662) as well as ChIP-seq of H3K4me3 (GSM945182), H3K36me3 (GSM945211), H3K9me3 (GSM1003519), H3K27me3 (GSM945231), Pol2 (GSM935543), and HNF4α (GSM935619) in HepG2 cells were retrieved from GEO DataSets for their visualization in the IGV software. Additionally, to determine the role of tissue-specific binding of HNF4α in the tissue-specific regulation of miRs, ChIP-seq data for DNA-binding of HNF4α in the mouse liver (GSM1390711) and small intestinal villus cells (GSM851120) were compared using the IGV software.

Generation of expression vectors for wildtype and mutant mouse Hnf4α1

The mouse Hnf4α1 cDNA was synthesized by Integrated DNA Technologies, Inc (IDT, Coralville, IA) and cloned into the pcDNA3 backbone to generate the expression vector for wildtype Hnf4α1, which was named as pcDNA3-Hnf4α1. The expression vector for the 304 serine to aspartic acid (S304D) mutant of Hnf4α1 was generated using pcDNA3-Hnf4α1 and the Q5® Site-Directed Muta-

genesis Kit (New England Biolabs), and verified by sequencing.

Generation of reporter constructs for the promoters of human miR-101-2 and mouse miR-101b

miR-101 is mainly transcribed from the human miR-101-2 and mouse miR-101b loci[35] which are located in the intron8-9 of RNA terminal phosphate cyclase-like 1 (*RCL1*) gene. The first base of the pre-miR-101-2 was assigned as chr9:4840297[35], located within intron5-6 of RCL1, around where prominent peaks of H3K4me3 and DNA-binding of Pol2 and HNF4α were identified. Thus, we PCR cloned a 739-bp fragment of miR-101-2 proximal promoter (-926 to -190 bp), located within the intron5-6 of RCL1, into the KpnI/MluI sites of pGL3-Basic reporter vector, which was named as pGL3-miR-101-2. In mice, miR-101b is predominantly expressed in the liver[35]. Similar to its human ortholog miR-101-2, we found prominent peaks of HNF4α, H3K4me3 and Pol2 that start at the intron5-6 of Rcl1 and extend to intron7-8 and intron8-9 of Rcl1. Thus, we PCR cloned a 933-bp fragment of the miR-101b promoter, located within intron5-6 of Rcl1 that contains the peaks of HNF4α and Pol2, into the KpnI/MluI sites of pGL3-Basic reporter vector, which was named as pGL3-miR-101b.

Generation of reporter constructs for the proximal and/or distal promoters of human and mouse miR-194-2/miR-192 cluster

Mouse miR-194-1/miR-215 and miR-194-2/miR-192 forms gene clusters in chromosome 1 and 19, respectively. The miR-194-1/miR-215 loci is expressed lowly in mouse liver[36]. In mouse liver, we found prominent peaks of DHSs, HNF4α, Pol2 and H3K4me3 located approximately 1.6 kb upstream of the miR-194-2. Thus, we PCR cloned a 1973 bp fragment (-1694 to + 279 bp) of the promoter of the mouse miR-194-2/miR-192 cluster into the MluI/XhoI site of pGL3-Basic reporter vector, which was named as pGL3-mmiR-194-2. The sequences of all the primers used for PCR cloning of miR promoters are listed in Supplemental Materials.

A previous study indicates that a single approximately 2.4 kb transcript contains the human pri-miR-194-2 transcript and a 5′ AK092802 cDNA. In the human colon cancer Caco-2 cells, HNF1α binds to a HNF1 site located between -70 and -52 bp upstream of the transcription start site (TSS) of AK092802 to activate the promoter of pri-miR-194-2[37]. The upstream genomic region close to the TSS of pri-miR-194-2 contains some highly conserved regions between humans and mice[37]. We found prominent peaks of DHSs, HNF4α, Pol2 and H3K4me3 within a 350 bp fragment from -329 to +21 bp upstream of the TSS of AK092802, which was PCR cloned into the KpnI/MluI sites of pGL3-Basic reporter vector and named as pGL3-hmiR-194-2-Dist. Genomic DNA prepared from C57BL/6 mouse liver and human embryonic kidney 293 cells were used as the PCR templates. In addition to the prominent peaks of HNF4α and Pol2 identified in approximately 2 kb upstream of the human miR-194-2 loci, smaller peaks of HNF4α and Pol2 were also found in the proximal promoter of human miR-194-2. A DNA fragment of 417 bp that contains 5′ KpnI and 3′ Hind Ⅲ restriction sites as well as a wild-type and mutant 405 bp human miR-194-2 promoter (from -405 to +1) were synthesized and verified by sequencing (GenScript United States Inc., Piscataway, NJ), and ligated into the KpnI/HindⅢ site in the pGL3-basic vector, which was named as pGL3-hmiR-194-2-Pro and pGL3-hmiR194-2 TriM. The mutant 405-bp human miR-194-2 promoter had mutations of 3 putative HNF4-binding sites predicted by software of NHR-scan[38] and HNF4 Binding Site Scanner[39] (for DNA sequences see Supplemental Materials).

Generation of reporter construct for the mouse miR-802 promoter

We PCR cloned a 2 kb fragment of the mouse miR-802 promoter (-2004 to -1 bp) into the MluI/XhoI sites of pGL3-Basic to generate the reporter vector for mouse miR-802 promoter, which was named as pGL3-mmiR-802 Pro.

Determination of effect of HNF4α on the promoter activities of human and mouse miRs

Human hepatocellular adenoma HepG2 cells were maintained in D-MEM with 5% FBS. Cells were added to 96-well plates and grown to approximately 80% confluence. Plasmid DNA including pGL3 reporter vectors, the pRL-CMV luciferase (as control for transfection efficiency), pCDNA3-HNF4α2 (Addgene), pCMV-CCAAT/enhancer-binding protein α (C/EBPα) (gift from Dr. Magnus Nord, Karolinska Institute), or pCDNA3 were complexed with Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) and applied to individual wells, according to the manufacturer's protocol. Transfected cells were lysed with passive lysis buffer (Promega) 24 h after transfection. Promoter activities of cell lysates were quantified by Dual-Glo™ luciferase assay (Promega) with the control values of pGL3-Basic *vs* pRL-CMV set at 1.0. To study the role of SP1 in mediating the transactivation of human miR-194-2 proximal promoter by HNF4α, the SP1 inhibitor mithramycin was added 1 h after transfection and cells were lysed 24 h after transfection for dual-luciferase assay.

Generation of reporter construct for the 3'UTR of mouse chromodomain helicase DNA binding protein 1 (Chd1) and H3f3 mRNAs

The chromatin remodeling factor Chd1 is required to maintain the open chromatin and pluripotency of mouse embryonic stem cells[40]. DNA sequence containing 48 bp of the 3'UTR of mouse Chd1 mRNA (NM_007690.3, 6708-6756, in bold), namely CTAGTGATTGGCTTT AATATAAAAACTGTTACAGTACACACTGATTGTATATA CGCGTA, and its antisense sequence AGCTTACGCG TATATACAATCAGTGTGTACTGTAACAGTTTTTATATTAAA

GCCAATCA were synthesized by IDT. DNA sequence containing 48 bp of the 3'UTR of mouse H3f3b mRNA (NM_008211.3, 1593-1639, in bold), namely CTAGTAA GTATCCTATTGAAGTTTTTAGGTCAATTATGTATGTTGA CTAAATACGCGTA, and its antisense sequence AGCTT ACGCGTATTTAGTCAACATACATAATTGACCTAAAAA CTTCAATAGGATACTTA were synthesized by IDT. The two sense and antisense oligos were annealed and ligated into the Spe Ⅰ/Hind Ⅲ site between the luciferase cDNA and SV40 polyA in pMIR-REPORT™ microRNA Expression Reporter Vector (Applied Biosystems/Ambion, Austin, TX), which was named pMIR-Chd1 and pMIR-H3f3, respectively. The correctness of pMIR-Chd1 and pMIR-H3f3 was verified by the unique restriction site (ACGCGT) for MluI that was introduced into the synthetic oligo.

Determination of effect of miR-194 and miR-192 on the stability of mouse Chd1 and H3f3 3'-UTR using dual-luciferase assay

HepG2 human hepatocellular adenoma cells were maintained in D-MEM with 5% FBS. Cells were added to 96-well plates and grown to approximately 80% confluence. Plasmid DNA including pmiR-Chd1 (or pmiR-H3f3), the pRL-CMV luciferase, and a synthetic mimic of miR-194/miR-192 (miScript miR-194/miR-192, QIAGEN Inc, Valencia, CA), or AllStars Negative Control siRNA (QIAGEN, as negative control for microRNAs) were co-transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol of DNA-RNAi co-transfection. Transfected cells were lysed with passive lysis buffer (Promega) 24 h after transfection. Promoter activities of cell lysates were quantified by Dual-Glo™ luciferase assay (Promega) with the control values of pmiR-Chd1/pmiR-H3f3 *vs* pRL-CMV set at 1.0.

Animal care and use statement: The animal protocol was designed to minimize the pain or distress to the mice. Age-matched young-adult HNF4α Liv-KO mice and their wild-type control littermates were fed rodent chow (#8064, Teklad; Harlan, Indianapolis, IN). Mice were housed at an ambient temperature of 22 ℃ with alternating 12-h light/dark cycles and allowed water and feed *ad libitum*.

Statistical analysis

Data are presented as mean ± SE. Differences between two groups were determined using Student's *t*-test. For multiple comparisons, analysis of variance was performed, followed by the Student-Newman-Keuls Method in SigmaPlot 12.5, with significance set at $P < 0.05$.

RESULTS

Results of microarray analysis of microRNAs in pooled young-adult male and female Hnf4α-LivKO mouse livers

Generally, there were few gender differences in hepatic expression of microRNAs in mice (Figure 1), which is similar to that in rats[41]. Hepatic expression of most microRNAs remained unchanged (< 50% differential expression among the 4 pooled samples) in *Hnf4a*-LivKO mice (data not shown). However, *Hnf4a*-LivKO mouse livers had up- or down-regulation of a small portion of microRNAs that are important in regulating cell proliferation, differentiation, and apoptosis (Figure 1). Thirty microRNAs were found to have ≥ 50% differential expression among the 4 pooled samples, namely male WT and *Hnf4a*-LivKO as well as female WT and *Hnf4a*-LivKO mice. Fourteen microRNAs had > 50% lower expression in *Hnf4α*-LivKO mice than in WT mice (Figure 1A). Among them, the 4 liver-predominant microRNAs miR-194, miR-192, miR-215 and miR-193 were 71%, 72%, 70% and 70% lower, respectively, in *Hnf4α*-LivKO male mouse livers than WT males (WTM). miR-101a and 101b, which are expressed moderately in liver, also decreased > 50% in male *Hnf4α*-LivKO mice. Female *Hnf4α*-LivKO mouse livers had very similar lower expression of these microRNAs than WT females (Figure 1A). In contrast, two microRNAs that are expressed highly in liver, namely miR-122 and miR-26a[42,43], had less than 50% differential expression in all the groups (Supplemental Table 1).

In contrast to the down-regulation of certain liver-predominant microRNAs, hepatic expression of 16 microRNAs were > 50% higher in *Hnf4α*-LivKO mice than in WT mice (Figure 1B). The tumor-suppressor miR-34a[44] was expressed at relatively low levels in wild-type mouse liver, but was induced 2.6 fold in male *Hnf4α*-LivKO mouse livers. Tumor-suppressor miR-29b and miR-195[45] were highly and modestly expressed in WT mouse livers, respectively, and were 90% and 70% higher, respectively, in male *Hnf4α*-LivKO mouse livers than WTM (Figure 1B).

The oncogenic miR-17-92 locus encodes a cluster of 7 microRNAs transcribed as a single primary transcript[46]. Four miR-17-92 members, namely miR-17, 19a, 19b and 20 tended to be higher in *Hnf4α*-LivKO mouse liver (Figure 1B).

Verification of changes in hepatic microRNAs in male Hnf4α-LivKO mice by real-time PCR

To verify the changes in microRNAs detected by microarray in the pooled liver samples, real-time PCR was used to quantify 12 microRNAs in individual samples from *Hnf4α*-LivKO mice (Figure 2). Because similar alterations of these microRNAs were found in male and female *Hnf4α*-LivKO mice (Figure 1), only individual male *Hnf4α*-LivKO liver samples were used in this study. The selection of these 12 microRNAs for verification was based on their relative expression levels (Supplemental Table 1) and their reported importance in cellular pathophysiology.

Compared to male WT mice, male *Hnf4α*-LivKO mice had markedly lower levels of miR-101b (7% of WT values), miR-192 (24%), miR-193a (24%), miR-194 (16%), miR-215 (59%) and miR-802 (33%) (Figure 2A-B), but higher levels of miR-29b (190%) and miR-34a (244%) (Figure 2C). In contrast, hepatic levels of miR-26a and miR-195 were similar between male WT and *Hnf4α*-LivKO mice (Figure 2C-D). Hepatic miR-122 was

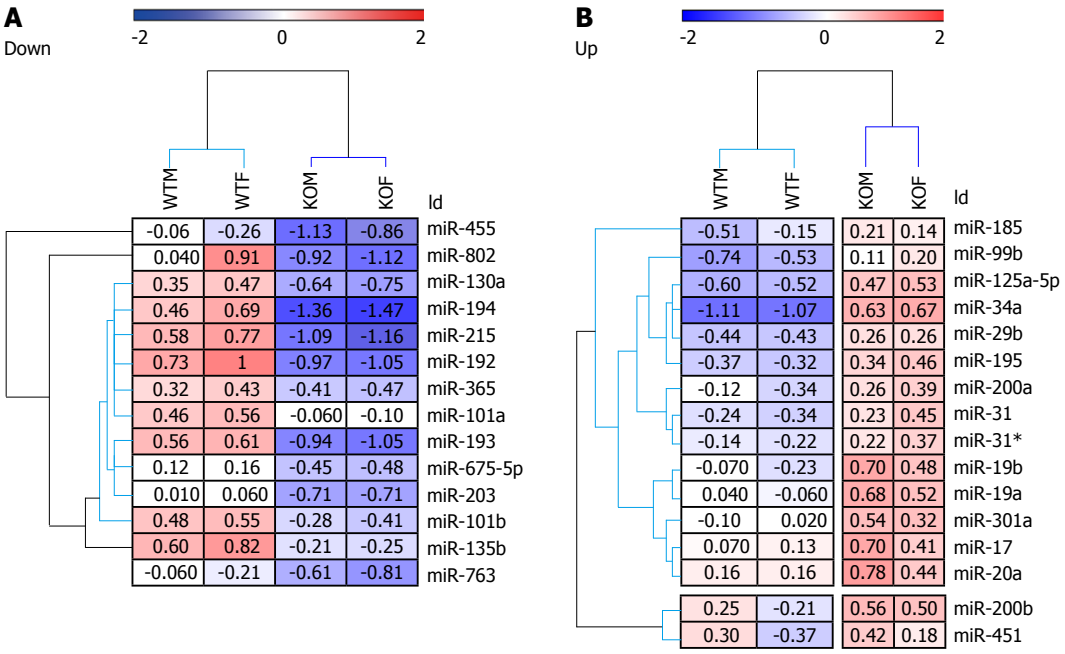

Figure 1 Heat map and unsupervised hierarchical clustering of hepatic microRNAs in male and female *Hnf4a*-LivKO mice. The heat map diagram shows the results of the 2-way hierarchical clustering of microRNAs and samples. Each row represents a microRNA and each column represents a pooled liver sample. The microRNA clustering tree is shown on the left, and the sample clustering tree appears at the top. The color scale shown at the top illustrates the relative expression level of a microRNA across all samples: Red color represents an expression level above mean, blue color represents expression lower than the mean. The clustering is performed on log2(Hy3/Hy5) ratios which passed the filtering criteria on variation across samples; LogMedianDRatios differencies > 0.58, corresponding to 50% differential expression. WTM: Wild-type male; WTF: Wild-type female; KOM: Knockout male; KOF: Knockout female.

modestly (30%) lower in male *Hnf4α*-LivKO mice than male WT mice (Figure 2D).

DNA-binding of HNF4α in mouse liver and small intestine as well as the chromatin status of microRNAs in mouse liver

To understand the mechanism of regulation of microRNA expression by HNF4α in mouse liver, we used IGV software to analyze the published genome-wide DNAse-seq and ChIP-seq data on DNA-binding of HNF4α as well as the presence of DHSs, Pol2 and active (H3K4me3 and H3K36me3) and suppressing (H3K9me3 and H3K27me3) epigenetic signatures, in the loci of several microRNAs in mouse liver and/or small intestine. Consistent with their high expression in mouse liver, miR-122a, miR-194-2/miR-192 and miR-101b had large peaks of DHSs in their gene loci, which were associated with sequential prominent peaks of HNF4α, Pol2, H3K4me3 and H3K36me3 downstream (Figure 3A-3C). This strongly suggests that the binding of HNF4α to the promoter of these miR genes causes the recruitment of Pol2 and the introduction of H3K4me3 and H3K36me3, the active marks of transcription initiation and elongation. Consistent with the liver-specific and liver-predominant expression of miR-122a and miR-101b, respectively, no binding of HNF4α to the promoters of miR-122a and miR-101b was found in mouse small intestine (Figure 3A and 3C). In contrast, large peaks of HNF4α were identified in the distal and proximal promoters of the miR-194-2/miR-192 cluster, consistent with their high expression in the mouse intestine[37].

Similarly, peaks of DHSs, HNF4α, Pol2 and H3K4me3 were also found in the gene loci of miR-193 and miR-802 (Figure 3D and E); however, the peaks were smaller and less sequential compared to those in the gene loci of miR-122, miR-194-2/miR-192 and miR-101b. In contrast, no clear peaks of H3K36me3 were found in regions that encode the mature transcripts of miR-193 and miR-802 (Figure 3D and E). Interestingly, the silencing mark H3K27me3 was found to span the whole locus of miR-802, whereas a peak of H3K9me3 was found 3' downstream of the miR-802 (Figure 3E). In summary, the data suggest that these five microRNAs might be directly regulated by HNF4α in mouse liver.

Much smaller peaks of HNF4α were found in the gene loci of miR-194-1/miR-215, miR26a-1 and miR26a-2, and DNA-binding of HNF4α was not associated with prominent peaks of Pol2 or H3K4me3 in mouse liver (Figure 4A-C). Conversely, although prominent peaks of DHSs, HNF4α, Pol2 and H3K4me3 were found in the miR-26b locus, the direction of HNF4α, Pol2 and H3K4me3 peaks was toward the upstream of miR-26b, rather than the transcription initiation of miR-26b (Figure 4D). These data suggest that HNF4α may not have a direct and/or important role in regulating hepatic expression of miR-194-1/miR-215, miR-26a and miR-26b. In contrast, large peaks of HNF4α were found in the distal and proximal promoter of the miR-194-1/miR-215 cluster in mouse small intestine (Figure 4A), suggesting that HNF4α may be important in regulating the high expression of the miR-194-1/miR-215 cluster in mouse small intestine[36].

It was reported that HNF4α binds to the proximal

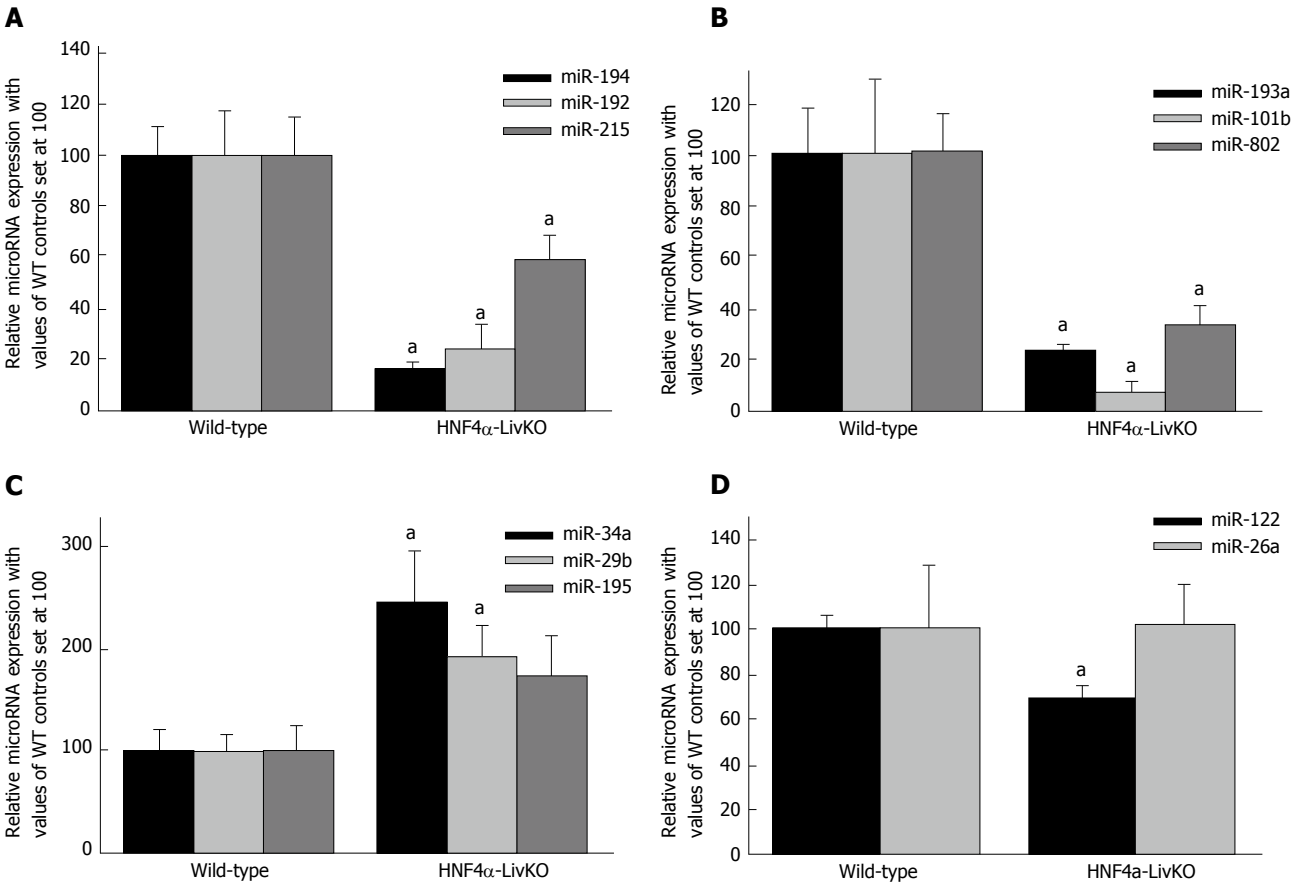

Figure 2 Hepatic microRNA expression in young-adult male mice with liver-specific deletion of *Hnf4a* (*Hnf4a*-LivKO) (A-D). microRNAs in total RNA from livers of Hnf4a-LivKO and wild-type (WT) control mice (n = 5-6) were determined by miRCURY LNA™ Universal RT microRNA PCR (Exiqon). Mean ± SE. $^{a}P < 0.05$ compared to WT control. HNF4α: Hepatocyte nuclear factor 4 alpha.

promoter of miR-29 a-b cluster in cultured mouse hepatocytes, and acute loss of HNF4α decreased the levels of miR-29a and miR-29b in isolated hepatocytes and livers from mice on a mixed background of SvJ129/FVB[47]. However, only a small peak of HNF4α was found within 10 kb of the mouse miR-29 a-b loci in adult liver from C57BL/6 mice, and the small HNF4α peak was not associated with peaks of Pol2 or H3K4me3 (Figure 5A). In contrast, a larger peak of HNF4α was found in the promoter of the miR-29 a-b loci in the small intestine (Figure 5A). Thus, the role of HNF4α in regulating hepatic expression of miR-29 a-b cluster in mice may be strain and/or cell-context dependent.

Recent studies indicate that HNF4α directly regulates miR-124 and miR-134 in human liver, and down-regulation of HNF4α is associated with reduction of miR-124 and miR-134 in human HCC[48,49]. However, our microarray data showed that miR-124 and miR-134 were expressed very lowly in mouse liver, and *Hnf4α* deficiency had no effect on hepatic expression of miR-124 and miR-134 in mice (Supplemental Table 1). Consistently, there were no clear peaks of HNF4α, Pol2, or the activating signatures H3K4me3, H3K36me3 in the loci of the 3 mouse miR-124 genes, namely miR-124a-1, 124a-2 and 124a-3 in livers of C57BL/6 mice (Figure 5B-D). In contrast, large peaks of the silencing mark H3K27me3 were found in the whole loci of miR-124a-1, 124a-2 and 124a-3, and a large peak of H3K9me3 was found in the miR-124a-1 locus (Figure 5B-D). Similarly, there were no prominent peaks of DHSs, HNF4α, Pol2, H3K4me3, or H3K36me3 detected in the locus of mouse *miR-134* gene, where the silencing mark H3K9me3 was found (Figure 5E). Taken together, the very low signal of miR-124s and miR-134 in the microarray data (Supplemental Table 1) and the lack of activating epigenetic signatures but enrichment of silencing epigenetic signatures in the loci of miR-124s and miR-134 strongly indicate that miR-124 and miR-134 are expressed very lowly in adult mouse liver, and they are not HNF4α-targe genes in mouse liver. Thus, there appear to be species differences between humans and mice in hepatic basal expression and regulation of miR-124 and miR-134 by HNF4α.

Because our data of microRNA expression and analysis of public database for ChIP-seq strongly suggest that HNF4α has a critical direct role in maintaining hepatic expression of miR-194/miR-192 and miR-101b in mice, we further examined DNA-binding of HNF4α and chromatin status in the gene loci of miR-194-2/miR-192 and miR-101-2 in the human hepatoma HepG2 cells using the data from public database (Figure 6). Very similar to the mouse miR-194-2/miR-192 cluster (Figure 3B), starting from approximately 2 kb upstream of the

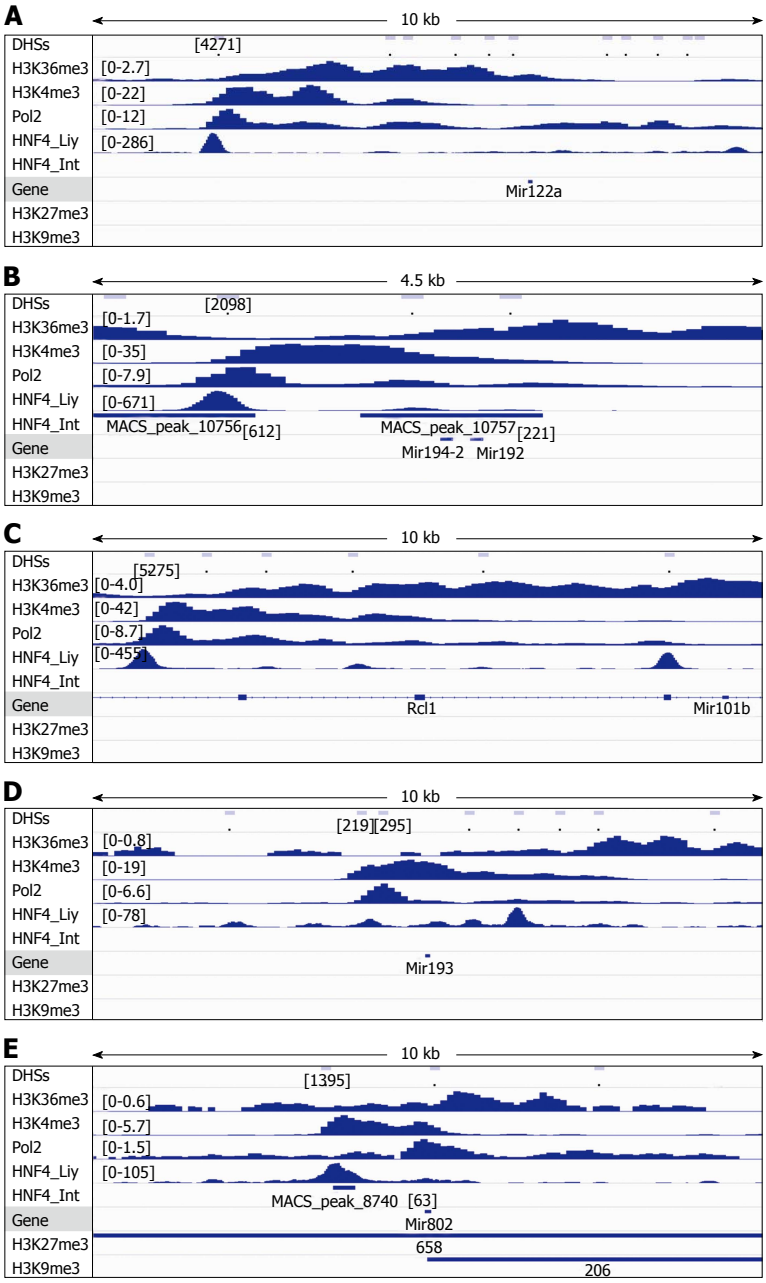

Figure 3 Analysis of DNAse-I hypersensitive sites as well as DNA-binding of HNF4α, RNA polymerase Ⅱ (Pol2), and methylated histones to loci of miR-122a (A), miR-194-2/miR-192 (B), miR-101b (C), miR-193 (D) and miR-802 (E) in wildtype mouse liver. DNA-binding of HNF4α to these microRNA loci in the mouse small intestine (HNF4α _Int) was compared to those in the mouse liver (HNF4α_Liv). Data of DHSs (determined by DNAse-seq) and DNA-binding of proteins (determined by ChIP-seq) were retrieved from the public database of GEO DataSets and visualized in the IGV software. The peak values/ranges for each mark were shown in square brackets or under the line mark. DHSs: DNAse-I hypersensitive sites; HNF4α: Hepatocyte nuclear factor 4 alpha; H3K36me3: H3 trimethylation at lysine-36; H3K4me3: H3 trimethylation at lysine-4; H3K27me3: H3 trimethylation at lysine-27; H3K9me3: H3 trimethylation at lysine-9; Pol2: Polymerase 2; HNF4α: Hepatocyte nuclear factor 4 alpha; ChIP-seq: Chromatin immunoprecipitation-sequencing; IGV: Integrative genomics viewer.

human pri-miR-194-2, prominent sequential peaks of DHSs, HNF4α, Pol2, H3K4me3 and H3K36me3 were identified in the human *miR-194-2/miR-192* gene cluster in HepG2 cells (Figure 6A). Very similar to the mouse miR-101b, the human *miR-101-2* gene body is located in the intron8-9 of the *RCL1* gene, and clear (but weaker than miR-194-2) sequential peaks of HNF4α, Pol2, H3K4me3 and H3K36me3 were identified in the intron5-6 of RCL1 (Figure 6B). These data strongly suggest that HNF4α may also have a direct critical role in regulating hepatic expression of miR-194-2/miR-192 and miR-101-2 in humans. In contrast, there were no clear peaks of HNF4α, Pol2, or H3K4me3 (Figure 6C) in the miR-122 locus which is known to be silenced in HepG2 cells[42].

Regulation of the mouse and human miR-194-2/miR-192 gene cluster by HNF4α

Hepatic expression of miR-194 is markedly down-regulated in mice null for Hnf1α[36], a down-stream target

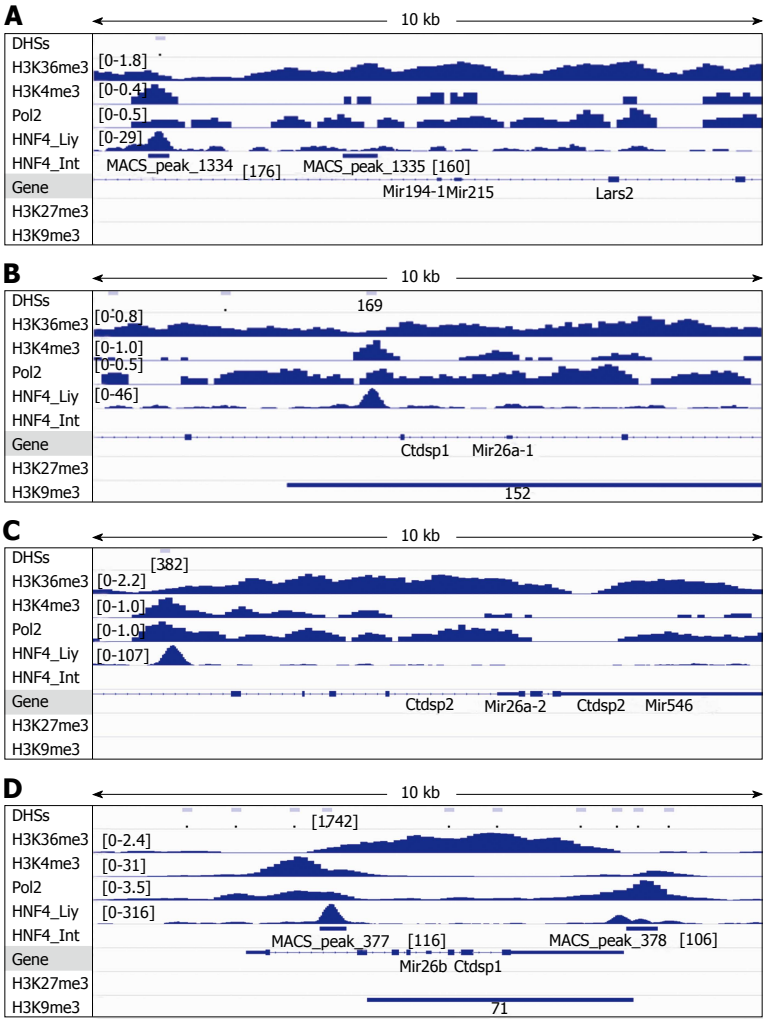

Figure 4 Analysis of DNAse-I hypersensitive sites as well as DNA-binding of HNF4α, RNA polymerase Ⅱ (Pol2), and methylated histones to loci of miR-194-1/miR-215 (A), miR-26a-1 (B), miR-26a-2 (C) and miR-26b (D) in wildtype mouse liver. DNA-binding of HNF4α to these microRNA loci in the mouse small intestine (HNF4α _Int) was compared to those in the mouse liver (HNF4α_Liv). Data of DHSs (determined by DNAse-seq) and DNA-binding of proteins (determined by ChIP-seq) were retrieved from the public database of GEO DataSets and visualized in the IGV software. The peak values/ranges for each mark were shown in square brackets or under the line mark. DHSs: DNAse-I hypersensitive sites; H3K36me3: H3 trimethylation at lysine-36; H3K4me3: H3 trimethylation at lysine-4; H3K27me3: H3 trimethylation at lysine-27; H3K9me3: H3 trimethylation at lysine-9; Pol2: Polymerase 2; HNF4α: Hepatocyte nuclear factor 4 alpha; ChIP-seq: Chromatin immunoprecipitation-sequencing; IGV: Integrative genomics viewer.

of HNF4α. In small intestine, miR-194 is transcriptionally up-regulated by Hnf1α[37]. Hepatic mRNA expression of Hnf1α decreased modestly in *Hnf4α*-LivKO mice[1]. We found that HNF1α and HNF4α modestly activated the reporter for the mouse *miR-194-2/miR-192* gene cluster 1.5 and 2.8 fold, respectively, and they synergistically activated mouse miR-194-2/miR-192 promoter 7.5 fold (Figure 7A). ChIP-seq results showed that HNF4α bound strongly to the distal promoter but weakly to the proximal promoter of human miR-194-2/miR-192 cluster (Figure 6A). To determine the role of HNF4α in regulating the *miR-194-2/miR-192* gene cluster in humans, we generated reporter vectors for the distal and proximal promoters of human miR-194-2/miR-192 cluster. Surprisingly, HNF4α only modestly activated the distal promoter 3 fold, but very strongly activated the proximal promoter of human miR-194-2/miR-192 cluster by 200 fold (Figure 7B). To identify the critical cis-elements responsible for the very strong transactivation of this proximal promoter by HNF4α, we engineered luciferase reporter constructs for the mutated 400-bp proximal promoter of human *miR-194-2* gene cluster. Surprisingly, mutations of the 3 putative HNF4-binding sites (HNF4-RE) within the 400-bp miR-194-2 promoter had little effects on the transactivation of this promoter by HNF4α (Figure 7C). HNF4α can transactivate the human p21 promoter *via* physically interacting with the general transcription factor SP1, independent of DNA-binding of HNF4α, because the S304D mutant of HNF4α which has markedly decreased DNA-binding activity[50], is equally active as the WT HNF4α in transactivating p21[51]. Thus, we tested the hypothesis that HNF4α can DNA-binding-independently transactivate the proximal human miR-194-2 promoter *via* interacting with SP1. We found that mithramycin, a widely used SP1 inhibitor[52], dramatically suppressed the HNF4α-transactivation of both the WT and HNF4RE-mutant miR-194-2 promoter by 94% and 95%, respectively (Figure 7C). Moreover,

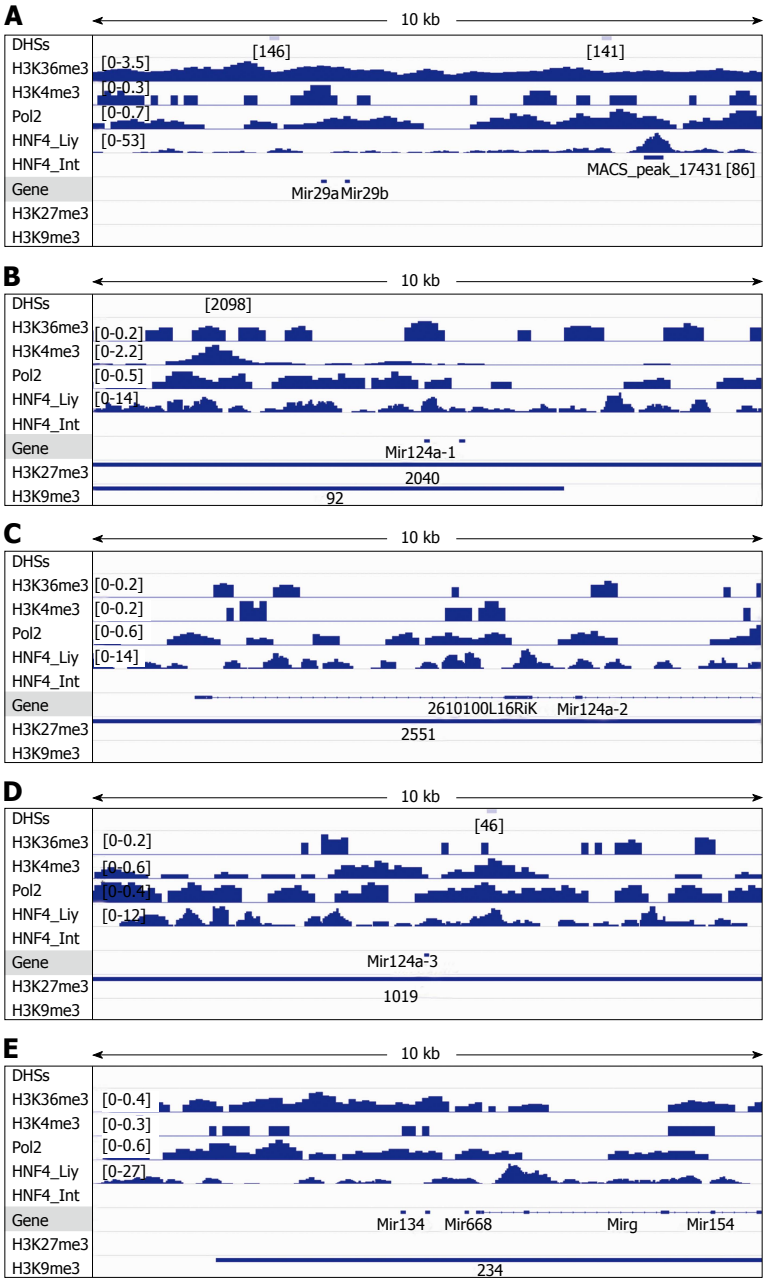

Figure 5 Analysis of DNAse-I hypersensitive sites as well as DNA-binding of HNF4α, RNA polymerase II (Pol2), and methylated histones to loci of miR-29a/miR-29b (A), miR-124a-1 (B), miR-124a-2 (C), miR-124a-3 (D) and miR-134 (E) in wildtype mouse liver. DNA-binding of HNF4α to these microRNA loci in the mouse small intestine (HNF4α _Int) was compared to those in the mouse liver (HNF4α_Liv). Data of DHSs (determined by DNAse-seq) and DNA-binding of proteins (determined by ChIP-seq) were retrieved from the public database of GEO DataSets and visualized in the IGV software. The peak values/ranges for each mark were shown in square brackets or under the line mark. DHSs: DNAse-I hypersensitive sites; H3K36me3: H3 trimethylation at lysine-36; H3K4me3: H3 trimethylation at lysine-4; H3K27me3: H3 trimethylation at lysine-27; H3K9me3: H3 trimethylation at lysine-9; Pol2: Polymerase 2; HNF4α: Hepatocyte nuclear factor 4 alpha; ChIP-seq: Chromatin immunoprecipitation-sequencing; IGV: Integrative genomics viewer.

the S304D-mutant of HNF4α was equally active as the WT HNF4α in transactivating the proximal human miR-194-2 promoter (Figure 7D). Taken together, these data strongly indicate that HNF4α can DNA-binding-independently transactivate the proximal human miR-194-2 promoter *via* interacting with SP1.

Regulation of mouse miR-101b and human miR-101-2 promoters by HNF4α

The mouse miR-101b promoter was moderately active in HepG2 cells (Figure 8A), whereas the human miR-101-2 promoter was largely inactive in HepG2 cells (Figure 8B). C/EBPα, a liver-enriched transcription factor, plays a key role in regulating liver-specific gene expression. The expression of C/EBPα is low in HepG2 cells, and re-expression of C/EBPα in HepG2 cells can reactivate certain liver-specific genes[53]. Our analysis of published ChIP-seq data for C/EBPα in mouse liver (GSM1037657) showed that C/EBPα bound to the miR-101b promoter, located in the Intron5-6 of Rcl1, in close proximity to

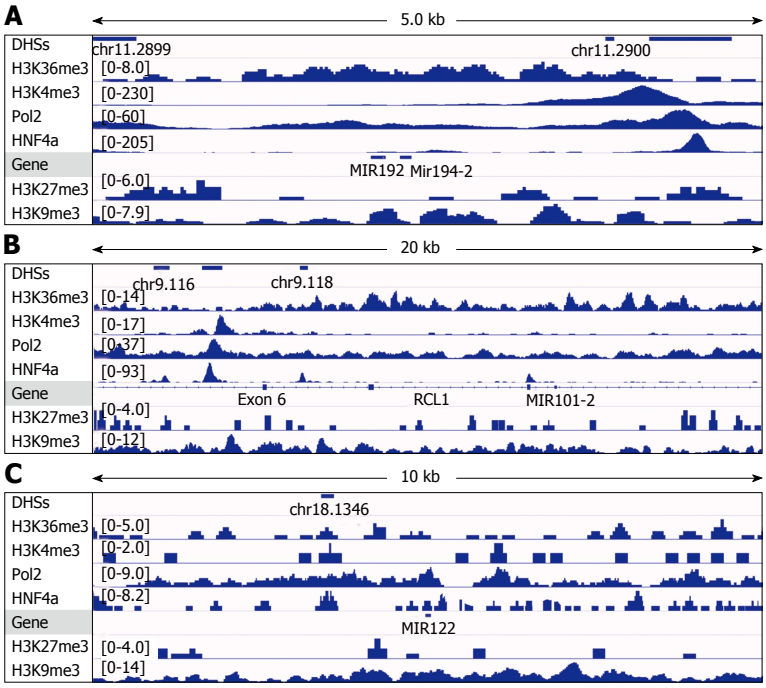

Figure 6 Analysis of DNAse-I hypersensitive sites as well as DNA-binding of HNF4α, RNA polymerase II (Pol2), and methylated histones to loci of miR-194-2/miR-192 (A), miR-101-2 (B) and miR-122 (C) in human hepatoma HepG2 cells. Data of DHSs (determined by DNAse-seq) and DNA-binding of proteins (determined by ChIP-seq) were retrieved from the public database of GEO DataSets and visualized in the IGV software. The peak values/ranges for each mark were shown in square brackets or under the line mark. DHSs: DNAse-I hypersensitive sites; H3K36me3: H3 trimethylation at lysine-36; H3K4me3: H3 trimethylation at lysine-4; H3K27me3: H3 trimethylation at lysine-27; H3K9me3: H3 trimethylation at lysine-9; Pol2: Polymerase 2; HNF4α: Hepatocyte nuclear factor 4 alpha; ChIP-seq: Chromatin immunoprecipitation-sequencing; IGV: Integrative genomics viewer.

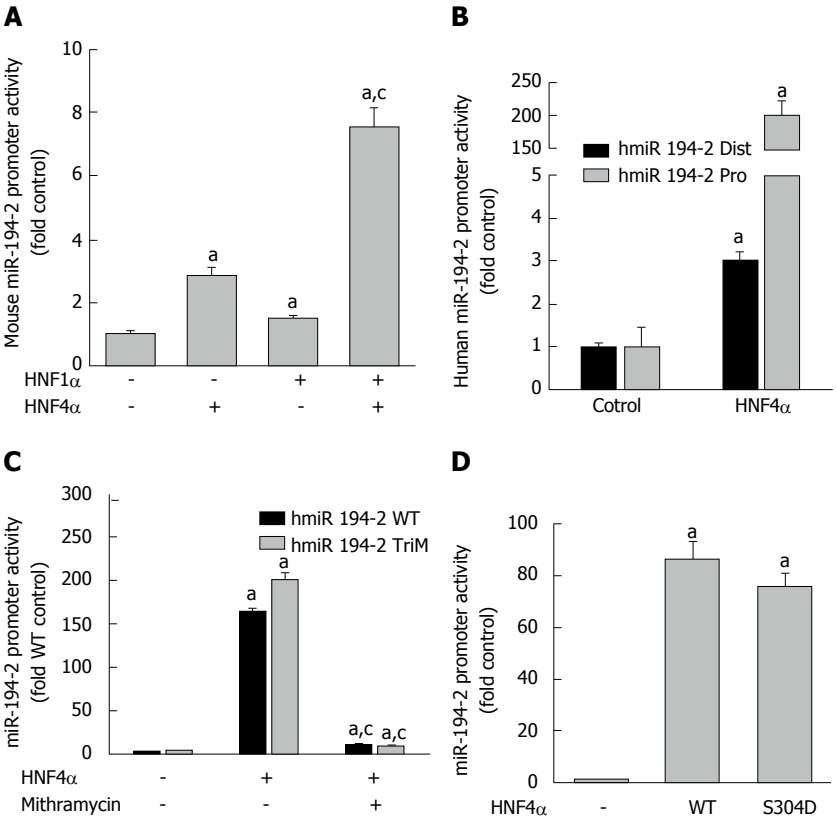

Figure 7 Activation of mouse (A) and human (B-D) miR-194-2/miR-192 promoter by HNF4α. Human hepatoma HepG2 cells were transfected with firefly luciferase vectors containing wild-type and mutant miR-194-2 promoter, pRL-CMV, and an expression vector for HNF4α/HNF1α. Dual-luciferase reporter assay was conducted 24 h after transfection. The y-axis represents relative luciferase activity for microRNA promoter normalized by the renilla luciferase. $n = 4$, Mean ± SE. $^{a}P < 0.05$ compared to vector control; $^{c}P < 0.05$ compared to HNF4α alone group. HNF4α: Hepatocyte nuclear factor 4 alpha.

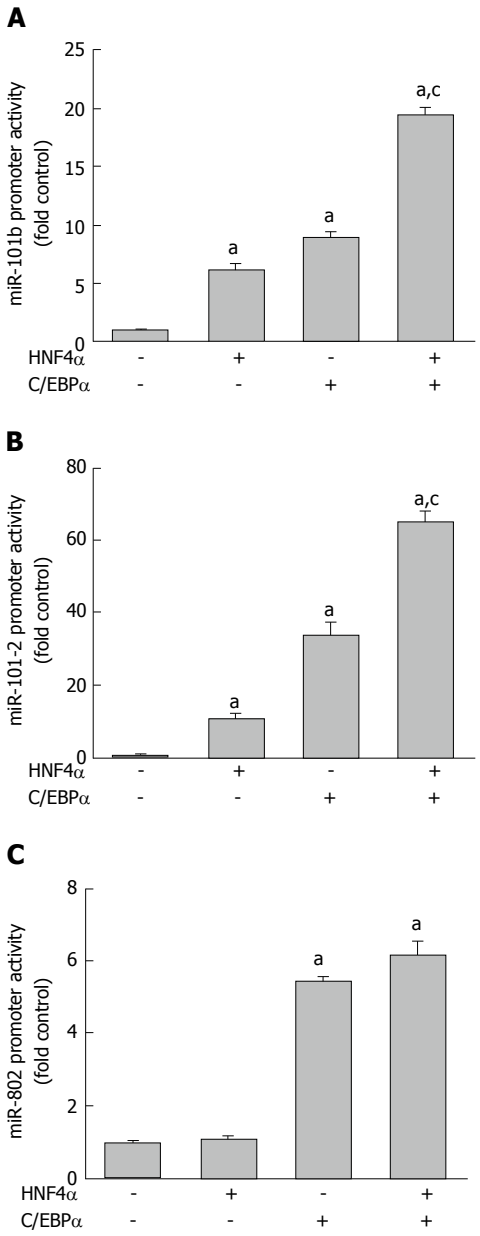

Figure 8 Activation of (A) mouse miR-101b, (B) human miR-101-2, and (C) mouse miR-802 promoter by HNF4α. Human hepatoma HepG2 cells were transfected with firefly luciferase vectors containing microRNA promoter, pRL-CMV, and an expression vector for HNF4α and/or C/EBPα. Dual-luciferase reporter assay was conducted 24 h after transfection. The Y-axis represents relative luciferase activity for microRNA promoter normalized by the renilla luciferase. n = 4, Mean ± SE. $^{a}P < 0.05$ compared to vector control; $^{c}P < 0.05$ compared to HNF4α alone group. HNF4α: Hepatocyte nuclear factor 4 alpha; C/EBPα: CCAAT/enhancer-binding protein α.

HNF4α. Moreover, putative C/EBP binding sites are highly enriched in the human miR-101-2 promoter, predicted by the Alibaba2 software. We found that HNF4α and C/EBPα activated the mouse miR-101b promoter 6.2 and 8.9 fold, respectively, and they synergistically activated the miR-101b promoter 19 fold in HepG2 cells (Figure 8A). Similarly, HNF4α and C/EBPα activated the human miR-101-2 promoter 11 and 33 fold, respectively, and they synergistically activated the miR-101-2 promoter 65 fold in HepG2 cells (Figure 8B).

Regulation of mouse miR-802 promoter by HNF4α

Different from miR-101, HNF4α had no effect on the 2 kb mouse miR-802 promoter, and HNF4α did not enhance the transactivation of the miR-802 promoter by C/EBPα in HepG2 cells (Figure 8C).

Regulation of mouse Chd1 and H3f3 by miR-194 and miR-192

TargetScan was used to identify potential targets of liver-predominant microRNAs down-regulated in *Hnf4α*-LivKO livers. miR-192/215 and miR-194 have a perfect match (8 mer) and very high context score percentile of 96%-99% with human and mouse histone H3f3b (H3.3b) and Chd1, respectively, indicating a very high likelihood of inhibition (Table 1). Therefore, we generated luciferase reporters for the 3'UTR of H3.3 and Chd1. Results of dual luciferase assay showed that miR-194 and miR-192 significantly decreased the luciferase activity for the 3' UTR of Chd1 (Figure 9A) and H3.3 (Figure 9B) by 37% and 36%, respectively, in HepG2 cells.

DISCUSSION

The present study demonstrates that Hnf4α is essential for hepatic expression of certain liver-predominant microRNAs, namely miR-101, miR-192, miR-193 and miR-194. HNF4α transactivates these miRs *via* direct DNA-binding to the promoters and/or interacting with the general transcription factor SP1. These miRs target essential epigenetic modifiers, such as EZH2 (by miR-101), histone H3.3 (by miR-192) and Chd1 (by miR-194) (Figure 10).

The present data provide the first evidence that HNF4α is essential for hepatic expression of miR-194 in mice, and likely in humans. In both mice and humans, miR-194 is expressed highly in kidney and GI tract including liver and small intestine[37]. The tissue distribution of miR-194 parallels that of HNF4α. In liver, miR-194 signals are detected in hepatocytes but not in non-parenchymal cells, and miR-194 is down-regulated during dedifferentiation of hepatocytes[54]. miR-194 inhibits the metastasis of mesenchymal-like liver cancer cells. Moreover, ChIP-seq results demonstrate direct binding of HNF4α to the distal and proximal promoters of mouse and human miR-194-2 (Figure 3B and 6A). Furthermore, results of reporter assays indicate that HNF4α potently activates the promoter of mouse and human *miR-194-2/miR-192* gene cluster (Figure 7). Taken together, these data strongly indicate that HNF4α plays a key role in maintaining hepatic expression of miR-194 in mice and humans.

Two recent studies of mice with inducible knockout of Hnf4α demonstrate that acute loss of Hnf4α in adult mouse liver triggers extensive hepatocyte proliferation, hepatomegaly, and increased HCC[55-57]. The increased intestinal cell proliferation in mice with specific loss of Hnf4α in the adult intestinal epithelium is ascribed to the activation of the Wnt/beta-catenin system[58]. miR-194 negatively control expression of frizzled-6, which activates the beta-catenin pathway[36]. Therefore, Hnf4α may

Table 1 Targeting of human and mouse genes by liver-predominant microRNAs predicted by TargetScan

	Predicted pairing of target region (top) and microRNA (bottom)	Seed match	Context score percentile
Position 1085-1091 of human H3F3B 3′UTR miR-192/215	5′ ...AUUUACUGAAGUUUUUAGGUCAA... ||||||| 3′ CCGACAGUUAAGUAUCCAGUC	8 mer	96
Position 1064-1070 of mouse H3f3b 3′UTR miR-192/215	5′ ...UCCUAUUGAAGUUUUUAGGUCAA... ||||||| 3′ CCGACAGUUAAGUAUCCAGUC	8 mer	99
Position 1109-1115 of human CHD1 3′UTR miR-194	5′ ...GACUUUUAAUAUAAACUGUUACA... ||||||| 3′ AGGUGUACCUCAACGACAAUGU	8 mer	99
Position 1100-1106 of mouse Chd1 3′UTR miR-194	5′ ...GCUUUAAUAUAAAAACUGUUACA... ||||||| 3′ AGGUGUACCUCAACGACAAUGU	8 mer	99

3′UTR: Untranslated regions.

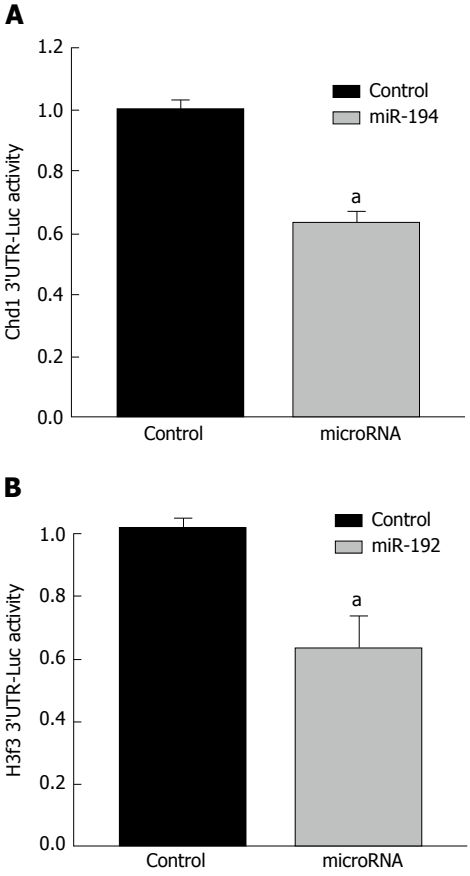

Figure 9 Effects of miR-194 and miR-192 on the activities of luciferase reporter vectors for the 3′UTR of mouse Chd1 and H3f3. Human hepatoma HepG2 cells were transfected with plasmid DNA including pmiR-Chd1 (or pmiR-H3f3), the pRL-CMV luciferase, and a synthetic mimic of miR-194/miR-192, or AllStars Negative Control siRNA (as negative control for microRNAs) using Lipofectamine 2000. Dual-luciferase reporter assay was conducted 24 h after transfection. The Y-axis represents relative luciferase activity for the 3′UTR of Chd1 or H3f3 normalized by the renilla luciferase. $n = 4$, Mean ± SE. $^{a}P < 0.05$ compared to control (AllStars Negative Control siRNA). 3′UTR: Untranslated regions.

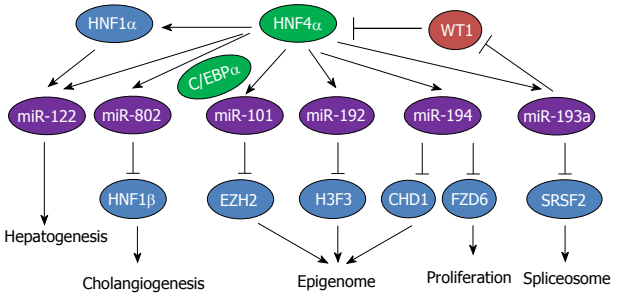

Figure 10 Diagram that illustrates the regulation of hepatic microRNA expression by Hnf4α in mouse liver. HNF4α: Hepatocyte nuclear factor 4 alpha; C/EBPα: CCAAT/enhancer-binding protein α.

inhibit cell proliferation through the miR-194→frizzled-6→beta-catenin signaling pathway.

The chromatin remodeling factor CHD1 is required to maintain the open chromatin and pluripotency of mouse embryonic stem cells[40]. CHD1 is required for chromatin incorporation of the histone variant H3.3, which is generally associated with active genes[59]. However, CHD1 may also repress gene expression *via* association with HDACs[60]. Overexpression of HNF4α in hepatoma cells dramatically decreased the "stemness" gene expression and the percentage of cancer stem cells in HCC[8]; however, the underlying mechanism is unknown. HNF4α, *via* regulating miR-194, might inhibit stemness gene expression by targeting the chromatin remodeling factor CHD1, which deposits the unmodified or altered histone H3.3 into chromatin and increases the stemness of *Hnf4α*-LivKO hepatocytes.

The present data indicate that Hnf4α is essential for hepatic expression of miR-192, and the histone variant H3.3 is a direct target of miR-192. Thus, down-regulation of miR-192 may be the underlying mechanism of hepatic induction of H3.3 in young-adult *Hnf4α*-LivKO mice[21]. The replacement H3 variant H3.3 is encoded by two genes termed H3.3A and H3.3B, both code for the same amino acid sequence, but differ in nucleotide sequences and gene organization[61]. H3.3 is the exclusive substrate for replication-independent deposition, which provides a mechanism for the immediate activation of genes that are silenced by histone modification[62,63], and H3.3 is important in epigenetic memory[64]. H3.3/H2A.Z double variant-containing nucleosomes mark "nucleosome-free regions" of active promoters and other regulatory

regions[65]. Deposition of H3.3 can rapidly derepress gene silencing[66]. Taken together, Hnf4α directly regulates miR-192, and the down-regulation of miR-192 in *Hnf4α*-LivKO livers may be the underlying mechanism of hepatic induction of H3.3, which contributes to the marked alteration of epigenome and transcriptome in *Hnf4α*-LivKO livers[21].

The present study indicates that Hnf4α is required for hepatic expression of the tumor-suppressor miR-101. miR-101 is predominantly expressed in the liver[35]. miR-101 is down-regulated in HCC[67] and miR-101 directly represses EZH2[68,69], a protooncogene that silences the expression of tumor-suppressors *via* H3K27me3. Down-regulation of miR-101 in *Hnf4α*-LivKO mouse livers might be the underlying mechanism of induction of EZH2 and increased H3K27me3 observed previously[21].

The present data indicate that Hnf4α is important for hepatic basal expression of the tumor-suppressor miR-193a. miR-193a and miR-365 closely cluster in chromosome 11 in mice. The tumor-suppressor miR-193a is down-regulated in the majority of HCC in humans[70] and miR-193a prevents the resistance of HCC to 5-fluorouracil *via* repressing the expression of serine/arginine-rich splicing factor 2 (SRSF2)[71]. Through maintaining hepatic expression of miR-193a, HNF4α might regulate expression of SRSF2 and the splicing of transcripts in liver. Interestingly, miR-193a also targets directly Wilms' tumor protein 1 (WT1)[72]. WT1 is overexpressed in cirrhotic liver and HCC[18,73], and induction of WT1 down-regulates HNF4α expression in liver[18]. The putative feedback regulatory loop of HNF4α→miR-193a→WT1 and its significance in liver cirrhosis and carcinogenesis warrant further investigation.

The present data provide the first evidence that Hnf4α is important for hepatic expression of mir-802 (Figure 2). Results of reporter assay (Figure 8C) suggest that HNF4α may indirectly regulate hepatic miR-802 expression *via* C/EBPα, whose DNA-binding activity decreased in *Hnf4α*-LivKO mice[27] and human hepatoma cells. Interestingly, the miR-802 locus is marked with both the activating signature H3K4me3 and the silencing signature H3K27me3, a feature of bivalent chromatin which allows a low basal expression but timely activation of developmentally-regulated genes[74]. Hnf1β is a direct target of mir-802[75], and Hnf1β is overexpressed in adult *Hnf4α*-LivKO mouse livers[4]. In mouse liver, mir-802 is expressed at 10-fold higher levels in hepatocytes than non-hepatocytes[75]. In contrast, Hnf1β is strongly expressed in cholangiocytes but weakly in hepatocytes, and Hnf1β plays a key role in bile-duct morphogenesis and glucose homeostasis[76]. Thus, the putative HNF4α →C/EBPα→mir-802→HNF1β pathway might play a role in controlling cell-specific expression of HNF1β and liver morphogenesis during liver development.

The tumor-suppressor microRNAs miR-34a, miR-192, miR-215 and miR-194 are all p53-inducible microRNAs[77]. The induction of the p53-target gene p21 in Hnf4α-nul mouse livers[13] suggests that p53 is activated by *Hnf4α* deficiency, which may contribute to the induction of the p53-target miR-34a and miR-29b (Figure 2B). However, hepatic expression of other p53-target microRNAs miR-192, mir-215 and miR-194 are markedly down-regulated in *Hnf4α*-LivKO mice. It is interesting that HNF4α can transactivate two p53-target genes, p21 and miR-194 (Figure 7), independent of DNA-binding of HNF4α to the promoter. The AMP-activated protein kinase (AMPK) phosphorylates HNF4α at S304, resulting in a marked decrease in the DNA-binding activity and decreased transactivation of apolipoprotein C3[50]. AMPK suppresses lipogenesis and carcinogenesis in liver[78,79]. The contribution of selective modulation of HNF4α-target lipogenic genes and tumor-suppressors (p21 and miR-194) to the physiological and pharmacological roles of AMPK in liver diseases warrants further investigation.

miR-29 is broadly expressed at high levels in normal tissues. miR-29 sensitizes cholangiocarcinoma cells to TNF-induced cytotoxicity[80] and miR-29 activates p53[81]. miR-29 induces global DNA hypomethylation and tumor suppressor gene reexpression in lung cancer and acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1[82,83]. miR-29 also directly inhibits Dnmt3a and Dnmt3b in mice[84]. Thus, induction of miR-29b might contribute to the lack of global changes in hepatic DNA methylation, despite an induction of Dnmt1, in the young-adult *Hnf4α*-LivKO mice[21]. Currently, the mechanism of induction of miR-29 in the young-adult *Hnf4α*-LivKO mice remains unknown. miR-29 can be transactivated by p53[85]. Thus, activation of p53 might contribute to hepatic induction of miR-29b in *Hnf4α*-LivKO mice.

The liver-specific miR-122 is important in regulating hepatic cholesterol and lipid metabolism[86,87], and down-regulation of miR-122 contributes to HCC malignancy[88-90]. HNF4α can directly activate the expression of miR-122 in mouse liver[91]. However, knockdown of HNF4α does not affect the high expression of miR-122 in a HCC cell line, although miR-122 expression correlates strongly with HNF4α[88]. In contrast, hepatic miR-122 expression is regulated by Hnf1α[88]. The moderate down-regulation of miR-122 in *Hnf4α*-LivKO mouse livers parallels the moderate decrease of Hnf1α in these mice[27]. Taken together, these data suggest that HNF4α has a positive but limited role in regulating hepatic expression of miR-122.

The present study demonstrates species differences between humans and mice in hepatic basal expression and regulation of miR-124 and miR-134 by HNF4α. Interleukin-6 (IL6) plays a key role in inflammation and hepatocarcinogenesis[92]. Interestingly, HNF4α exerts anti-inflammatory effects in human hepatocytes *via* the miR-124-IL6R-STAT3 pathway; knockdown of HNF4α in human hepatocytes leads to down-regulation of miR-124, induction of IL6R and IL6, and activation of STAT3[49]. However, there is no induction of IL-6 or activation of STAT3 in adult mice with acute loss of HNF4α[56]. Thus, there may be species difference between humans and mice regarding the interaction of HNF4α with miR-regulated inflammatory and carcinogenic pathways in the liver.

Our previous study found that Hnf4α deficiency in

young-adult mice causes marked alteration of histone modifications, which is associated with induction of epigenetic modifiers such as Ezh2 and histone H3.3[20]. However, ChIP-seq data reveal no direct binding of Hnf4α to these epigenetic modifiers in adult mouse livers, suggesting that these epigenetic modifiers may not be directly regulated by Hnf4α. The present study provides the first evidence of the essential role of Hnf4α in maintaining hepatic expression of certain microRNAs, including miR-101, miR-192, miR-193a, miR-194 and miR-802. These microRNAs target certain key proteins in gene regulation and epigenetic modifications, such as WT1 (by miR-193a)[72], HNF1β (by miR-802)[75], CHD1 (by miR-194) (Figure 9), EZH2 (by miR-101)[69], SRSF2 (by miR-193a)[71], and histone H3.3 (by miR-192) (Figure 9). Establishment and maintenance of hepatic expression of these microRNAs by HNF4α may play a key role in the indirect regulation of hepatic transcriptome and epigenome by HNF4α (Figure 10).

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COMMENTS

Background

Hepatocyte nuclear factor 4 alpha (HNF4α) is a liver-enriched master regulator of liver development and function. HNF4α plays a key role in regulating hepatic transcriptome and epigenome. However, little was known about the role of HNF4α in regulating hepatic expression of microRNAs, essential modulators of the transcriptome and epigenome. Additionally, HNF4α deficiency causes marked induction of a large number of genes in mouse liver; however, the mechanism of suppression of hepatic gene expression by HNF4α remains poorly understood.

Research frontiers

Previous studies demonstrate that HNF4α regulates hepatic expression of miR-122, miR-124 and miR-29.

Innovations and breakthroughs

This is the first study to use microarray and liver-specific knockout mice to determine the genome-wide role of HNF4α in the regulation of hepatic expression of microRNAs in mice. The key changes in hepatic microRNA expression induced by HNF4α deficiency were verified by real-time polymerase chain reaction. Moreover, hepatic microRNA expression were correlated with chromatin accessibility as well as DNA-binding of HNF4α, RNA polymerase Ⅱ, and activating/silencing epigenetic signatures to determine the role of HNF4α in regulating hepatic expression of these microRNAs. The novel key role of HNF4α in regulating liver-predominant expression of miR-101-2/miR-101b and the miR-194-2/miR-192 cluster was confirmed by luciferase reporter assay.

Applications

Results from this study uncover species differences and similarities between humans and mice in the role of HNF4α in regulating hepatic expression of certain important microRNAs. Such novel knowledge will help understand the role of HNF4α in post-transcriptional regulation of gene expression and maintenance of the normal epigenome and physiology in mouse and human liver.

Terminology

Epigenetic signatures/marks are modifications of the genome that do not change the underlying DNA sequence but can switch genes on and off and thus affect how cells express genes. Typical epigenetic signatures/marks include DNA methylation and histone modifications.

Peer-review

The study appears to be properly conducted and written. No major criticisms and/or weaknesses were noted.

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ORIGINAL ARTICLE

Basic Study

Antioxidant effects of aqueous extract of Salep on Paraquat-induced rat liver injury

Shekoufeh Atashpour, Hossein Kargar Jahromi, Zahra Kargar Jahromi, Sanaz Zarei

Shekoufeh Atashpour, 2nd Department of Pharmacology, Jahrom University of Medical Sciences, Jahrom 7414846192, Iran

Shekoufeh Atashpour, Hossein Kargar Jahromi, Research Center for Non-Communicable Disease, Jahrom University of Medical Sciences, Jahrom 7414846192, Iran

Hossein Kargar Jahromi, 2nd Zoonoses Research Center, Jahrom University of Medical Sciences, Jahrom 7414846192, Iran

Zahra Kargar Jahromi, Zoonoses Research Center, Jahrom University of Medical Sciences, Jahrom 7414846192, Iran

Sanaz Zarei, Student Research Committee, Jahrom University of Medical Sciences, Jahrom 7414846192, Iran

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Correspondence to: Hossein Kargar Jahromi, PhD, Research Center for Non-Communicable Diseases, Jahrom University of Medical Sciences, Shahid Motahari Street, Jahrom 7414846192, Iran. hossein.kargarjahromy@yahoo.com
Telephone: +98-71-54340405
Fax: +98-71-54340405

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Abstract

AIM

To evaluate the effects of aqueous extract of Salep on Paraquat-mediated liver injury.

METHODS

In this experimental study, 56 adult male Wistar rats were divided randomly to 7 groups as control, sham, and 5 experimental groups. In control group, rats did not receive any substance during experiment. In Sham group, rats were given distilled water according to their body weight and in experimental groups, Paraquat alone and with different doses of Salep aqueous extract

(40, 80, 160 and 320 mg/kg) was given intraperitoneal daily for 14 d. After that, liver biochemical parameter and histologic changes were analyzed and compared in different groups.

RESULTS

Paraquat compared to control and sham groups, significantly ($P < 0.05$) increased serum level of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin, malondialdehyde (MDA) and total oxidant capacity (TOC); while level of total protein, albumin and total antioxidant capacity (TAC) were remarkably decreased by Paraquat. Salep at doses of 80, 160 and 320 mg/kg significantly decreased serum level of ALT, AST, ALP, bilirubin, MDA and TOC and significantly increased total protein, albumin and TAC level as compared to Paraquat exposed group in dose dependent manner. Aqueous extract of Salep at doses of 40 mg/kg made no significant changes in serum level of mentioned biochemical parameters. Liver microscopic observation revealed that Paraquat could cause hepatocyte necrosis, degenerative changes, proliferation and activation of Kupffer cells (sporadically) which were reduced by Salep treatment.

CONCLUSION

Salep possesses remarkable hepatoprotection activity against Paraquat-induced hepatic injury by having antioxidant activity and reducing lipid peroxidation and oxidative stress.

Key words: Salep; Paraquat; Liver injury; Antioxidant; Oxidative stress

Core tip: Oxidative stress has a key role in triggering Paraquat-mediated liver injury. Paraquat causes oxidative stress *via* modulation of redox cycling, generation of free radicals and reduction of endogenous antioxidant levels. Salep from orchid family (Orchidaceae) used in traditional medicine as a healing agent in the treatment of breast disorders, gastrointestinal disorders, tuberculosis, diarrhea, Parkinson, cancer, fever, and impotency. Salep is used in food engineering for preparation of ice cream and drinks. This study showed that Salep could have a protective effect against Paraquat-induced hepatic injury *via* reinforcing endogenous antioxidant systems, reduction of lipid peroxidation and free radical scavenging. The antioxidant and protective effect of Salep could be due to presence of flavonoids and polyphenols such as Quercetin, Ferulic Acid and Glucomannan.

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INTRODUCTION

With the increasing population of human societies, providing nourishment without the use of advanced scientific farming is impossible. In this modern agriculture, using pesticides, herbicides and chemical fertilizers for more and higher quality crop is inevitable and may be toxic to man and animals. Paraquat (1, 1'dimethyl-4, 4'-bipyridylium-dichloride) is a widely used herbicide for broadleaf weed control[1] and is extremely poisonous for humans and animals and many cases of acute poisoning and death have been reported over the past few decades[2].

Paraquat is a bipyridyl compound with high toxicity for lungs, kidney, brain and liver[3]. When it is given in acute dose (50 mg/kg) in mice, liver necrosis and inflammation will develop[4]. Paraquat toxicity is due to oxidative damage to cells and generation of free radicals[5]. Herbicidal activity of paraquat can be explained by its interfering with photosynthesis and intracellular electron transfer system in plants and prevention of NADP reduction to NADPH. This could disrupt important NADPH-dependent biochemical processes[6,7]. In addition, Paraquat radical forms superoxide anion in presence of oxygen which leads to production of more toxic reactive oxygen species like hydrogen peroxides and hydroxyl radical and would cause oxidative stress[1,8]. Superoxide anion may also attack unsaturated lipids of membrane to form fatty acid hydroperoxide, resulting in lipid peroxidation, membrane injury, cell death and multi-system toxicity[9].

Due to the role of oxidative stress mechanisms in Paraquat toxicity and the lack of an effective antidote, researchers are currently focused on the importance of antioxidant in Paraquat poisoning management[10]. Many herbal compounds have antioxidant properties and can protect the liver from damaging agents like Paraquat. One of these plants is Salep from orchid family (Orchidaceae) which has different species worldwide[11]. Salep contains Quercetin, Nitrogenic materials, Ferulic acid, starch, protein, Glucomannan, Glucose, Daucosterol, Cirsilineol and steroids[11-13]. This plant is used in traditional medicine as a healing agent in the treatment of breast disorders, gastrointestinal disorders, tuberculosis, diarrhea, Parkinson, cancer, fever, and impotency. Salep is used in food engineering for preparation of ice cream and drinks[13-15].

Polyphenols, especially flavonoids such as quercetin, are important antioxidants found in Salep[12]. These compounds have hepatoprotective effects against liver damage caused by toxins and free radicals[16] and can also protect cells against depletion of glutathione by increasing the capacity of antioxidant enzymes such as glutathione reductase, glutathione peroxide and catalase[17]. Furthermore, glucomannan can inhibit oxidative stress and

effectively reduce alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels[18]. Therefore, the aim of this survey was to evaluate the effects of aqueous extract of Salep on Paraquat - induced hepatotoxicity.

MATERIALS AND METHODS

Chemicals

Paraquate was purchased from Ara Shimi-Iran Company. Paraquat was dissolved in distilled water and animals were given intraperitoneal injection in each case. Malondialdehyde (MDA), total antioxidant capacity (TAC) and total oxidant capacity (TOC) measurement kits were purchased from Diametra Company (Italy), ALT, AST, alkaline phosphatase (ALP), albumin and bilirubin and total protein (TP) measurement kits were purchased from Pars Azmoon Company (Iran).

Collection and extraction of Salep

Salep plants were obtained from farmlands around Yasouj (a city in the southwest of Iran). Salep roots were washed and dried in laboratory and mixed with Ethanol 96° in 1 to 5 proportions, mixed for 24 h at room temperature and a homogeneous mixture was obtained. Then, the uniform solution was filtered and dried for 48 h to obtain solid extract without ethanol. The final dried extract was dissolved with distilled water[12].

Experimental animals

Fifty-six adult male Wistar rats (180-200 g) were obtained from the Animal House of Jahrom University of Medical Sciences. The animal house temperature was maintained at 22 ℃ ± 2 ℃ with a 12 h light/dark cycle. All animals were kept for two weeks prior to experiment and had free access to food and water. All ethical points regarding working with laboratory animals were considered in this research (Ethical Code: IR.JUMS.1394.722).

Experimental design

The rats were divided randomly to 7 groups, 8 rats each, as followed: Control group: Rats did not receive any substance during experiment; Sham group: Rats were given distilled water according to their body weight during the experiment; Experimental group 1: Rats were given Paraquat 2 mg/kg per BW; Experimental groups 2, 3, 4 and 5: Rats were given Paraquat at a dose of 2 mg/kg per BW daily and Salep at doses of 40, 80, 160 and 320 mg/kg per BW, respectively. Salep doses were selected based on previous studies done on this herbal treatment[12]; Paraquat and Salep aqueous extract were administered intraperitoneally daily for 14 d in all 5 groups.

Blood sampling and liver function evaluation

At the end of the study (day 15) after weighing the animals, blood sample were taken directly from their hearts using 5 cc syringes (rats were anesthetized by barbiturate) and blood serum was collected after centrifugation (15 min, 3000 rpm) and stored at -20 ℃ until they were tested. Biochemical measurement kits (made in Iran and Italy) using the colorimetric method and an autoanalyzer machine (Selectera XL model made in Holland) were used for assessment of biochemical factors including ALT, AST, ALP, TP, albumin, bilirubin, MDA, TOC and TAC.

Histological examination

After drawing the blood, for histological examination a small part of liver was separated, fixed by 10% formalin and embedded in paraffin wax. Paraffin sections with thickness of 5 μm were prepared, stained employing the haematoxylin and eosin and Masson Trichrome stain methods and histological and pathological changes were studied using a light microscope. Furthermore, The Degree of inflammation in the portal zone, liver necrosis and inflammatory cell infiltration were evaluated in the form of semiquantitative scale, double-blind, according to the method described by Frei *et al*[19] in 1984. Severity of damage were ranked from zero to four (zero: No damage, 1: Minimum damage, 2: Mild damage, 3: Average damage, 4: Severe damage). Scoring was performed in five microscopic fields of each cut, randomly, with magnification of × 100.

Statistical analysis

All values were given as mean ± SEM. Statistical analysis was carried out using SPSS 21, One-way analysis of variance followed by Duncan *post hoc* test. Statistical *P*-value less than 0.05 was considered significant.

RESULTS

Biochemical measurement

Paraquat compared to control and sham groups significantly ($P < 0.05$) increased serum level of liver factors including ALT, AST and ALP, Bilirubin, MDA and TOC; while serum level of Total Protein, Albumin and TAC were considerably lower in group receiving Paraquat (Tables 1 and 2).

Paraquat treatment groups with aqueous extract of Salep at doses of 80, 160 and 320 mg/kg significantly decreased serum level of ALT, AST, ALP, Bilirubin, MDA and TOC and significantly increased elevated Total Protein, Albumin and TAC serum level as compared to Paraquat treatment group alone (Tables 1 and 2). Aqueous extract of Salep at doses of 40 mg/kg made no significant changes in serum level of mentioned biochemical parameters while the greatest effect is related to the dose of 320 mg/kg of Salep.

Histopathological examination

Microscopic examination of liver tissue of control and sham groups showed that liver tissue structure was normal and healthy (normal structure of lobules with

Table 1 The serum levels of liver enzymes (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase), bilirubin, malondialdehyde and total oxidant capacity in different study groups

Group/parameter	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Bilirubin (mg/dL)	MDA (nmol/L)	TOC (IU/mL)
Control	183.4 ± 2.19	95.4 ± 2.52	138.4 ± 4.55	0.8 ± 0.02	0.13 ± 0.005	0.18 ± 0.01
Sham	185.5 ± 2.19	94.7 ± 3.00	138.9 ± 5.01	0.8 ± 0.02	0.13 ± 0.007	0.18 ± 0.01
Paraquat at 2 mg/kg	563.2 ± 11.43[a]	265.5 ± 7.48[a]	736.1 ± 4.21[a]	2.4 ± 0.05[a]	3.36 ± 0.06[a]	2.02 ± 0.05[a]
Paraquat + Salep at 40 mg/kg	536.4 ± 14.44[b]	252.5 ± 6.25[b]	730.0 ± 9.30[b]	2.3 ± 0.04[b]	3.27 ± 0.03[b]	1.98 ± 0.05[b]
Paraquat + Salep at 80 mg/kg	517.2 ± 7.30[b,c]	234.7 ± 7.14[b,c]	709.0 ± 9.14[b,c]	2.0 ± 0.04[b,c]	2.92 ± 0.13[b,c]	1.77 ± 0.05[b,c]
Paraquat + Salep at 160 mg/kg	460.5 ± 12.01[b,c]	209.4 ± 4.55[b,c]	626.4 ± 6.74[b,c]	1.7 ± 0.03[b,c]	2.48 ± 0.05[b,c]	1.55 ± 0.04[b,c]
Paraquat + Salep at 320 mg/kg	376.5 ± 12.07[b,c]	166.0 ± 3.75[b,c]	428.1 ± 7.25[b,c]	1.1 ± 0.03[b,c]	1.22 ± 0.04[b,c]	1.20 ± 0.03[b,c]

[a]Significant difference between Sham and Paraquate- exposed Group; [b]Significant difference between Sham and Paraquate + Salep treated Group; [c]Significant difference between Paraquate-exposed and Paraquate + Salep Treated Group (Based on Duncan's test). The means are presented in the form of Mean ± SEM. $P < 0.05$ is considered statistically significant. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; MDA: Malondialdehyde; TOC: Total oxidant capacity.

Table 2 The serum levels of albumin, total protein and total antioxidant capacity in different study groups

Group/parameter	Total protein (g/dL)	Albumin (g/dL)	TAC (IU/mL)
Control	8.0 ± 0.19	5.0 ± 0.13	1.11 ± 0.02
Sham	7.9 ± 0.26	5.1 ± 0.18	1.6 ± 0.04
Paraquat at 2 mg/kg	4.2 ± 0.09[a]	2.5 ± 0.06[a]	0.40 ± 0.02[a]
Paraquat + Salep at 40 mg/kg	4.1 ± 0.05[b]	2.6 ± 0.5[b]	0.43 ± 0.02[b]
Paraquat + Salep at 80 mg/kg	5.0 ± 0.10[b,c]	3.1 ± 0.04[b,c]	0.66 ± 0.03[b,c]
Paraquat + Salep at 160 mg/kg	5.7 ± 0.09[b,c]	3.6 ± 0.07[b,c]	0.82 ± 0.05[b,c]
Paraquat + Salep at 320 mg/kg	6.6 ± 0.11[b,c]	5.0 ± 0.07[b,c]	1.10 ± 0.03[b,c]

[a]Significant difference between Sham and Paraquate-exposed Group; [b]Significant difference between Sham and Paraquate + Salep treated Group; [c]Significant difference between Paraquate-exposed and Paraquate + Salep treated Group (Based on Duncan's test). The means are presented in the form of Mean ± SEM. $P < 0.05$ is considered statistically significant. TAC: Total antioxidant capacity.

normal central venous, sinusoids and Kupffer cells and normal distribution of glycogen and lack of lymphocytic infiltration and congestion in the blood vessels) (Figure 1A-D).

In study group 3 (rats were given Paraquat alone), microscopic observation revealed hepatocyte necrosis, degenerative changes, proliferation and activation of Kupffer cells (sporadically), increased infiltration of inflammatory cells around the portal vein and in sinusoid space, formation of fibrotic inflamed bridges between liver lobules, and sever cellular ballooning and blood congestion in the sinusoids. In this group, progressive liver fibrosis had occurred as evidenced by presence of collagen fibers in the liver parenchyma, the portal space and around the central vein in the centrilobular region (Figure 1E-J).

Treatment with aqueous extract of Salep reduced the damaging effect of Paraquat on liver tissue. This reduction in destructive effect of Paraquat on liver tissue was mild with Salep at doses of 80 mg/kg, moderate at doses of 160 mg/kg and highest at doses of 320 mg/kg as compared to study group that received Paraquat alone (Figure 1K-R). A microscopic observation of liver tissue of rats under study have been brought to a quantitatively in Table 3.

DISCUSSION

Oxidative stress has a key role in triggering Paraquat-mediated liver injury[20]. Paraquat causes oxidative stress *via* modulation of redox cycling, generation of free radicals and reduction of endogenous antioxidant levels[21-23]. Furthermore, generation of nitric oxide and reactive oxygen species like superoxide also play a crucial role in Paraquate - induced hepatotoxicity[24]. Salep could have protective effect against chemical induced liver injury *via* reinforcing endogenous antioxidant systems and free radical scavenging[12,16]. As liver is one of the major sites of Paraquat toxicity, this study was done in order to evaluate protective potential of Salep against Paraquat- induced liver injury.

Remarkable increase of ALT, AST, ALP and bilirubin and significant decrease of total protein and Albumin levels were observed in Paraquat - exposed group in comparison with control group, which confirmed the hepatotoxic potential of Paraquat. These results were in concurrence with previous studies on evaluation of Paraquat induced liver toxicity, which showed increase in serum level of liver enzymes[21,25]. Significant reduction of increased level of ALT, AST, ALP and bilirubin and marked increased in level of Albumin and Total Protein in Paraquat + Salep treated groups showed that Salep

Table 3 The effect of aqueous extract of Salep roots on Paraquate - induced rat liver injury

Group/damage score	Control	Sham	Paraquat 2 mg/kg	Paraquat + Salep at 40 mg/kg	Paraquat + Salep at 80 mg/kg	Paraquat + Salep at 160 mg/kg	Paraquat + Salep at 320 mg/kg
Portal congestion and inflammation							
Score 0	8	8	0	0	0	0	1
Score 1	0	0	0	0	0	0	5
Score 2	0	0	0	1	2	3	1
Score 3	0	0	2	2	2	1	1
Score 4	0	0	6	5	4	4	0
Necrosis							
Score 0	8	8	0	0	0	0	1
Score 1	0	0	0	0	0	1	4
Score 2	0	0	1	2	2	2	3
Score 3	0	0	2	1	2	2	0
Score 4	0	0	5	5	4	3	0
Interstitial infiltration of inflammatory cells							
Score 0	8	8	0	0	0	0	1
Score 1	0	0	0	0	0	1	4
Score 2	0	0	1	1	1	3	2
Score 3	0	0	1	2	3	2	1
Score 4	0	0	6	5	4	2	0

Zero: No damage; 1: Minimum damage; 2: Mild damage; 3: Average damage; 4: Severe damage. Scoring was performed in five microscopic fields of each cut, randomly, with magnification of × 100.

could have protective effect against Paraquat-mediated hepatic injury in dose dependent manner. These results are supported by a previous report which also revealed the protective effect of Salep on liver function[12].

Liver pathological examination showed hepatocyte necrosis, proliferation and activation of Kupffer cells, increased infiltration of inflammatory cells around the portal vein and in sinusoid space, formation of fibrotic inflamed bridges between liver lobules, and sever cellular ballooning and blood congestion in the sinusoids in Paraquat - exposed group, which was in accordance with previous reports[3,20]. Remarkable recovery toward normal liver histology in Paraquat + Salep treated groups also favored the protective activity of Salep against Paraquat-induced liver injury.

As oxidative stress has a crucial role in Paraquat-induced liver injury, in this study we evaluated serum level of TOC, which precisely shows the oxidant status of blood, and TAC, as indicator of blood, cells and tissues defense system against free radicals, measures the antioxidant capacity of all antioxidants in a biological sample and not just the antioxidant capacity of a single compound. Measurement of TAC can provide information on overall antioxidant status, which may include those antioxidants not yet recognized or not easily measured[20,26]. Significant augmentation of TOC and reduction of TAC were observed in Paraquat-exposed groups, which confirmed the role of free radical generation and attenuation of antioxidant level in Paraquat-medicated hepatic injury. Significant reduction of TOC and marked increased of TAC in Paraquate + Salep treated groups demonstrated that Salep could have protective effects against Paraquat toxicity by possessing antioxidant activity. These results were supported by a previous study done by Pourahmad *et al*[12]. The antioxidant effect of Salep could be due to the presence of flavonoids and polyphenols such as Quercetin, Ferulic Acid and Glucomannan[11,16,18]. The two latter components of Salep could also reduce serum level of liver enzymes such as ALT and AST[27,28]. Zhang *et al*[16] showed that Quercetin could have hepatoprotective and antioxidant activity by decreasing lipoxygenase, free radical scavenging, enhancing the expression of antioxidant transcription factor and antioxidant enzyme such as Thioredoxin and Peroxiredoxin.

Furthermore, part of Paraquat hepatotoxicity is related to lipid peroxidation due to free radical generation including Oxygen Reactive Species[24]. MDA works as an indicator of lipid peroxidation and oxidative stress assessment[29]. MDA level was significantly augmented in Paraquat - exposed group which was in accordance with previous studies[6]. Serum level of MDA was substantially decreased in Paraquat + Salep treated groups as compared with Paraquat - exposed group in dose dependent manner. This result also supported the protective activity of Salep against oxidative stress and lipid peroxidation caused by Paraquat.

In conclusion, based on our results, it could be concluded that Salep possesses remarkable hepatoprotection activity against Paraquat-induced liver injury and could reduce the damaging effect of Paraquat on liver by having antioxidant activity and reducing lipid peroxidation and oxidative stress. Further studies are required to evaluate protective and antioxidant effect of Salep in human.

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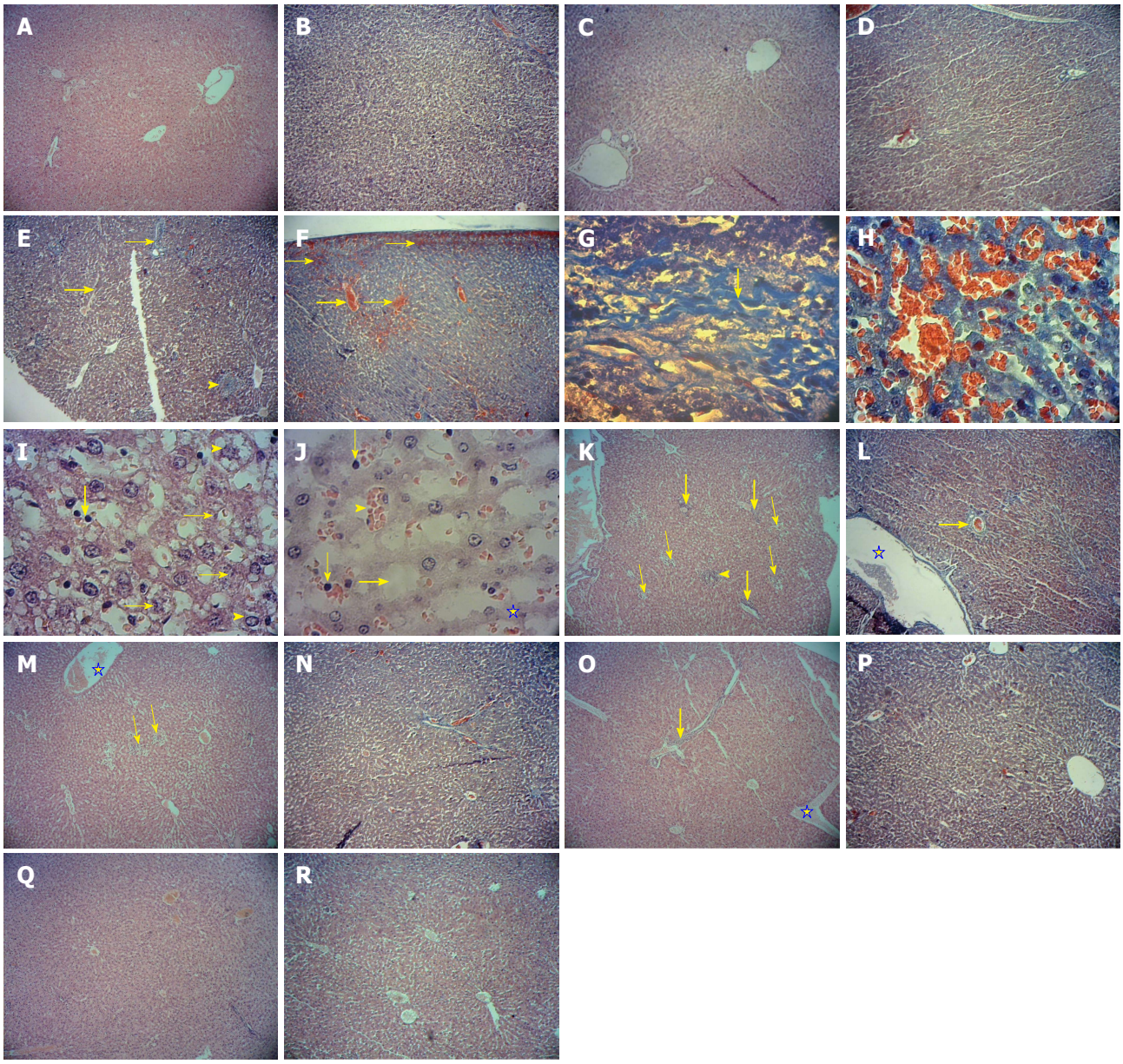

Figure 1 Microscopic views of the liver tissue in study Groups. Five micron paraffin sections were prepared, stained employing the haematoxylin and eosin stain and histological and pathological changes were studied using a light microscope. A: Control: With a natural structure (Hematoxylin-eosin, 40 × magnification); B: Control: With a natural structure and leukocyte infiltration and congestion cannot be seen (Masson trichrome, 40 × magnification); C: Sham: With a natural structure and Central venous congestion cannot be seen (Hematoxylin-eosin, 40 × magnification); D: Sham: With a natural structure and Collagen fibers cannot be seen. (Masson trichrome, 40 × magnification); E: Paraquat 2 mg/kg: Formation of fibrotic inflamed bridges between liver lobules (thin arrow), the loss of cellular order toward the center (wide arrow), accumulation of collagen fibers and inflammatory cells around the centrilobular vein (arrowhead) (Masson trichrome, 40 × magnification); F: Paraquat 2 mg/kg: Enlarged and congested centrilobular vein (wide arrow), congestion in the sinusoids (thin arrow) (Masson trichrome, 40 × magnification); G: Paraquat 2 mg/kg: Accumulation and progressive of collagen fibers in the liver parenchyma (Masson trichrome, 400 × magnification); H: Paraquat 2 mg/kg: Sever congestion in the sinusoids (Masson trichrome, 400 × magnification); I: Paraquat 2 mg/kg: Sever cellular ballooning (arrowhead), degenerative changes (thin arrow), proliferation and activation of Kupffer cells (wide arrow), (Hematoxylin-eosin, 400 × magnification); J: Paraquat 2 mg/kg: Activation of Kupffer cells (thin arrow), sever congestion in the sinusoids (arrowhead), degenerative changes (asterisk), enlargement of sinusoids spece(wide arrow), (Hematoxylin-eosin, 400X magnification); K: Paraquat + Salep at 40 mg/kg: Infiltration of inflammatory cells around the centrilobular vein (wide arrow), Infiltration of inflammatory cells around the portal space (arrowhead), degenerative changes (thin arrow), (Hematoxylin-eosin, 40 × magnification); L: Paraquat + Salep at 40 mg/kg: Enlargement and congested centrilobular vein (asterisk), accumulation of collagen fibers around the portal space (wide arrow) (Masson trichrome, 40X magnification); M: Paraquat + Salep at 80 mg/kg: Degenerative changes (thin arrow), congested centrilobular vein (asterisk) (Hematoxylin-eosin, 40 × magnification); N: Paraquat + Salep at 80 mg/kg: Decreased infiltration of inflammatory cells around the portal, decreased congestion in the sinusoids and more regular cellular order toward the center (Masson trichrome, 40 × magnification); O: Paraquat + Salep at 160 mg/kg: Infiltration of inflammatory cells around the portal space (wide arrow), congested centrilobular vein (asterisk), (Hematoxylin-eosin, 40 × magnification); P: Paraquat + Salep at 160 mg/kg: More regular cellular order toward the center, more decreased congestion of sinusoids and more decreased infiltration of inflammatory cells in the liver parenchyma (Masson trichrome, 40 × magnification); Q: Paraquat + Salep at 320 mg/kg: Its tissues seem relatively healthy, without any certain pathological changes (Hematoxylin-eosin, 40 × magnification); R: Paraquat + Salep at 320 mg/kg: Its tissues seem relatively healthy, without any certain pathological changes (Masson trichrome, 40 × magnification).

COMMENTS

Background

Paraquat is a common herbicide used in agriculture and could cause severe damage to the lungs, liver and other tissues in mammals. Oxidative stress has a key role in triggering Paraquat-mediated hepatotoxicity. Salep could have protective effect against chemical induced hepatotoxicity *via* reinforcing endogenous antioxidant systems and free radical scavenging.

Research frontiers

In the present study, the authors found that Salep possesses remarkable hepatoprotection activity against Paraquat-induced liver injury and could reduce damaging effect of Paraquat on liver by having antioxidant activity and reducing lipid peroxidation and oxidative stress.

Innovations and breakthroughs

This is the first study evaluating the effect of Salep on Paraquat-induced liver injury. This study investigates the protective and antioxidant effect of salep on liver damage caused by Paraquat. The results of current study demonstrated that Salep could ameloriate paraquate-mediated liver injury by having antioxidant activity and reducing lipid peroxidation and oxidative stress.

Applications

Salep aqueous extract could reduce damaging effect of Paraquat on liver tissue by having significant antioxidant activity. Therefore, the results of this study showed that Salep can be introduced as an alternative to chemical agents as potential therapeutic strategies for Paraquate-induced liver injury.

Terminology

Oxidative stress is essentially an disturbance in balance between the production of free radicals and the ability of the body to counteract or detoxify their harmful effects through neutralization by antioxidant which could cause tissue damange including liver. Lipid peroxidation is a crucial step in the pathogenesis of several disease states in adult and infant patients. Lipid peroxidation is a process mainly caused by the effect of reactive oxygen species including hydroxyl radical and hydrogen peroxide. These reactive oxygen species readily attack the polyunsaturated fatty acids of the cell membrane, initiating a self-propagating chain reaction. The destruction of membrane lipids and the end-products of such lipid peroxidation reactions are dangerous for the viability of cells, even tissues.

Peer-review

The paper by Atashpour *et al* has an interesting rationale and a good background. The results presented are consistent with the effects of the different components of the Salep.

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ORIGINAL ARTICLE

Clinical Trials Study

Phase 3 trial of first generation protease inhibitor therapy for hepatitis C virus/human immunodeficiency virus coinfection

Kenneth E Sherman, Minhee Kang, Richard Sterling, Triin Umbleja, Kristen Marks, Jennifer J Kiser, Beverly Alston-Smith, Wayne Greaves, Adeel A Butt; the ACTG 5294 BIRTH Study Team

Kenneth E Sherman, Division of Digestive Diseases, University of Cincinnati College of Medicine, Cincinnati, OH 45267, United States

Minhee Kang, Triin Umbleja, Harvard T.H. Chan School of Public Health, Boston, MA 02115, United States

Richard Sterling, Department of Gastroenterology, Virginia Commonwealth University, Richmond, VA 23284, United States

Kristen Marks, Division of Infectious Disease, Weill Cornell Medical School, New York, NY 10065, United States

Jennifer J Kiser, University of Colorado School of Pharmacy, Aurora, CO 80045, United States

Beverly Alston-Smith, NIAID-Division of AIDS, Rockville, MD 20852, United States

Wayne Greaves, Merck and Co, Inc., Kenilworth, NJ 07033, United States

Adeel A Butt, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, United States

Adeel A Butt, Hamad Healthcare Quality Institute, Doha, Qatar

Author contributions: Sherman KE, Sterling R and Butt AA conceived of the study, managed study implementation, provided critical review of the analysis, and wrote the first draft of the manuscript; Kang M and Umbleja T managed study implementation, data collection and data analysis; Sterling R, Marks K, Kiser JJ, Alston-Smith B, Greaves W and Butt AA provided study oversight, critical review of the analysis and manuscript; and all authors read and approved the final manuscript.

Institutional review board statement: The study was performed in the NIH AIDS Clinical Trials Group network (ACTG, National Institutes of Health Registration number NCT01482767) with enrollment of participants at 42 sites across the United States. The study was conducted with approval of Institutional Review Boards (IRB) at each individual site.

Clinical trial registration statement: This study is registered at ClinicalTrials.gov. The registration identifier is NCT01482767.

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

Conflict-of-interest statement: Sherman KE serves on a Merck Advisory Board (paid to institution); the other authors declare no conflict of interests.

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Correspondence to: Kenneth E Sherman, MD, PhD, Gould Professor of Medicine, Division of Digestive Diseases, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati, OH 45267, United States. kenneth.sherman@uc.edu
Telephone: +1-513-5583918
Fax: +1-513-5581744

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Abstract

AIM

To evaluate efficacy/safety of hepatitis C virus (HCV) protease inhibitor boceprevir with pegylated interferon (PEG-IFN) alfa and weight-based ribavirin (RBV) in a phase 3 trial.

METHODS

A prospective, multicenter, phase 3, open-label, single-arm study of PEG-IFN alfa, weight-based RBV, and boceprevir, with a PEG-IFN/RBV lead-in phase was performed. The HCV/human immunodeficiency virus coinfected study population included treatment naïve (TN) and treatment experienced (TE) patients. Treatment duration ranged from 28 to 48 wk dependent upon response-guided criteria. All patients had HCV Genotype 1 with a viral load > 10000 IU/mL. Compensated cirrhosis was allowed. Sample size was determined to establish superiority to historical (PEG-IFN plus RBV) rates in sustained viral response (SVR).

RESULTS

A total of 257 enrolled participants were analyzed (135 TN and 122 TE). In the TN group, 81.5% were male and 54.1% were black. In the TE group, 76.2% were male and 47.5% were white. Overall SVR12 rates (HCV RNA < lower limit of quantification, target not detected, target not detected) were 35.6% in TN and 30.3% in TE. Response rates at SVR24 were 28% in TN and 10% in TE, and exceeded those in historical controls. The highest rate was observed in TN non-cirrhotic participants (36.8% and the lowest in TE cirrhotics (26.3%). Cirrhotic TN participants had a 27.8% SVR12 rate and 32.1% of TE non-cirrhotics achieved SVR12. Significantly lower response rates were observed among black participants; in the TE, SVR12 was 39.7% in white participants but only 13.2% of black subjects (P = 0.002). Among the TN, SVR12 was 42.1% among whites and 27.4% among blacks (P = 0.09).

CONCLUSION

The trial met its hypothesis of improved SVR compared to historical controls but overall SVR rates were low. All-oral HCV treatments will mitigate these difficulties.

Key words: Human immunodeficiency virus; Hepatitis C virus; Boceprevir; Pegylated interferon alfa; Ribavirin

Core tip: Approval of first generation hepatitis C virus (HCV) protease inhibitors has initiated a change in care of HCV infected patients. Phase 2 trials in HCV/human immunodeficiency virus coinfected patients have suggested improved efficacy and tolerability for regimens that combined pegylated interferon (PEG-IFN) + ribavirin (RBV) with either boceprevir or telaprevir. We evaluated an HCV treatment regimen using a first generation HCV protease inhibitor (boceprevir) with PEG-IFN, and weight-based RBV in a phase 3 treatment trial, including HCV treatment-naïve and treatment-experienced coinfected subjects. While sustained viral response rates were low overall they did exceed historical PEG-IFN/RBV rates. Use of new interferon-free direct acting antiviral agents modalities in this population is indicated.

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INTRODUCTION

Hepatitis C virus (HCV) coinfection is a major cause of morbidity and mortality among those with human immunodeficiency virus (HIV) infection[1-4]. Prior to the emergence of new HCV targeted direct acting antiviral agents (DAAs) in 2011, response to standard therapy with pegylated interferon (PEG-IFN) and ribavirin (RBV) was poor, both in terms of efficacy and medication tolerability[5]. The approvals of first generation serine protease inhibitors of HCV replication initiated a revolution in terms of the care and management of HCV infected patients. Phase 2 trials in HCV/HIV coinfected patients suggested improved efficacy with moderate drug tolerability for treatment regimens that combined either boceprevir or telaprevir with PEG-IFN + RBV[6,7]. In an effort to define treatment efficacy with response- and cirrhosis-guided regimens in HCV/HIV coinfected, we conducted a prospective, multicenter, open-label Phase 3 trial in both HCV treatment naïve and treatment experienced participants with comparison to historical controls in the same clinical trials network.

MATERIALS AND METHODS

The study was performed in the NIH AIDS Clinical Trials Group network (ACTG, National Institutes of Health Registration number NCT01482767) with enrollment of participants at 42 sites across the United States. All participants provided informed consent and the study was conducted with approval of Institutional Review Boards at each site. The study was monitored by an independent, NIH-chartered data safety and monitoring board.

The overall study design is shown in Figure 1. Briefly, treatment naïve (TN) participants (Group A) were treated with PEG-IFN alfa 2b 1.5 μg/kg subcutaneously with weight-based ribavirin (800-1400 mg/d) for 4 wk (lead-in). Then boceprevir 800 mg tid was added to the treatment regimen. Cirrhotic participants received 44 wk of triple therapy. Among non-cirrhotics, the week 8 serum HCV RNA was used to determine total duration of therapy. Those who had undetectable HCV RNA at week

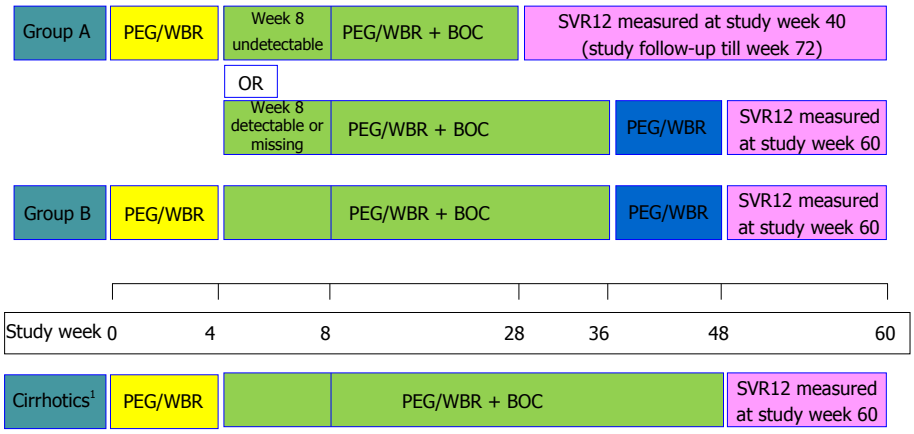

Figure 1 Overall study design. Group A refers to treatment naïve participants while Group B refers to treatment experienced participants. PEG/WBR treatment is pegylated-interferon alfa 2b (PEG-IFN) and weight-based ribavirin (WBR). [1]Cirrhotic participants received 44 wk of triple therapy. SVR12: HCV RNA < LLOQ, target not detected at 12 wk post treatment discontinuation; BOC: Boceprevir; SVR: Sustained viral response; HCV: Hepatitis C virus; LLOQ: Lower limit of quantification.

8 completed therapy at week 28. Those with detectable HCV RNA at week 8 received 32 wk of triple therapy followed by 12 additional weeks of double-drug therapy with PEG-IFN/RBV. Treatment experienced participants (TE) (Group B) also had lead-in followed by 32 wk of triple therapy and 12 wk of PEG-IFN/RBV double therapy if non-cirrhotic, or by 44 wk of triple therapy if cirrhotic. Treatment was to be discontinued due to failure if: (1) HCV RNA $\geqslant$ 100 IU/mL at week 12; (2) detectable HCV RNA at week 24; or (3) confirmed HCV RNA > 1000 IU/mL any time after week 12. HCV RNA was determined to be undetectable if below the lower limit of quantification (LLOQ) and target not detected (TND) by Roche COBAS® TaqMan® HCV Test v2.0.

Key inclusion criteria included HCV genotype 1 with HCV RNA $\geqslant$ 10000 IU/mL. All participants underwent either liver biopsy or non-invasive marker (FibroSure®) testing to determine whether or not cirrhosis was present. Cirrhotics were confirmed to have stage A Child-Pugh disease. HIV RNA viral load was required to be < 50000 copies/mL for participants not on antiretroviral therapy, or less than 50 copies/mL for those on an approved antiretroviral regimen. A $CD4^+$ T-cell count of > 200 cells/mm^3 was also required within 42 d of study entry. Approved regimens included efavirenz, raltegravir, lopinavir/ritonavir, atazanavir/ritonavir or darunavir/ritonavir plus a dual nucleoside reverse transcriptase inhibitor backbone that did not include zidovudine or didanosine. Key exclusion criteria were those with mixed HCV genotypes, prior use of HCV protease or polymerase inhibitors or the presence of decompensated liver disease. Also excluded were other known causes of significant liver disease including HBV, HAV, hemochromatosis, or alpha-1 antitrypsin deficiency.

Data were centrally submitted and analyzed using SAS 9.4 (SAS Institute, Cary, NC, United States). The key outcome measure was sustained viral response in each study group and how the estimates compared to those in historical controls from a prior study of PEG-IFN plus RBV therapy (ACTG 5178). The study was powered to conclude that sustained viral response (SVR) is greater than 28% in TN and 10% in treatment experienced participants, based on A5178 results on HCV genotype 1 participants. The SVR proportions were estimated with two-sided 95% Wilson confidence intervals (CI), and Fisher's exact tests were conducted for comparisons between groups. The analyses included all participants who met the eligibility criteria and initiated the study treatment.

RESULTS

The baseline characteristics of the TN and TE participants as well as the historical controls are shown in Table 1. A total of 257 enrolled participants were analyzed: 135 TN (Group A) and 122 TE (Group B). The study included primarily middle-age males. There was a high representation of black/African-American participants, and this was accompanied by a similarly high percentage of IL28b genotypes carrying the "T" allele. Median CD4 counts were above 600 cell/mm^3 in both groups, corresponding to the high rate of active antiretroviral therapy (> 95%). There were more participants with cirrhosis in TE than in TN, in both A5294 and historical controls.

Overall SVR12 (HCV RNA < LLOQ, TND (target not detected) at 12 wk post treatment discontinuation) rates were 35.6% (95%CI: 28.0-43.9%) in TN and 30.3% (95%CI: 22.9%-39.0%) in TE (Table 2). Rates of response exceeded SVR24 in historical controls: 28% in TN and 10% in TE. The highest rate was observed in TN non-cirrhotic participants (36.8%, 95%CI: 28.6%-45.8%) and the lowest in TE cirrhotic participants (26.3%, 95%CI: 15.0%-42.0%). Cirrhotic TN participants had a 27.8% (95%CI: 12.5%-50.9%) SVR12 rate and 32.1% (95%CI: 23.1%-42.7%) of TE non-cirrhotics achieved SVR12. Race was a significant factor in treatment outcome. Indeed, among TE, SVR12 was noted to occur in 39.7% of white participants but in only 13.2% of those identified as black (P = 0.002). Among TN, SVR12 was 42.1% among whites and 27.4% among blacks (P =

Table 1 Demographic and laboratory characteristics *n* (%)

Characteristic	A5294		Historical controls	
	Treatment naïve (*n* = 135)	Treatment Exp (*n* = 122)	Treatment naïve (*n* = 183)	Treatment Exp (*n* = 87)
Age (yr)				
Median	51	53	48	48
Q1, Q3	44, 57	49, 57	41, 52	42, 51
Sex				
Male	110 (81.5)	93 (76.2)	151 (82.5)	74 (85.1)
Female	25 (18.5)	29 (23.8)	32 (17.5)	13 (14.9)
IV drug history				
Never	71 (52.6)	70 (57.4)	73 (39.9)	39 (44.8)
Currently	0	0	4 (2.2)	1 (1.1)
Previously	64 (47.4)	52 (42.6)	106 (57.9)	47 (54.0)
Race				
Asian	2 (1.5)	2 (1.6)	1 (0.5)	0
Black or African American	73 (54.1)	53 (43.4)	91 (49.7)	24 (27.6)
White	57 (42.2)	58 (47.5)	79 (43.2)	59 (67.8)
American Indian	0	2 (1.6)	3 (1.6)	1 (1.1)
More than One Race	2 (1.5)	2 (1.6)	5 (2.7)	0
Unknown	1 (0.7)	5 (4.1)	4 (2.2)	3 (3.4)
BMI (kg/m^2)				
Median	26.3	27.5	25.7	26
Q1, Q3	22.6, 29.6	25.0, 31.0	22.9, 29.4	23.6, 30.1
Missing	1	0	0	0
IL28b genotype (RS 12979860)				
c/c	32 (25.2)	31 (27.2)	38 (33.9)	19 (31.1)
c/t	61 (48.0)	39 (34.2)	51 (45.5)	30 (49.2)
t/t	34 (26.8)	44 (38.6)	23 (20.5)	12 (19.7)
Missing	8	8	71	26
CD4 ($cells/mm^3$)				
Median	646	621.5	495	520
Q1, Q3	462, 818	488.5, 858.5	373, 697	368, 706
Missing	2	2	0	0
HIV RNA quantitation				
Unquantifiable	133 (100.0)	113 (92.6)	129 (70.5)	71 (81.6)
Quantifiable	0	9 (7.4%)	54 (29.5)	16 (18.4)
Missing	2	0	0	0
HCV RNA (log_{10} IU/mL)				
Median	6.7	6.9	6.5	6.6
Q1, Q3	6.2, 7.1	6.5, 7.3	6.1, 6.8	6.3, 7.0
Missing	1	0	0	0
Cirrhosis				
Yes	18 (13.3)	38 (31.1)	20 (10.9)	18 (20.7)
No	117 (86.7)	84 (68.9)	163 (89.1)	69 (79.3)
Baseline cART regimen				
No ART	2 (1.5)	6 (4.9)	40 (21.9)	11 (12.6)
EFV + 2 NRTIs	58 (43.0)	51 (41.8)	NA	NA
RAL + 2 NRTIs	47 (34.8)	45 (36.9)	NA	NA
LPV/RTV + 2 NRTIs	4 (3.0)	4 (3.3)	NA	NA
ATV/RTV + 2 NRTIs	18 (13.3)	10 (8.2)	NA	NA
DRV/RTV + 2 NRTIs	6 (4.4)	6 (4.9)	NA	NA

NA: A5178 did not have cART restrictions; Exp: Experienced; Q1, Q3: First quartile (Q1), third quartile (Q3); BMI: Body mass index; rs: Reference sequence for single nucleotide polymorphism; ART: Antiretroviral therapy; EFV: Efavirenz; NRTIs: Nucleoside reverse transcriptase inhibitors; RAL: Raltegravir; LPV: Lopinavir; RTV: Ritonavir; ATV: Atazanavir; DRV: Darunavir; cART: Combination antiretroviral therapy.

0.09). Treatment discontinuation rates were high in all groups and were attributed to a mix of treatment failure per HCV viral load criteria or due to adverse events. Among TN, there was one death unrelated to the study, 42 (31%) treatment failures leading to early discontinuation, and additional 22 (16%) premature treatment discontinuations due to adverse events. In TE, there were 52 treatment failures (43%), additional 16 (13%) premature treatment discontinuations due to adverse events, and no deaths. The most commonly reported adverse events of grade 3 or higher included hematologic laboratory events (44% in TN and 48% in TE), and general body (chills, fatigue, pain, weight loss; 23% in TN and 22% in TE), gastrointestinal (4% in TN and in 3% TE) and neurologic (7% in TN and 5% in TE) symptoms. HIV breakthrough was rare and only two study participants (both on raltegravir regimen) met predetermined criteria for this event.

Among TN, the highest SVR rates were observed among participants whose cART regimen included rito-

Table 2 Sustained viral response rates

A5294 participants (n = 257)	% SVR12
Treatment naïve (n = 135)	35.6
Non-cirrhotic (n = 117)	36.8
Cirrhotic (n = 18)	27.8
Treatment experienced (n = 122)	30.3
Non-cirrhotic (n = 84)	32.1
Cirrhotic (n = 38)	26.3

navir - boosted atazanavir with a 2 nucleoside/nucleotide backbone. Overall SVR12 rate in this group (n = 18) was 61.1% (95%CI: 38.6%-79.7%) which was significantly higher than SVR12 rates among participants receiving other cART regimens combined (P = 0.018) in a post-hoc analysis. However, we note that this was an exploratory analysis on a small subset not adjusted for baseline covariates, and this effect was not observed in TE.

DISCUSSION

HCV/HIV coinfection remains a serious medical problem characterized by a high global disease burden (4-5 million) of patients who are at risk for increased fibrotic progression, cirrhosis, and hepatocellular carcinoma[8]. Coinfected patients also have significant non-hepatic complications including increased cardiovascular risk[9]. Therefore, HCV cure is a priority in the management of coinfected HCV/HIV patients. The emergence of new DAAs for HCV has been a rapid and turbulent process which followed years of stagnation in the field. It is not surprising that new therapeutic regimens have been under investigation, even as earlier regimens were entering confirmatory clinical trials. The primary Phase 2 trial for boceprevir/PEG-IFN/RBV was initiated in 2010 and results were reported in July 2013[6]. Planning for the Phase 3 trial reported in this publication began in 2011, and the study completed in early 2015. During this brief interlude, even more effective, shorter duration regimens were studied and brought to the marketplace.

Despite this rapid advancement in therapy, the Phase 3 trial met its primary goals and moved the field forward in a number of key aspects. First, it again demonstrated the importance of Phase 3 trials which often reveal efficacy levels that fall short of their Phase 2 predecessors. The Phase 2 HCV/HIV coinfection trial of the boceprevir/PEG-IFN/RBV regimen yielded an SVR rate of 63%. This is significantly higher than what we observed in the Phase 3 trial which enrolled a population more representative of the United States HCV/HIV population at large in terms of racial distribution. Indeed, the proportion of black participants in this study (49%) is higher than the imputed racial distribution of HCV/HIV coinfected patients in United States (23%-33%) based upon a 2002 analysis[10]. It also exceeds the black representation in the previously reported Phase 2 trial[6]. Our treatment population was more male, more non-white, with a higher representation of the IL28b T allele and with more advanced fibrosis/cirrhosis than the population enrolled in the previously reported Phase 2 study. Our findings of a lower SVR in this population is similar to that reported in "real world" analyses using first generation protease inhibitors[11].

Interestingly, we observed a higher SVR12 among treatment-naïve subjects whose cART regimen consisted of ritonavir boosted atazanavir + a dual NRTI backbone. Pharmacokinetic data indicates that boceprevir AUC was reduced 32% when administered with ritonavir-boosted darunavir while atazanavir AUC decreased only 5%[12]. While we cannot categorically state that this difference affected overall SVR, we suspect it represents an important factor in treatment outcomes among treatment naïve patients. The lack of this finding in treatment experienced participants may represent the overall decreased effectiveness of the PEG-IFN component in that group which masks more subtle effects related to HCV protease inhibitor pharmacokinetics.

Interferon-based therapy is difficult to tolerate and this is clearly demonstrated by the high drop-out rate seen in our study cohort. Though some guidelines and insurers still encourage use of PEG-IFN in some treatment groups, this approach may be particularly detrimental in the HIV-infected patient where tolerability to interferon-based regimens seems to be lower than that observed in comparable Phase 3 trials in monoinfected patients.

Though the treatments utilized in this Phase 3 multicenter trial will not be utilized in general practice, our study provided several important principles and observations that may guide future trials in the field. First, we provide additional support to the concept that Phase 3 trials represent a more accurate representation of true response rates compared to Phase 2 trials. We also note that outcomes in HCV/HIV coinfected patients may be related to the background HIV antiretroviral regimen and that this effect may be a drug effect rather than a class effect. Finally, we note the systematic delays in initiation of clinical trials for those with underlying HIV infection *vs* those without HIV. Phase 3 trials of first generation HCV protease inhibitors lagged significantly behind drug approvals in HCV monoinfected patients. More recent drug development programs have attempted to remedy this situation, but the HIV research community should remain vigilant to reduce this bias going forward, particularly in rapidly moving developmental fields.

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COMMENTS

Background

Hepatitis C virus (HCV) coinfection is a major cause of morbidity and mortality among those with human immunodeficiency virus (HIV) infection. Prior to the emergence of new HCV targeted direct acting antiviral agents in 2011, response to standard therapy with pegylated interferon (PEG-IFN) and ribavirin (RBV) was poor, both in terms of efficacy and medication tolerability. The approvals of first generation serine protease inhibitors of HCV replication initiated a revolution in terms of the care and management of HCV infected patients. Phase 2 trials in HCV/HIV coinfected patients suggested improved efficacy with moderate drug tolerability for treatment regimens that combined either boceprevir or telaprevir with PEG-IFN + RBV. In an effort to define treatment efficacy with response- and cirrhosis- guided regimens in HCV/HIV coinfected, the authors conducted a prospective, multicenter, open-label Phase 3 trial in both HCV treatment naïve and treatment experienced participants with comparison to historical controls in the same clinical trials network.

Research frontiers

The treatment of hepatitis C is a rapidly moving and dynamic field. Introduction of new agents has led to expansion of indications prior to completion of comprehensive Phase 3 trials in some cases. This study provides data regarding a large Phase 3 trial of a first generation protease inhibitor of HCV which was utilized in combination with PEG-IFN and RBV in HCV/HIV coinfected patients.

Innovations and breakthroughs

This is the largest study to investigate the efficacy and safety of this first generation protease inhibitor therapy in HCV/HIV coinfected patients. The treatment was not optimal, but it did meet criteria for treatment success compared to historical controls treated with PEG-IFN plus RBV.

Applications

While this study demonstrates efficacy of a first generation HCV protease inhibitor in the treatment of HCV/HIV coinfected patients, the regimen is unlikely to be widely used due to rapid development of all-oral regimens that have supplanted the used of PEG-IFN-based regimens. The importance of conducting Phase 3 trials was emphasized by the lower rates of efficacy than were observed in Phase 2 trials that included highly selected patients.

Terminology

Treatment naïve patients are those who have never been treated with a hepatitis C active agent while treatment experienced are those who may have been exposed to interferon or PEG-IFN with or without RBV in the past. Therapies for HIV are collectively called cART which includes combinations of drugs used for antiretroviral therapy.

Peer-review

The authors report data on efficacy and safety of HCV protease inhibitor boceprevir with PEG-IFN alfa and weight-based RBV in a phase 3 trial in patients with HCV plus HIV. The result, in terms of RBV, is similar to that reported by other studies in the real world and reflects the limits of this treatment. The authors, correctly, described the chronology of their trial, born before the entry in the clinical practice of the new treatments.

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Robotic liver surgery is the optimal approach as bridge to transplantation

Paolo Magistri, Giuseppe Tarantino, Roberto Ballarin, Andrea Coratti, Fabrizio Di Benedetto

Paolo Magistri, Department of Medical and Surgical Sciences and Translational Medicine, Sapienza - University of Rome, 00185 Rome, Italy

Paolo Magistri, Giuseppe Tarantino, Roberto Ballarin, Fabrizio Di Benedetto, Hepato-Pancreato-Biliary Surgery and Liver Transplantation Unit, University of Modena and Reggio Emilia, 41124 Modena, Italy

Andrea Coratti, Department of Oncology, Division of Oncological and Robotic General Surgery, Careggi University Hospital, 50134 Florence, Italy

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Correspondence to: Paolo Magistri, MD, Hepato-Pancreato-Biliary Surgery and Liver Transplantation Unit, University of Modena and Reggio Emilia, Via del Pozzo 71, 41124 Modena, Italy. paolo.magistri@uniroma1.it
Telephone: +39-059-4224740
Fax: +39-059-4223765

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Abstract

The role of minimally invasive liver surgery as a bridge to transplantation is very promising but still underestimated. However, it should be noted that surgical approach for hepatocellular carcinomas (HCC) is not merely a technical or technological issue. Nowadays, the epidemiology of HCC is evolving due to the increasing role of non-alcoholic fatty-liver-disease, and the emerging concerns on direct-acting antivirals against hepatitis C virus in terms of HCC incidence. Therefore, a fully multidisciplinary study of the cirrhotic patient is currently more important than ever before, and the management of those patients should be reserved to tertiary referral hepatobiliary centers. In particular, minimally invasive approach to the liver showed several advantages compared to the classical open procedure, in terms of: (1) the small impact on abdominal wall; (2) the gentle manipulation on the liver; (3) the limited surgical trauma; and (4) the respect of venous shunts. Therefore, more direct indications should be outlined also in the Barcelona Clinic Liver Cancer model. We believe that treatment of HCC in cirrhotic patients should be reserved to tertiary referral hepatobiliary centers, that should offer patient-tailored approaches to the liver disease, in order to provide the best care for each case, according to the individual comorbidities, risk factors, and personal quality of life expectations.

Key words: Hepatocellular carcinomas; Liver transplant; Robotic surgery; Bridge to transplantation; Da vinci; Barcelona Clinic Liver Cancer; Patient safety

Core tip: We read with great interest the manuscript

by Dr. Memeo *et al*. The role of minimally invasive liver surgery as a bridge to transplantation is very promising but still underestimated. In particular, minimally invasive approach to the liver showed several advantages compared to the classical open procedure in cirrhotic patients, and currently it deserves more direct indications that should be outlined also in the Barcelona Clinic Liver Cancer model.

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TO THE EDITOR

We read with great interest the paper by Memeo *et al*[1], recently published on *World Journal of Hepatology* and titled "Innovative surgical approaches for hepatocellular carcinoma". In their well written and complete analysis of surgical planning and treatment for hepatocellular carcinomas (HCC), the authors affirm that the well-known advantages of minimally invasive liver surgery (MLS) compared to the classic "open" approach (OLS) may result in an easier access to the abdomen in case of future liver transplantation (LT). We completely agree and compliment them for highlighting this issue, which is currently underestimated. In July 2014 we started a robotic program at University of Modena and Reggio Emilia and in a period of two years 69 procedures have been performed. A total of 47 robotic liver procedures were ruled out, and among those 24 resection for HCC in cirrhotic patients. In this cohort of patients there were no conversions to laparotomy, mean operative time was 318 min (docking time included), and the mean in-hospital stay was 5.1 d. No readmission nor recurrences were observed. Our robotic cohort of HCC patients is included in an ongoing study funded by "Regione Emilia Romagna" (Regional Public Health System) that aims to investigate the role of robotic surgery in bridging patients with HCC to LT. Up to now, in our Institution two patients successfully underwent LT after MLS and four are on the waiting list.

The robotic platform is expanding its field of application on liver surgery for HCC including the so-called "difficult segments", and should be considered as a valuable tool for bridging patients to LT[2-6]. Although OLS has been classically limited to a strictly selected population of patients, several studies demonstrated that MLS is safe, feasible and particularly effective for parenchyma-sparing procedures, as needed in cirrhotic patients[7]. However, it should be noted that surgical approach for HCC is not merely a technical or technological issue. Nowadays, the epidemiology of HCC is evolving due to the increasing role of non-alcoholic fatty-liver-disease and direct-acting antivirals against hepatitis C virus[8]. Therefore, a fully multidisciplinary study of the cirrhotic patient is currently more important than ever before, and the management of those patients should be reserved to tertiary referral hepatobiliary centers. Moreover, it should be taken into account that the intraoperative management as well is not only a matter of individual ability to perform certain procedures. MLS seems more effective than OLS in patients affected by HCC within a cirrhotic liver due to several reasons. First of all, in a setting of reduced liver function and reduced functional reserve, we can benefit from less impact on the abdominal wall, gentle manipulation on the liver, respect of the venous shunts and limited surgical trauma. In addition, the perioperative perspiration is consistently less with MLS compared to OLS: Consequently, fluids administration can be more conservative since generous substitutions are not needed. Finally, a better control of post-operative pain and early mobilization of the patient after MLS reduce respiratory complications by enhancing respiratory movements[9]. Currently, there is no formal evidence of the superiority of robotic approach *vs* conventional laparoscopy and also oncological results are similar[10]. The correct timing and criteria for choosing between liver resection or LT is still debated, and optimizing organ allocation is still our priority[11]. MLS offers an opportunity to safely treat HCC patients even with a Child A-B cirrhotic liver, with lower rates of overall morbidity when compared to OLR, and lower incidence of local recurrence when compared to radiofrequency ablation[12]. In conclusion, minimally invasive liver procedures can be considered as an independent field of surgery, with particular indication for Child A and B patients and parenchima-sparing procedures, that should be better classified in the classical Barcelona Clinic Liver Cancer model[13-15].

We compliment again the Authors for their work and their effort as a referral center of technological innovation to improve both surgical performances and patients' safety. We believe that a modern hepatobiliary center should offer patient-tailored approaches to the liver disease, in order to provide the best care for each case, according to the individual comorbidities, risk factors and personal quality of life expectations.

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