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TOPIC HIGHLIGHT

2016 Hepatocellular Carcinoma: Global view

Usefulness of staging systems and prognostic scores for hepatocellular carcinoma treatments

Xavier Adhoute, Guillaume Penaranda, Jean Luc Raoul, Patrice Le Treut, Emilie Bollon, Jean Hardwigsen, Paul Castellani, Hervé Perrier, Marc Bourlière

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Abstract

Therapeutic management of hepatocellular carcinoma (HCC) is quite complex owing to the underlying cirrhosis and portal vein hypertension. Different scores or classification systems based on liver function and tumoral stages have been published in the recent years. If none of them is currently "universally" recognized, the Barcelona Clinic Liver Cancer (BCLC) staging system has become the reference classification system in Western countries. Based on a robust treatment algorithm associated with stage stratification, it relies on a high level of evidence. However, BCLC stage B and C HCC include a broad spectrum of tumors but are only matched with a single therapeutic option. Some experts have thus suggested to extend the indications for surgery or for transarterial chemoembolization. In clinical practice, many patients are already treated beyond the scope of recommendations. Additional alternative prognostic scores that could be applied to any therapeutic modality have been recently proposed. They could represent complementary tools to the BCLC staging system and improve the stratification of HCC patients enrolled in clinical trials, as illustrated by the NIACE score. Prospective studies are needed to compare these scores and refine their role in the decision making process.

Key words: Scoring system; Hepatocellular carcinoma; Barcelona Clinic Liver Cancer staging system; NIACE; Transarterial chemoembolization

Core tip: Different scores or classification systems have been proposed to refine hepatocellular carcinoma prognosis and better guide medical treatment. The Barcelona Clinic Liver Cancer (BCLC) system has become the reference classification in Western countries. Its treatment algorithm is based on randomized studies, but only offers one recommendation for BCLC stages B and C, whereas they include a broad spectrum of tumors. In clinical practice, many patients are treated out of the scope of these recommendations. In this context, alternative scores or classifications, which have been opposed for a long time, could be complementary tools for the benefit of the treatment.

Adhoute X, Penaranda G, Raoul JL, Le Treut P, Bollon E, Hardwigsen J, Castellani P, Perrier H, Bourlière M. Usefulness of staging systems and prognostic scores for hepatocellular carcinoma treatments. *World J Hepatol* 2016; 8(17): 703-715 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i17/703.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i17.703

INTRODUCTION

Most hepatocellular carcinomas (HCC) develop upon chronic diseases of the liver, mainly B or C viral hepatitis. HCC is a frequent and serious cancer, often diagnosed at an inoperable stage[1]. It is singular as its prognosis not only relies on the tumor characteristics but also on the underlying liver disease, frequently at a cirrhotic stage. The tumor-node-metastasis (TNM) classification of solid tumors failed to impose itself as the reference system for such a dual pathology, despite its recognized prognostic value even for non-operated tumors[2]. In order to refine the prognosis and provide better medical care, different scores or classifications originating from Asian or Western countries have been published recently. Most of them use regression models based on the prognostic variables of the studied populations. If they all share common parameters including liver function, tumor characteristics, age-related clinical consequences, comorbidities or cirrhosis (Figure 1), there is no universally recognized score or classification to date.

In the first part, we will focus on the main scores and classification systems published in the recent years, following a chronological order and revealing the differences between Western and Asian countries, the corresponding affected populations, treatment modalities and recommendations being distinct. The second part highlights the complementarity between the two systems in the decision making process (excluding graft), as successively exemplified by the sorafenib, the transarterial chemoembolization (TACE), the radiofrequency ablation (RFA) and the surgical resection treatments.

HCC PROGNOSIS: SCORES OR CLASSIFICATIONS?

The OKUDA score, published in the eighties, was the first to combine tumor-associated parameters (more *vs* less than 50% of invaded parenchyma) and liver function (ascites, albumin, bilirubin) (Tables 1 and 2). It classifies patients into three stages [lowly (Ⅰ), moderately (Ⅱ) or highly advanced (Ⅲ)] with different outcomes, depending on their number of positive variables (0 *vs* 1-2 *vs* 3-4, respectively). This score was initially validated on a population of 850 patients, either non-treated or treated according to the modalities applicable at that time (surgery, intra-arterial or systemic chemotherapy, arterial embolization)[3]. Although approximative and hardly differentiating the less advanced patients (*e.g.*, the median survival of stage Ⅰ patients was 11.5 mo independently of the treatment *vs* 25.6 mo when operated), this score has been widely used.

Published in the late nineties, the Italian Cancer of the Liver Italian Program (CLIP) score was calculated from the prognostic values of 435 patients originating from 16 centers (Tables 1 and 2)[4]. It includes other tumor-linked parameters such as portal vein thrombosis or alpha-fetoprotein (AFP) serum levels and better estimates the liver function using the Child-Pugh score. Easy to calculate (4 variables to add), it is well correlated with survival (CLIP 0, 1, 2, 3, 4, 5-6: 42.5 *vs* 32 *vs* 16.5 *vs* 4.5 *vs* 2.5 *vs* 1.0 mo). The CLIP score was first assessed on a prospective cohort[5,6] and subsequently validated on Asian cohorts[7]. Still recently ranked first for its ability to predict survival[8], it was criticized for its lack of treatment offer, approximation in tumor morphology and extension, for the absence of clinical status consideration and its inability to classify intermediate stages. Another issue is that studies evaluating the CLIP system mainly included patients with scores only ranging from 0 to 2[7-9].

French speaking teams have created the GRETCH score in 1999. Quite similar to the CLIP, it further includes the patients' overall condition but lacks tumor morphology information[10]. Also determined from a multivariate analysis including 761 patients (mainly non-treated) from 24 centers, it identifies 3 different groups (A: 0, B: 1 to 5 and C: 6 to 11 points) with distinct prognosis [overall survival after a year: A (72%), B (34%), C (7%), respectively]. Less evaluated than the CLIP, it faces the same limitations.

The BCLC classification was published at the same time[11]. Differently built as it is not based on a regression model but results from the combination of different studies, it distinguishes 4 different stages [A: (very) early, B: intermediate, C: advanced, D: terminal] with different prognosis, according to the liver function, the

Table 1 Hepatocellular carcinoma scores and staging systems published in the recent years

Scores and classifications	Liver function	AFP	PS	Tumor spread
Okuda 1985	Ascites, albumin, bilirubin	No	No	Hepatic spread 50%< *vs* > 50%
CLIP 1998	Child-Pugh score	< 400 ng/mL *vs* ⩾ 400 ng/mL	No	Nodule(s), hepatic spread 50% ⩽ *vs* > 50% Portal vein thrombosis
GRETCH 1999	Bilirubine, phosphatases alcalines	< 35 ng/mL *vs* ⩾ 35 ng/mL	Yes	Portal vein thrombosis
BCLC 1999	Child-Pugh score	No	Yes	Nodule(s), size Portal vein thrombosis
c-JIS 2003	Child-Pugh score	No	No	TNM LCSGJ
bm-JIS 2008	Child-Pugh score	Yes (+ AFP-L3 + DCP)	No	TNM LCSGJ
TIS 2010	Child-Pugh score	< 400 g/mL *vs* ⩾ 400 ng/mL	No	Total tumor volume
HKLC 2014	Child-Pugh score	No	Yes	Nodule(s), size Portal vein thrombosis

BCLC: Barcelona Clinic Liver Cancer; CLIP: Cancer of the Liver Italian Program; JIS: Japan Integrated Staging; HKLC: Hong Kong Liver Cancer; TIS: Taipei Integrated Scoring System; AFP: Alpha-fetoprotein; PS: Performance Status; bm-JIS: Biomarker combined JIS; DCP: Des-gamma-carboxy prothrombin; LCSGJ: Liver Cancer Study Group of Japan; TNM: Tumor-node-metastasis.

Table 2 Definitions of the Okuda score and the Cancer of the Liver Italian Program score

	Okuda score		CLIP score		
Parameters	**(+) 1 point**	**(-) 0 point**	**0 point**	**1 point**	**2 points**
Tumor spread	> 50%	< 50%			
Albumin, g/dL	< 3	> 3			
Bilirubin, mg/dL	> 3	< 3			
Ascites	Yes	No			
Child-Pugh score			A	B	C
Tumor spread			Unipolar and hepatic spread ⩽ 50%	Multinodular and hepatic spread ⩽ 50%	Massive or hepatic spread > 50%
Portal vein thrombosis			No	Yes	
AFP, ng/dL			< 400	⩾ 400	

AFP: Alpha-fetoprotein; CLIP: Cancer of the Liver Italian Program.

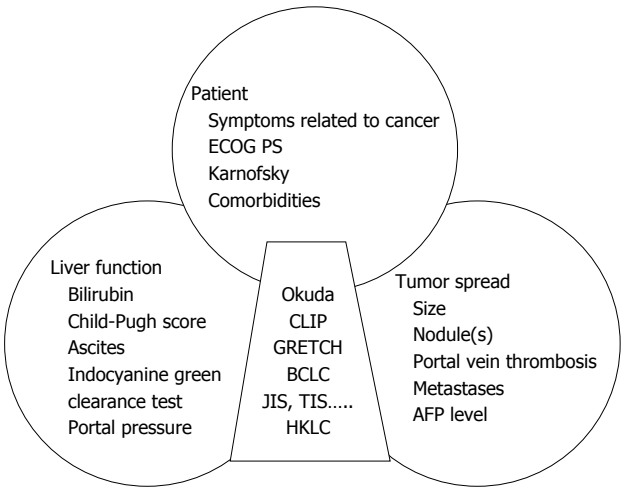

Figure 1 Common parameters between hepatocellular carcinoma classifications and scores. AFP: Alpha-fetoprotein; CLIP: Cancer of the Liver Italian Program; BCLC: Barcelona Clinic Liver Cancer; JIS: Japan Integrated Staging; HKLC: Hong Kong Liver Cancer; TIS: Taipei Integrated Scoring System; ECOG (PS): Eastern Cooperative Oncology Group (performance status).

extent of the tumor and its consequences (Figure 2). As opposed to the previous scores, the early stages are well defined according to the number and size of nodules, the associated comorbidities and the portal vein pressure. The BCLC staging system was assessed on Western and Asian cohorts[12,13] and demonstrated a better ability to predict survival than most other scores[9,14]. This classification has imposed itself from its practical aspect and for being the only one linked to a treatment algorithm relying on a high level of evidence for each modality. Endorsed by both the European Associations for the Study of the Liver (EASL)[15] and the American Associations for the Study of the Liver (AASLD)[16], it has become the reference classification in Western countries and is being used in day-to-day practice and clinical trials.

However, BCLC is not the reference classification in Asia, notably as HCC treatment modalities differ according to the countries (*e.g.*, external radiotherapy, intra-arterial and systemic chemotherapy or TACE being indicated for advanced HCC despite a low level of evidence[17]). Such recommendations are based on studies but, as opposed to the BCLC, also rely on personal experience, experts advice and consensus conferences. Alternative scores or classifications have thus been proposed.

The Japan Integrated Staging (JIS) score was published in 2003 (Table 3)[18]. Also easy to calculate, it associates the Child-Pugh score and the Japanese TNM,

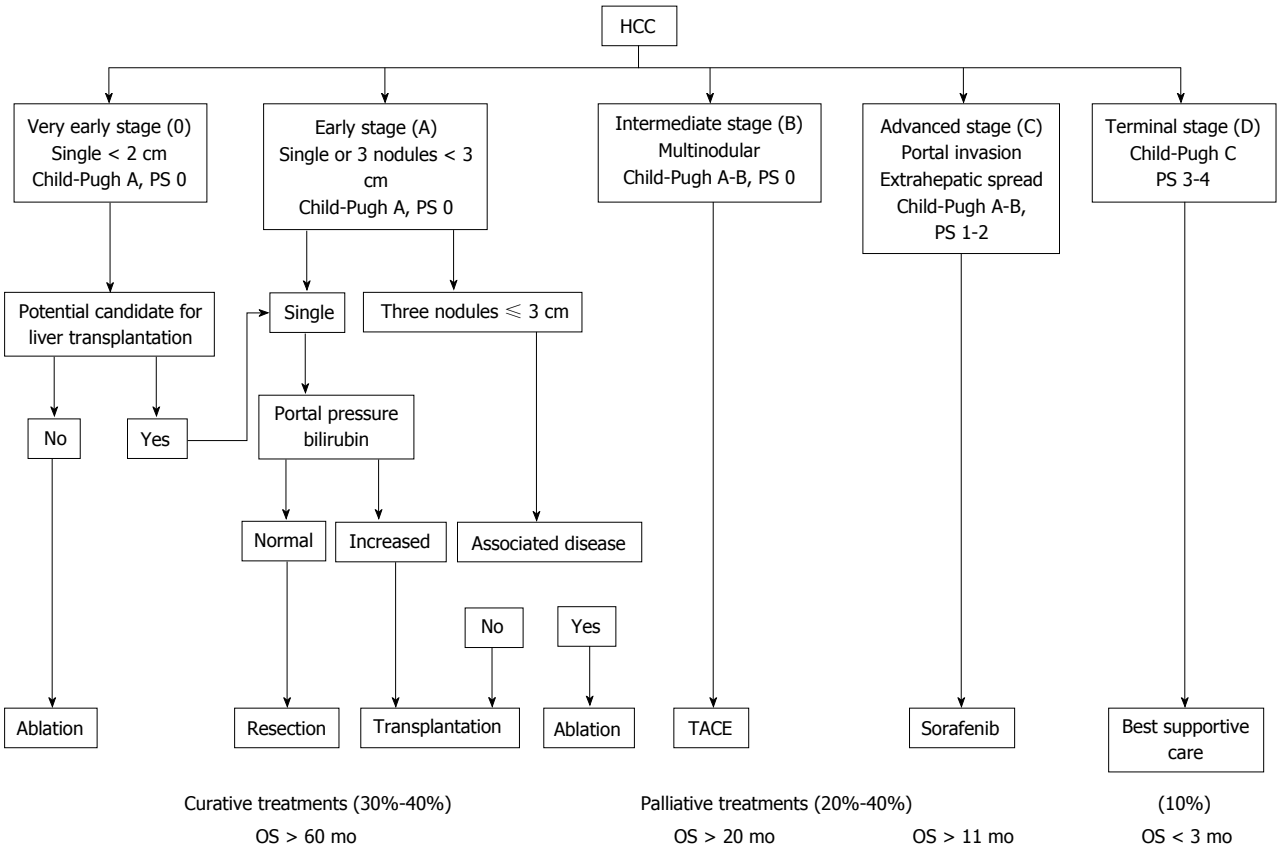

Figure 2 Barcelona Clinic Liver Cancer system. HCC: Hepatocellular carcinoma; PS: Performance status; TACE: Transarterial chemoembolization.

Table 3 Definitions of the c-Japan Integrated Staging score and the biomarker combined Japan Integrated Staging score

	0 point	1 point	2 points	3 points
Score c-JIS				
Child-Pugh stage	A	B	C	
TNM stage by LCSGJ[1]	I	II	III	IV
Score bm-JIS				
Child-Pugh stage	A	B	C	
TNM stage by LCSGJ[1]	I	II	III	IV
Elevated tumor markers, *n* (AFP, AFP-L3, DCP)	0	1	2 or 3	

[1]Definitions of the TNM stage by the LCSGJ; Stage Ⅰ: T1 (fulfilling 3 T factors) N0 M0; Stage Ⅱ: T2 (fulfilling 2 T factors) N0 M0; Stage Ⅲ: T3 (fulfilling 1 T factor) N0 M0; Stage Ⅳ: T4 (fulfilling 0 T factor) N0 M0 or any T N0 - 1 M1; T factor: (1) single; (2) < 2 cm; and (3) no vascular involvement. LCSGJ: Liver Cancer Study Group of Japan; bm-JIS: Biomarker combined-Japan Integrated Staging.

which is based on three parameters (vascular invasion, unique *vs* multiple nodules, diameter ⩽ *vs* > 20 mm) determined from a population of 13772 operated patients. It defines six groups with different prognosis (excluding JIS 4-5). This score has demonstrated a better ability to predict survival than the CLIP and was further improved a few years later with the modified-JIS[19], in which the encephalopathy item is replaced by the indocyanine green clearance, due to an early HCC screening in Japan and a preferred surgical orientation. In 2008, the JIS score became the biomarker combined JIS with the inclusion of three HCC serum markers [AFP, AFP-L3 (AFP-Lens culinaris agglutinin-reactive) and des-gamma-carboxy prothrombin], which allowed better survival predictions (Table 3)[20]. However, two of those markers are not frequently used in Western countries where HCC is also often being diagnosed at more advanced stages. Thus, this score, without treatment guidelines, has not been evaluated on patients from Western countries.

The Taipei Integrated Scoring system (TIS) was published in 2010[21] arised from the lack of a reference classification and the opposite results from studies regarding the performance of classification systems. TIS is a point scoring system combining AFP levels (< 400 *vs* > 400 ng/mL: 0 *vs* 1 point), Child-Pugh score (A, B and C : 0, 1 and 2 points, respectively) and the sum of the volume of each tumor (total tumor volume), calculated from the following formulae: [(4/3) × 3.14 × (radius of tumor in cm)^3], and which defines 4 different groups (< 50 cm^3, 50-250 cm^3, 250-500 cm^3, > 500 cm^3: 0, 1, 2 or 3 points, respectively). From a cohort of 2030 patients, mainly with viral hepatitis (hepatitis B virus 51%, hepatitis C virus 27%), the score identified six distinct prognostic groups, with a score evolution inversely correlated to survival. The predictive ability of the TIS score was better than the JIS and the BCLC for the whole cohort, independently of the treatment modality (curative or palliative), but not as good as the CLIP for the 936 patients treated with curative intent.

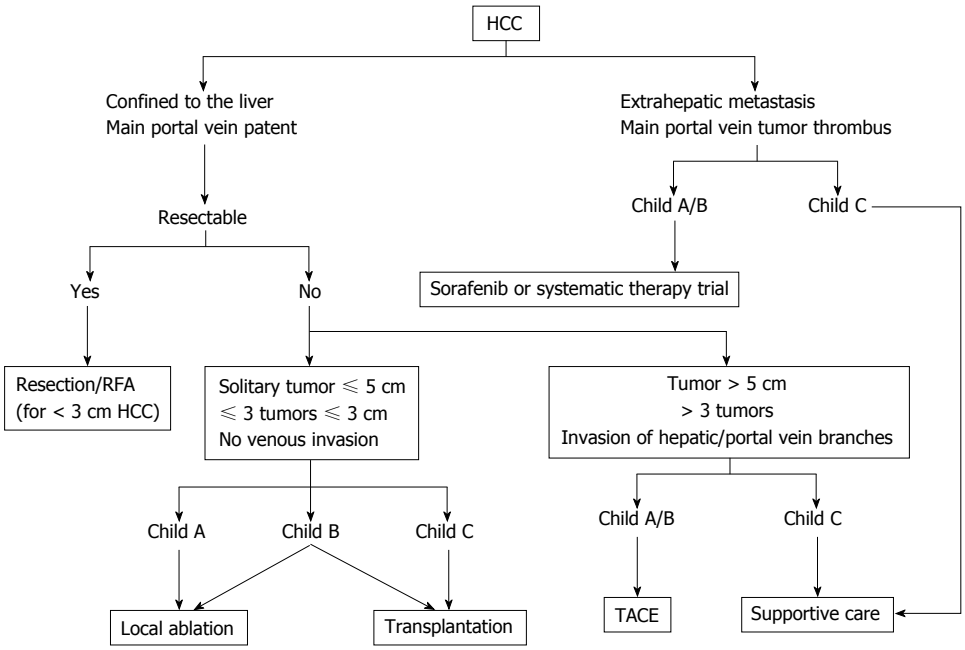

Figure 3 Asian Pacific Association for the Study of the Liver guidelines on the treatment algorithm for hepatocellular carcinoma. HCC: Hepatocellular carcinoma; TACE: Transarterial chemoembolization; RFA: Radiofrequency ablation.

Vascular invasion that was observed in 36.7% of the patients is taken into account in the CLIP but not in the TIS, which probably participates in this discrepancy. Again, this score appears promising, but lacks a linkage to any treatment decision choice and has not been validated on any Western country patient.

In 2011, an Asian experts meeting has suggested to adopt a common classification and common recommendations. TACE was then proposed for HCC with limited vascular invasion, despite a low level of evidence (Figure 3)[17]. The competing Hong Kong Liver Cancer (HKLC) classification, which is close to the BCLC system, was published in 2014[22]. Built from a population of 3856 patients (median age: 58 years old), mainly affected by viral hepatitis B, with Child-Pugh A scores (73%), it identifies five groups and nine sub-groups to further refine the prognosis (Figure 4). The associated treatment algorithm recommended surgery at more advanced stages and subsequently increased survival according to the authors. However, its prognostic value was comparable to the BCLC system for a European cohort of HCC linked to viral hepatitis C or alcohol, the ⅡaⅡb, ⅢbⅣa, ⅣbⅤb subgroups presenting similar survival[23], which limits the impact of such a stratification within this population. A prospective study is currently on-going to further evaluate this score.

Overall, the BCLC classification has become the reference in Western countries and has replaced the other prognostic scores. Limitations have however been highlighted since several years. The intermediate BCLC B stage, which gathers multifocal tumors lacking vascular invasion and excludes unique and large HCC, now part of the BCLC A group in newer version of the BCLC classification[24], remains heterogeneous[25]. Thus, a diffuse multinodular HCC or four nodules of one centimeter in size within the same lobe are categorized within the same BCLC B group, and only a single therapeutic option is offered (*i.e.*, chemoembolization). Advanced (BCLC C) stages encompass a broad spectrum of tumors, including cancers with or without symptoms, metastatic or locally advanced diseases, eventually associated with portal thrombosis, nodular or infiltrating tumors, uni- or multi-nodular tumors, associated with Child-Pugh A or B grade, which are, again, only associated with a single treatment (sorafenib)[24]. It has thus been suggested to extend the indication for surgery[26-28] or chemoembolization to some advanced stages[29,30]. Stage C HCC were defined using a population limited to 102 patients[31]. Furthermore, comparative studies have shown lower prognostic ability for the BCLC than the CLIP score regarding advanced HCC[32-34], and several studies have suggested a possible stratification for the BCLC C HCC[35-37]. For example, Yau *et al*[36] have proposed a new score called Advanced Liver Cancer Prognostic System (ALCPS), separating 3 groups according to their survival after 3 mo, and aiming at improving patients selection before their enrollment into clinical trials (Table 4). However, the ALCPS score is too complex for daily clinical practice as it includes eleven variables with different coefficients, as is the Chinese University Prognostic Index score[37].

Conversely, the recently published NIACE score[38] (Table 5) was determined from a population of advanced HCC and validated using an external Asian cohort, independently of the BCLC stage[39]. Easy to calculate and well correlated to survival, it distinguishes 2 subgroups with different prognosis within BCLC stage C patients. Advanced HCC are classified according to their morphology as infiltrating or diffuse (hardly delimited lesion, with a heterogeneous enhancement, more easily

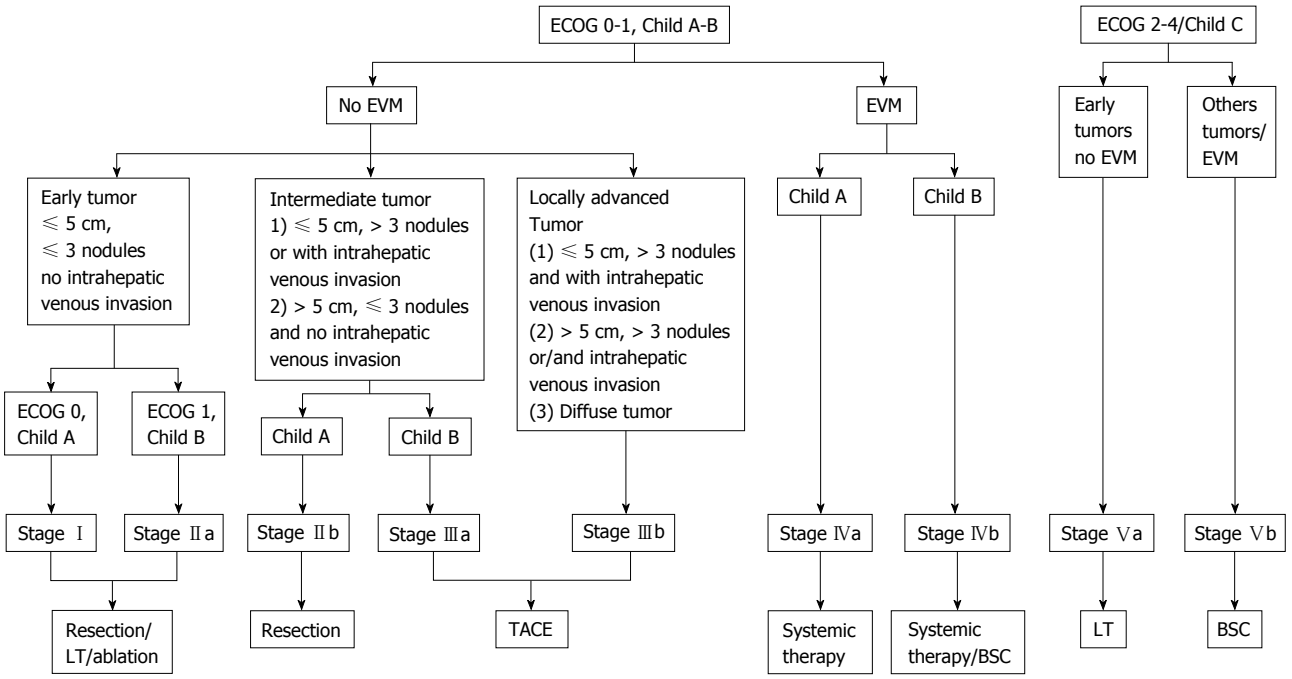

Figure 4 Hong Kong Liver Cancer classification. EVM: Extrahepatic vascular invasion/metastasis; BSC: Best supportive care; TACE: Transarterial chemoembolization; ECOG: Eastern Cooperative Oncology Group.

Table 4 Barcelona Clinic Liver Cancer C hepatocellular carcinoma, a broad spectrum of tumors; example of the Advanced Liver Cancer Prognostic System score[36]

Parameters	Points
Ascites	2
Abdominal pain	2
Weight loss	2
Child-Pugh grade A/B/C	0/2/5
alkaline phosphatase, UI/L > 200	3
Bilirubin, mcmol/L ⩽ 33/> 33-⩽ 50/> 50	0/1/3
Urea, mmol/L > 8.9	2
Portal vein thrombosis	3
Tumor size: Diffuse/> 5 cm/⩽ 5 cm	4/3/0
Lung metastases	3
AFP, ng/mL > 400	4
Probability of patients surviving at least 3 mo estimated by the ALCPS score[36]	
Score ⩽ 8 points: 82.0% (95%CI: 76.5%-87.5%)	
Score 9-15 points: 53.4% (95%CI: 48.3%-57.7%)	
Score ⩾ 16 points: 18.9% (95%CI: 14.7%-23.3%)	

Probability of patients surviving at least 3 mo estimated by the ALCPS score[36]. AFP: Alpha-fetoprotein; ALCPS: Advanced Liver Cancer Prognostic System.

Table 5 NIACE score[38]

Score NIACE	Points
Nodules < 3	0
Nodules ⩾ 3	1
Infiltrative HCC: No	0
Infiltrative HCC: Yes	1.5
AFP < 200 ng/mL (at baseline)	0
AFP ⩾ 200 ng/mL (at baseline)	1.5
Child-Pugh grade A	0
Child-Pugh grade B	1.5
ECOG PS 0	0
ECOG PS ⩾ 1	1.5

AFP: Alpha-fetoprotein; HCC: Hepatocellular carcinoma; ECOG (PS): Eastern Cooperative Oncology Group (Performance Status).

characterized using magnetic resonance imaging[40] and frequently associated with portal vein thrombosis[41] or bile duct invasion), as opposed to the nodular HCC meeting the EASL/AASLD diagnosis criteria[42]. It also considers the AFP level (± 200 ng/mL), whose prognostic value has been demonstrated independently of the stage of the disease[4,10,43]; those two last criteria missing from the BCLC system.

The predictive value of the NIACE score has been compared to those of the CLIP score and both the BCLC and HKLC classifications using a French multicenter HCC cohort of 1102 patients, of 68 (60-74) years of age, mostly with cirrhosis (81%), often linked to alcohol (41%) or hepatitis C (28%) or B (6%) viruses; most of the patients with Child-Pugh A and BCLC C scores, and treated according to the following modalities: Curative treatment in 22% of the cases (surgical resection or RFA), palliative treatment in 66% of the cases (TACE, sorafenib) and supportive care in 12% of the cases[44]. Each scoring system identified different prognosis subgroups ($P < 0.0001$), with scores and classifications correlated with survival. The NIACE score showed the best homogeneity (LR $\chi^2 = 532.0369$, $P < 0.0001$), the best discriminative ability (LT $\chi^2 = 91.6906$, $P < 0.0001$), the lowest Akaike information criterion (AIC 10648.198) and the highest C-index [C-index 0.718 (0.688-0.748)] (Table 6). Using a threshold value of 1 or 2.5, the NIACE score identified 2 distinct prognosis groups within the

Table 6 Comparison of prognostic performance of the NIACE, Barcelona Clinic Liver Cancer, Hong Kong Liver Cancer, and Cancer of the Liver Italian Program systems[44]

Score	Discriminatory ability linear trend test		Homogeneity likelihood ratio test		Akaike information criterion	C-index (95%CI)
	LT (χ^2)	*P* value	LR (χ^2)	*P* value		
NIACE	91.6906	< 0.0001	532.0369	< 0.0001	10648.198	0.718 (0.688-0.748)
BCLC	79.0342	< 0.0001	380.4100	< 0.0001	10805.825	0.674 (0.645-0.704)
HKLC	71.8861	< 0.0001	455.3169	< 0.0001	10740.918	0.698 (0.673-0.731)
CLIP	87.2785	< 0.0001	430.3872	< 0.0001	10749.848	0.716 (0.687-0.746)

BCLC: Barcelona Clinic Liver Cancer; CLIP: Cancer of the Liver Italian Program; HKLC: Hong Kong Liver Cancer; LR: Likelihood ratio; LT: χ^2 linear trend test.

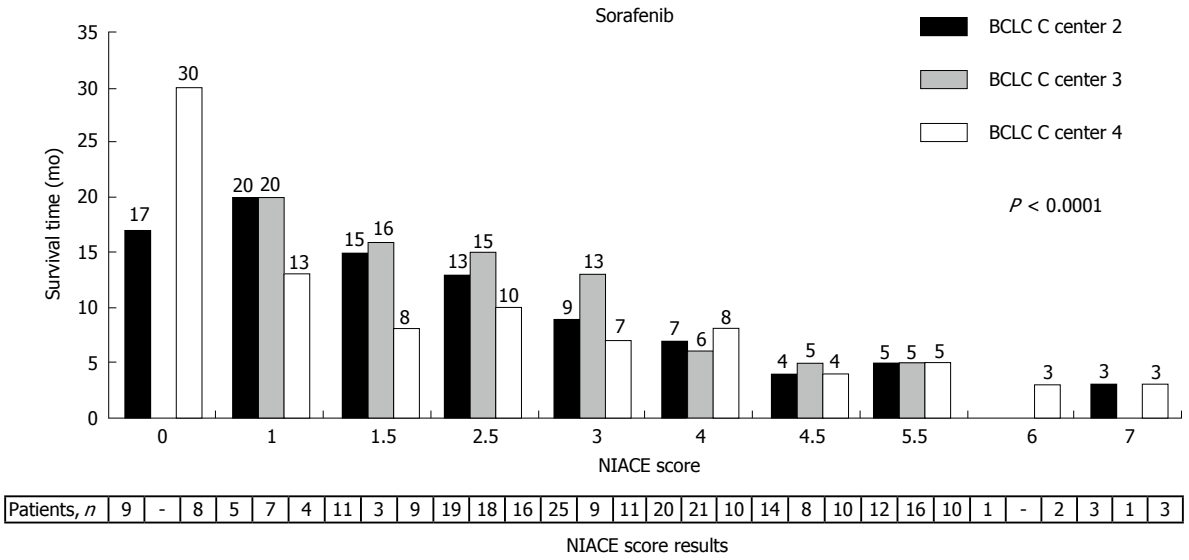

Figure 5 Evolution of the median overall survival according to the NIACE score in Barcelona Clinic Liver Cancer stage C patients from a French multicenter study, treated by sorafenib (black bars center 2, grey bars center 3, white bars center 4)[38]. BCLC: Barcelona Clinic Liver Cancer.

CLIP 0, 1, 2 and 3 groups ($P < 0.0001$). As opposed to the HKLC, when applied to the various HKLC groups with similar survival (*i.e.*, ⅡaⅡb, ⅢbⅣa, ⅣbⅤb), the NIACE score highlighted 2 different prognosis subgroups using a threshold value of 3 ($P < 0.0001$). The same results were obtained when investigating the HKLC Ⅰ group using a threshold value of 1 ($P < 0.0001$)[45].

In conclusion, the use of additional prognostic scores improves the stratification of HCC selected according to the BCLC system.

HCC CLASSIFICATION AND PROGNOSTIC SCORES: A USEFUL COMPLEMENTARITY FOR TREATMENT CHOICE

Prognostic scores benefit in HCC treatment: Before sorafenib

Sorafenib is recommended for BCLC stage C HCC[46,47] and is also a possible alternative for some BCLC stage B HCC being either progressive or confronted with chemoembolization contraindication[48]. The NIACE score allows to further stratify the BCLC stage C patients treated with sorafenib (Figure 5), by separating two distinct groups with different survival using a threshold value of 3[38]. The survival of patients with a NIACE score > 3 is limited to around 5.0 mo, despite a median treatment duration of 2 mo. Thus, this population does not seem to really benefit from the treatment and the NIACE score could be helpful in the treatment choice process or even earlier, to better classify patients before their enrollment into clinical trials.

Prognostic scores benefit in HCC treatment: Before chemoembolization

As chemoembolization is mainly recommended for intermediate BCLC stage B HCC[24], the usefulness of any additional prognostic score for such cases appears limited to some experts. However, if TACE remains the main treatment modality in most countries confronted with this disease[1], it is controversial. Its validation relies on two randomized studies with limited patients groups, mainly including intermediate and advanced HCC, and each offering a different treatment option[49,50]. Metadata analyses show contradictory results[51,52] and, despite the improvement of the selection criteria, the radiological response (according to the EASL or the mRECIST criteria)[53,54], the existing contraindications[55]

Table 7 Prognostic scores before the first transarterial chemoembolization

HAP (0 to 4 points)		NIACE (0 to 7 points)		STATE
Before the first TACE				
Albumin < 36 g/dL	1 point	⩾ 3 nodules	1 point	Albumin (g/L)
Bilirubin > 17 mcmol/L	1 point	infiltrative HCC *vs* nodular HCC	1.5 points	-12 (tumour load exceeding the up-to-7 criteria)
			0	
AFP > 400 ng/mL	1 point	AFP ⩾ 200 ng/mL	1.5 points	
		Child-Pugh A *vs* Child-Pugh B	0	
			1.5 points	
Size of dominant tumour > 70 mm	1 point	ECOG PS ⩾ 1	1.5 points	-12 (if CRP ⩾ 1 mg/dL)
No chemoembolization				
⩾ 2 points		> 3 points		< 18 points

HAP: Hepatoma arterial-embolisation prognostic; AFP: Alpha-fetoprotein; TACE: Transarterial chemoembolization; HCC: Hepatocellular carcinoma; ECOG: Eastern Cooperative Oncology Group; PS: Performance status; CRP: C reactive protein; STATE: Selection for transarterial chemoembolisation treatment.

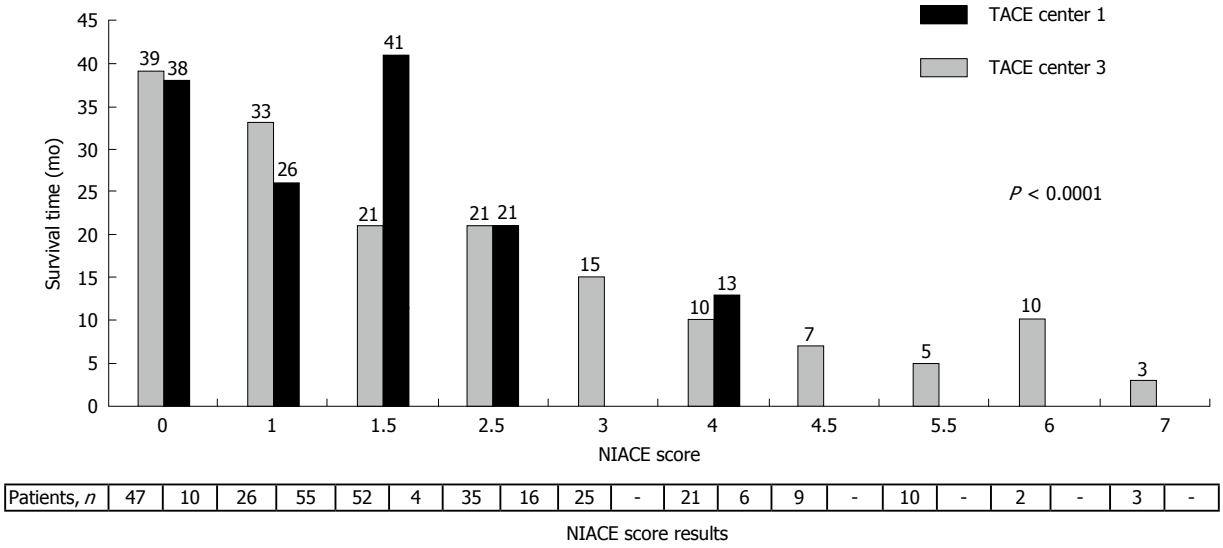

Figure 6 Evolution of the median overall survival according to the NIACE score in hepatocellular carcinoma patients from a French multicenter study treated by transarterial chemoembolization (grey bars center 1, black bars center 2)[60]. TACE: Transarterial chemoembolization.

or treatment termination criteria[56], there is still no consensus regarding the treatment strategy (on-demand or sequential), the number of treatments before reassessment[57], the overall aim (stability or response)[55,56] or concerning the TACE mode (using conventional techniques or calibrated drug-eluting beads). An additional score could thus facilitate the treatment strategy choice.

Before the first treatment

Several scores have been proposed recently to improve candidate patient selection (Table 7), as TACE is a potentially toxic treatment, with limited survival benefit. Among these pre-therapeutic scores, the Hepatoma Arterial-embolisation Prognostic (HAP) and the selection for transarterial chemoembolisation treatment (STATE) scores were determined from the prognostic variables of around a hundred of BCLC stage A, B (HAP, STATE) or even C (HAP) patients treated by TACE[58,59]. The NIACE score was also evaluated on two cohorts adding up 321 BCLC A, B or sometimes C (with distal portal vein thrombosis) patients treated by TACE. Using a threshold value of 3, the NIACE score identified two groups presenting a significantly different survival (NIACE ⩽ 3:27 mo (24-31) *vs* NIACE > 3:7 mo (6-10), $P < 0.0001$), even without any stage C patients (Figure 6)[60]. It also separated two subgroups with distinct prognosis from an Asian cohort of patients treated by TACE[39], as opposed to the HAP score which failed to prove its ability to select all the "good" candidates for TACE from a multicenter European cohort (with similar survival between the subgroups)[61]. Such a result could be anticipated as the same rating (1 point) is attributed to each variable and only HCC > 70 mm are taken into account, whereas the efficiency of the TACE treatment relies on the size (generally < 50 mm) and the number of nodules. The more recent STATE score, which mainly focuses on multinodular (BCLC B) HCC, still needs to be evaluated. The list presented here is not exhaustive and some relatively new scores now include indocyanine green clearance to better evaluate the liver function before TACE[29], but often at the expense of simplicity, which should remain a priority.

The continuation of a TACE treatment is determined by the radiological response (which is correlated to

Table 8 Pronostic scores before retreatment with transarterial chemoembolization

ART (0 to 8 points)		ABCR (-3 to 6 points)	
Before the second, the third TACE…..			
No radiological response	1 point	AFP < 200 ng/mL	0
		AFP ⩾ 200 ng/mL	1 point
AST increased > 25%	4 points	BCLC A/B/C	0/2/3 points
Child-Pugh increased: 1 point	1.5 points	Child-Pugh increased ⩾ 2 points	2 points
Increased: ⩾ 2 points	3 points	Radiological response	-3 points
No chemoembolization			
ART ⩾ 2.5 points		ABCR > 2 points	

TACE: Transarterial chemoembolization; ART: Assessment for retreatment with TACE; AST: Aspartate aminotransferase; AFP: Alpha-fetoprotein; BCLC: Barcelona Clinic Liver Cancer.

survival after TACE[53]), a decrease in AFP levels and the impact of the treatment on the liver function.

After the first TACE: Two scores easy to calculate were proposed to improve the selection of patients before repeating the treatment: The Assessment for Retreatment with TACE (ART) and the ABCR scores, both defined using regression models[62,63]. The ART score associates its higher coefficient with a possible increase in ASAT levels (4 points), the lower being associated with the radiological response (1 point). It is recommended not to repeat the treatment in case of a score worsening ⩾ 2.5 points (Table 8). Conversely, the ABCR system assigns a higher coefficient to the radiological response (-3 points), which is correlated to survival after TACE and to the initial stage of the disease (BCLC A/B/C: 0/2/3 points). The associated threshold value is a score worsening > 2 points. Both scores are usable after the second treatment. From a European multicenter cohort, the ART score calculated before the second or the third TACE failed to orientate the treatment option for all the patients[61,63]. If, unlike the ABCR, it did discriminate two different prognosis subgroups, the evolution of the ART score was not correlated with survival. As expected, patients with an ART score of 1 (*i.e.,* no radiological response) presented a lower survival than the ART 4 (ASAT levels increase > 25%) patients. Among the ABCR score limitations stands the possible absence of radiological response after the first TACE, which affects almost 25% of the "late responders", depending on the series[64]. The score being contributory after the second TACE, it is recommended to repeat the treatment in the absence of obvious progression and in case of worsening hepatic function.

The prognostic ability of the ABCR score was higher than the HAP and ART systems on both Western[65] and Asian cohorts[66].

Overall, these pre-chemoembolization scores are not able to embrace all the patients or situations and cannot replace a multidisciplinary meeting. However, owing to the high number of patients treated following this modality, the heterogeneity of HCC and day-to-day practices, such scores could help in the therapy decision making process (Figure 7).

Prognostic scores benefit in HCC treatment: Before surgical resection or radiofrequency

Surgical resection and radiofrequency ablation are curative treatments for HCC. In such cases, a score is not meant to exclude patients from the treatment when they meet the Barcelona criteria, early (BCLC A) stages being more homogeneous (single nodule or 3 nodules ⩽ 3 cm), but to further evaluate their prognosis (overall survival and recurrence), in the prospect of a possible complementary treatment. This is illustrated by the nomogram recently proposed by Liu *et al*[67] which orientates stage A HCC towards surgery or RFA according to the risk of recurrence (Figure 8). However, some experts have proposed to extend the indication for surgery beyond the Barcelona criteria to some intermediate or advanced HCC, which are more heterogeneous[27]. Despite some interesting results, only a proper randomized comparative study could address this question using a prognostic score to improve patient classification.

The NIACE score was tested on two French cohorts, both including around one hundred BCLC A/B and even C (single nodule with segmental portal vein thrombosis or above) HCC patients treated by surgery, thus beyond the scope of the BCLC recommendation, but in agreement with day-to-day practice. Using the more stringent threshold value of 1, it identified two different prognosis groups regarding the median overall survival (NIACE ⩽ 1:61 mo (36-81) *vs* NIACE > 1:18 mo (9-73), $P = 0.0005$) and the mean time to progression (NIACE ⩽ 1, 26.9 ± 16.3 mo *vs* NIACE > 1, 9.2 ± 9.7 mo, $P < 0.0001$)[68]. The score evolution was inversely correlated to survival (Figure 9). Similar results were observed using an Asian cohort comprising around one hundred BCLC A/B/C HCC patients treated by surgery[39].

When tested on a group of BCLC A HCC patients treated by surgery, selected from a French multicenter cohort, the NIACE score also highlighted two subgroups with distinct prognosis (median OS NIACE ⩽ 1:80 (58-81) mo *vs* NIACE > 1:39 (28-58) mo, $P = 0.0011$), notably among patients with a single tumor exceeding 50 mm in the longest axis (median OS NIACE ⩽ 1:80 (58-80) mo *vs* NIACE > 1:35 (18-58) mo, $P = 0.0024$)[44].

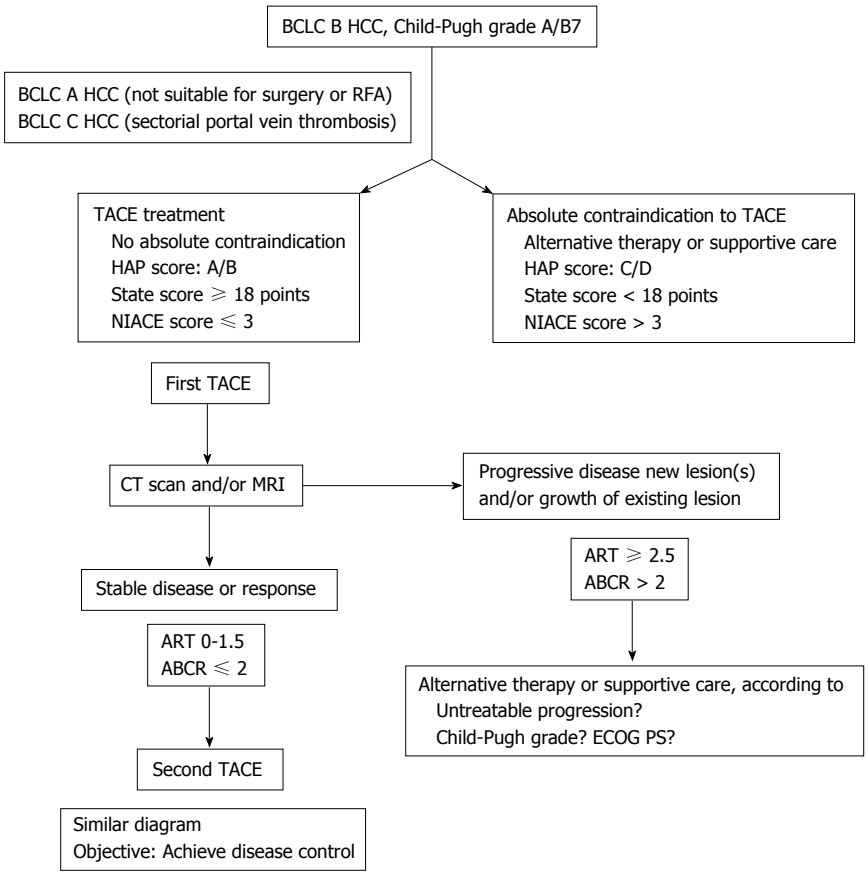

Figure 7 Prognostic scores designed to transarterial chemoembolization, an aid to the decision making process: In practice. BCLC: Barcelona Clinic Liver Cancer; HCC: Hepatocellular carcinoma; TACE: Transarterial chemoembolization; CT: Computed tomography; ART: Assessment for Retreatment with TACE; ECOG (PS), Eastern Cooperative Oncology Group (Performance Status); RFA: Radiofrequency ablation; HAP: Hepatoma Arterial-embolisation Prognostic; MRI: Magnetic resonance imaging.

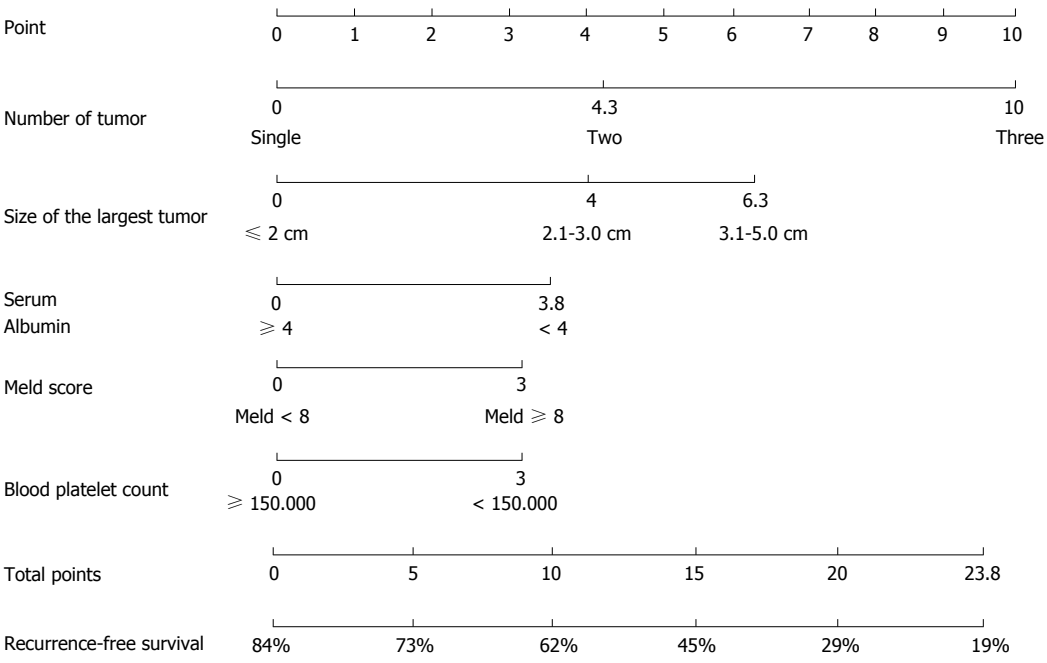

Figure 8 Nomogram for hepatocellular carcinoma recurrence after radiofrequency ablation[67].

These results should be further confirmed by a prospective study but, again, an additional prognostic score could provide complementary information to the BCLC system.

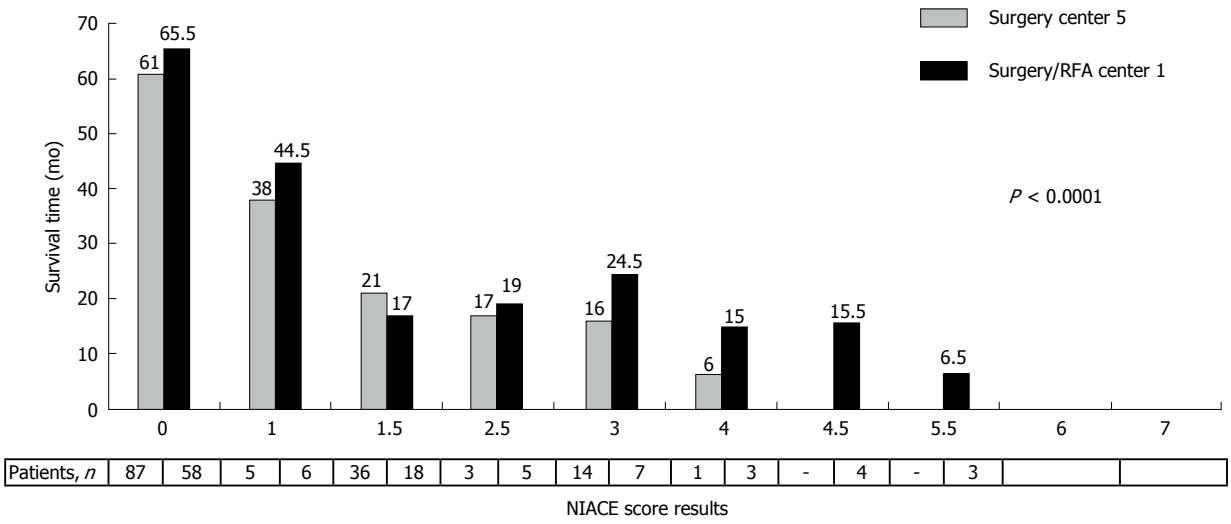

Figure 9 Evolution of the median overall survival according to the NIACE score in hepatocellular carcinoma patients from a French multicenter study treated by surgery/radiofrequency ablation (grey bars center 5, black bars center 1)[68]. RFA: Radiofrequency ablation.

CONCLUSION

HCC prognostic scores or classifications competed against each other until recently. A straightforward distribution and the corresponding treatment guide have allowed the BCLC classification to impose itself as the reference system in Western countries, and the HKLC system might do as well in Asian countries. However, owing to the heterogeneity of HCC, patients and daily practices, alternative scores such as NIACE, which includes different prognostic variables, could provide complementary tools to clinicians to better anticipate the disease evolution and optimize the stratification of patients within clinical trial or in the treatment decision making itself.

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TOPIC HIGHLIGHT

2016 Hepatitis B Virus: Global view

Innate immune targets of hepatitis B virus infection

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Abstract

Approximately 400 million people are chronically infected with hepatitis B virus (HBV) globally despite the widespread immunization of HBV vaccine and the development of antiviral therapies. The immunopathogenesis of HBV infection is initiated and driven by complexed interactions between the host immune system and the virus. Host immune responses to viral particles and proteins are regarded as the main determinants of viral clearance or persistent infection and hepatocyte injury. Innate immune system is the first defending line of host preventing from virus invasion. It is acknowledged that HBV has developed active tactics to escape innate immune recognition or actively interfere with innate immune signaling pathways and induce immunosuppression, which favor their replication. HBV reduces the expression of pattern-recognition receptors in the innate immune cells in humans. Also, HBV may interrupt different parts of antiviral signaling pathways, leading to the reduced production of antiviral cytokines such as interferons that contribute to HBV immunopathogenesis. A full comprehension of the mechanisms as to how HBV inactivates various elements of the innate immune response to initiate and maintain a persistent infection can be helpful in designing new immunotherapeutic methods for preventing and eradicating the virus. In this review, we aimed to summarize different branches the innate immune targeted by HBV infection. The review paper provides evidence that multiple components of immune responses should be activated in combination with antiviral therapy to disrupt the tolerance to HBV for eliminating HBV infection.

Key words: Hepatitis B virus; Infection; Targets; Innate immune response; Signaling pathway

Core tip: The pathogenesis of hepatitis B virus (HBV) infection is initiated and driven by complicated interplays between the virus and the host immune system. HBV DNA and different HBV proteins have various effects on different arms of innate immune system. The extent of HBV replication as well as the amounts of circulating

HBV antigens and different source of HBV proteins have heterogenous effects on innate immune responses and antiviral signaling pathways. Other factors, such as liver inflammation may also have impact on innate immune response. Multiple components of immune responses should be activated in combination with antiviral therapy to disrupt the tolerance to HBV for eliminating HBV infection.

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INTRODUCTION

Though hepatitis B virus (HBV) vaccine has been available for several decades and much progress has been made on anti-HBV therapeutics, there are still more than 350 million people chronically infected with HBV worldwide. The immunopathogenesis of HBV infection is initiated and propelled by complicated interactions between the host immune system and the virus[1].

It is recognized that host immune responses to HBV antigens are the major determinants of HBV pathogenesis and hepatocytes damage in the liver. On the contrary, viruses also exert immune regulatory effects to favor their replication. HBV has evolved active tactics to escape innate immune recognition and induce immunosuppression[2]. This has been displayed through the fact that HBV particles and proteins can be detectable around 5 wk postinfection, after which viral loads reach a logarithmic amplification stage[3]. The reason of the lag of viral replication is that the virus manages to evade being sensed by the innate immune system in the early phase of infection when the adaptive immune system has not been fully activated. HBV genome is 3.2 kbp in length and contains four overlapping genes that encode for the nucleocapsid (precore and core), polymerase, envelope (pre S and S), and hepatitis X proteins. The abundant HBV particles and viral proteins in the circulation in chronic HBV-infected patients allow multiple interactions among the virus, its viral proteins, and the immune system. HBV DNA and different HBV proteins have various effects on different parts of host immune systems, including immune cells and signaling pathways. The extent of HBV replication as well as the amounts of circulating HBV antigens, especially surface antigen (HBsAg), leads to heterogeneous profiles of the immune response, particularly in the context of chronic infection manifested as patients' different clinical profiles[4-6]. The immune tolerance phase has the highest level of serum HBsAg and hepatitis B e antigen (HBeAg) quantitation[7]. High levels of viremia, particularly high amounts of HBsAg, not only suppress innate immune cells, including monocytes, dendritic cells (DCs), natural killer (NK) cells, and NKT cells, through direct interaction, but also lead to exhaustion of cytotoxic T lymphocytes (CTLs) and helper T (Th) cells[8]. HBsAg mutations which enhanced the capability to avoid immune response were associated with HBV reactivation in a quite different clinical profiles[9]. Also, reduced viremia through antiviral therapy partially restores the impaired immune response[10] and the restored immune response status correlated with the levels of HBV infection parameters[11], which indirectly demonstrated the immune suppressive effect of HBV and its proteins.

A full understanding of the mechanisms as to how the virus inactivates various components of the immune system to maintain a persistent infection can help establish a new theory for designing novel immunotherapeutic methods and aid the eradication of the virus in chronic HBV infection.

Innate immune pathways are the targets of HBV to evade host antiviral responses contributing to chronicity of infection. The components of the innate immune system targeted by HBV include pattern-recognition receptors (PRRs), DCs, NKs, NKT cells, and antiviral signaling pathways[2]. In addition to directly regulating the innate immune response, HBV also modulates the innate immunity through alteration of the expression of microRNAs (miRNAs)[12].

PRRs

PRRs, including toll-like receptors (TLRs), retinoic acid-inducible gene Ⅰ (RIG-Ⅰ) - like receptors, and NOD-like receptors (NLRs), are crucial for sensing invading pathogens, initiating innate immune responses, restricting the spread of infection, and facilitating effective adaptive immune responses[13]. The early inhibition of the innate immune response by HBV is mainly through TLR-3 and RIG-Ⅰ/melanoma differentiation-associated gene 5 (MDA5) signaling pathways, which leads to decreased expression of several proinflammatory and antiviral cytokine genes[14]. In the setting of chronic HBV infection, reduced TLR expression and interference of PRRs signaling pathways lead to the impairment of host innate immune response.

TLRs

TLRs sense pathogen-associated molecule patterns (PAMPs), including nucleic acid sequences in degraded viral particles, and activate antiviral mechanisms, including intracellular antiviral pathways, production of antiviral effector interferons (IFNs) and proinflammatory cytokines, and initiation of adaptive immunity[15]. TLR signaling pathways are important parts of the innate immune response in HBV infection. It has been demonstrated that TLR ligands could suppress HBV replication[16]. Also, the activation of TLRs plays an important part in preventing intrauterine HBV transmission[17]. Accumulating evidence has consistently shown that the expression and function of TLRs in immune cells reduced during chronic HBV infection[18]. Expressions of TLR2 mRNA and protein were remarkably reduced

in peripheral blood monocytes (PBMCs) derived from chronic hepatitis B (CHB) patients[19].

HBV virions or proteins such as HBsAg and HBeAg may reduce TLR expression and abrogate TLR-induced antiviral activity. The inhibitory mechanisms include suppressing IFN-β production and induction of IFN-stimulated genes (ISG) and transcription factors, such as IFN regulatory factor 3 (IRF3) and nuclear factor-kappa B (NF-κB)[20]. HBsAg, HBeAg, and HBV particles could inhibit the activation of nonparenchymal liver cells by TLR3 ligands[20]. Jiang *et al*[21] demonstrated that TLR-induced the expression of IFN-γ, ISGs, and proinflammatory cytokines in murine Kupffer cells (KCs) and liver sinusoidal endothelial cells (LSECs), and the activation of NF-κB, IRF3, and mitogen-activated protein kinases (MAPKs) in hepatocytes were strongly suppressed by HBsAg. TLR3-stimulated KCs and LSECs mediated T-cell activation was also suppressed by HBsAg. Visvanathan *et al*[22] first showed that TLR2 expression on liver cells, KCs, and PBMCs significantly reduced in HBeAg-positive CHB patients compared with HBeAg-negative CHB and controls. TLR2 detects several microbial PAMPs and subsequently activates NF-κB in a myeloid differentiation primary response gene 88 (MyD88)-dependent manner. Therefore, decreased TLR2 expression may lead to impairment of immune responses to HBV infection[23]. In addition to directly inhibits the TLR2-mediated c-Jun N-terminal kinase/MAPK pathway, HBsAg may also induce interleukin (IL)-10 production in monocytes indirectly[24,25]. Thus, TLR2 is an important immune target of HBV infection.

Toll/IL-1 receptor (TIR) domain-containing adapter protein inducing IFN-β (TRIF) is an important component in innate immune signaling pathways. It is one of the main intracellular adapter proteins required for TLR3 and TLR4 signaling. Ayoobi *et al*[26] suggested that the expression of TRIF significantly decreased in PBMCs isolated from CHB patients compared with those isolated from healthy subjects. TRIF protein was also downregulated in human hepatoma cell lines and liver tissue specimens infected with HBV[27]. HBeAg interacted with TRIF-related adaptor molecule (TRAM), Mal, and TLR2 at the subcellular level, and mutated HBeAg not only may disrupt the interaction between Mal and MyD88 but also ablate homotypic TIR:TIR interaction, which is crucial for TLR-mediated signaling[28]. Furthermore, HBeAg can suppress TIR and IL-1β-mediated activation of the inflammatory transcription factors, such as NF-κB and inhibit NF-κB and IFN-β promoter activity[29]. These results suggest the presence of intracellular precore protein in addition to secreted extracellular HBeAg.

Hepatitis B virus X (HBx) and polymerase (Pol) are the proteins that interfere with the PRRs pathways most frequently[2,30]. For instance, HBx reduced TRIF protein expression *via* the proteasomal pathway in a dose-dependent manner[31]. However, no direct convincing evidence indicating that HBV RNAs, DNAs and proteins are authenticly recognized by TLRs is available up to date. The interplay between HBV proteins and TLRs should be verified directly *in vivo* through further investigation[15].

RIG-Ⅰ - MDA5 pathway

MDA5 and RIG-1 as the PRRs play important roles in viral mRNA recognition. HBx and HBV Pol are involved most frequently in the inactivation of the RIG-Ⅰ pathways and ultimately impaired IFN production. HBx is a pivotally protein involved in HBV-associated liver diseases. Studies[32] indicate that HBx can interact with the mitochondrial membrane protein virus-induced signaling adapter (VISA), which is a key adapter protein downstream RIG-Ⅰ and MDA5, and interrupts the association of VISA with its upstream and downstream parts. This inhibits the induction of type Ⅰ IFNs through the activation of transcription factors, including NF-κB and RF3. Human cell line studies[33] have also suggested that adapter protein mitochondrial antiviral signaling (MAVS) is another target for HBx. The RIG-Ⅰ/MDA5 pathway and IFN-β induction is inhibited due to degradation of MAVS promoted by HBx. A recent study[34] showed that mRNA levels of MDA5 and RIG-Ⅰ dramatically decreased in CHB patients in comparison with healthy controls. However, these mRNA levels have little alteration among CHB patients with different states of HBeAg and HBV DNA viral loads. Moreover, RIG-Ⅰ could also offset the interaction of HBV Pol with the 5′-ε region, which suggest that RIG-Ⅰ dually actions as an HBV sensor activating innate signaling and counteracting viral Pol in human liver cells[35]. Therefore, the mechanism underlying the downregulation of MDA5 may attribute to several reasons in patients with CHB[34]. DDX3, an HBV Pol binding protein, belonging to the DEAD-box RNA helicase family, is associated with mRNA metabolism. HBV Pol blocks PRRs signaling *via* interaction with DDX3[36]. This may explain the mechanism of how HBV evading the innate immune response.

In contrast, Luangsay *et al*[14] found that the early inhibition of dsRNA-mediated response resulted from the HBV inoculum, but not HBsAg or HBeAg itself. Whereas, the significance of these results in the human needs to be confirmed.

DCs

DCs are key cells in the initiation of adaptive immune responses because of their ability of processing foreign antigens and presenting them to effector cells. Also, mature DCs can efficiently induce T-cell polarization to Th1 and generate HBcAg-specific CTLs[37]. A long-lasting debate exists on the functionality and phenotypes of DCs in chronic HBV infection. Several studies demonstrated that DCs functions were impairment in CHB patients, which included decreased expression of co-stimulatory molecules, defective cytokine production, and reduced allostimulatory capacity compared with healthy people[38,39].

However, Gehring *et al*[40] suggested that the fre-

quency and function of myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) were largely intact *ex vivo* in HBV infected patients except for the reduced IFN-α production compared with those of healthy donor DCs. They found that reduced IFN-α production did not correlate with viral titer, which suggested that viral antigens had slight impact on DCs function. The major confusion about the function of DCs resulted from studies which indicated that function of healthy donor DCs was impaired exposing to various sources of HBV *in vitro*, which are in contrast to the results obtained from CHB patients. Tavakoli *et al*[41] also demonstrated that phenotypes and functionality of circulating total DCs, mDCs, or pDCs are unaffected in chronically HBV infected patients whether experimented *ex vivo* or after *in vitro* activation and maturation. They demonstrated that isolated mDCs and pDCs from chronic HBV carriers showed the similar expression of co-stimulatory molecules and alloreactive T cell stimulation as that of the control DCs.

However, other studies showed that pDCs were the targets of HBV. Both HBV virions and purified HBsAg have immune modulatory functions and may directly contribute to the impairment of mDCs functions in chronic HBV infection[42]. HBV particles and HBsAg were capable of abrogating TLR9-induced *IFN-α* gene transcription *via* combining to TLR9-triggered pDC directly[43]. HBV not only directly interfered with pDC function, but also indirectly disturbed monocyte-pDC interaction. In addition, the ability of inducing the cytolytic activity of NK cells by TLR9-activated pDCs from CHB patients were also compromised[44]. Virus-like particles (VLPs) comprising small HBV envelope protein (HBsAgS) impaired IFN-α production of pDCs in response to CpG *in vitro*[45]. Op den Brouw *et al*[42] suggested that in the presence of HBV or HBsAg, cytokine-induced maturation resulted in a more tolerogenic mDC phenotype, as demonstrated by a significantly reduced upregulation of co-stimulatory molecules and a decreased T-cell stimulatory capacity, as demonstrated by T-cell proliferation and production of IFN-γ and IL-12. It has been shown that DCs from immune tolerant patients showed a prominently lower expression of CD80, CD86, and HLA-DR and demonstrated an injured stimulatory capacity in mixed lymphocyte reactions and decreased production of IL-12, compared with those in the inactive HBsAg carrier state. Also, no remarkable difference was observed between the indexes from inactive carrier and healthy controls[46]. Several studies[47,48] revealed that mDC frequency could return to the level of healthy donors, IL12p70 production increased, and lower expression of phenotypic molecules was restored with antiviral therapy of adefovir and lamivudine. These results indirectly demonstrated the suppressive effect of high loads of HBV particles and proteins on DCs.

Though with suppressive effect on DCs functions, HBsAg is also a component of HBV vaccine. HBsAg-pulsed DCs might promote HBV-specific immune response in CHB patients[49]. HBsAgS VLPs can deliver an antigen to both major histocompatibility complex (MHC)-Ⅰ and MHC-Ⅱ in primary DCs and facilitate cytotoxic and helper T-cell priming[45]. Also, Ag-Ab immune complexes could be easily captured and taken up by DCs[50], and could efficiently induce HBs-specific T cells. A clinical study showed that the immunity was enhanced by autologous HBsAg-activated DC-cytokine-induced killer cells as adoptive immunotherapy[51]. Martinet *et al*[52] showed that the vaccination of Hepato-HuPBL mice with the HBc/HBs peptide-loaded pDCs induced HBV-specific T cells with specific ability of lysing the transfected hepatocytes. In addition, HBeAg might have a negative effect on the generation of DCs from bone morrow precursors[53].

The mechanism underlying the suppressive influence of HBV and HBV proteins on the function of DCs has not been fully elucidated. Some reports indicated that HBV and HBsAg can enter the DCs and cause damage, leading to a decline in the number of DCs and functional impairment[45,54]. However, Tavakoli *et al*[41] found that viral mRNA was not detectable by reverse transcription-polymerase chain reaction in both DC populations, which argues against viral replication in DCs.

The arguments regarding the functions and phenotypes of DCs result from the heterogeneous source of HBV antigens, variability of patients, and assay methods of DC maturation and cytokine production *in vitro* across studies[40]. In addition, liver pathology also likely affects the function of pDCs. Studies show that IFN-α production by pDCs is negatively correlated with the serum alanine aminotransferase (ALT) level in patients with CHB[39,43]. Furthermore, the expression of inhibitory molecule programmed death-ligand 1 (PD-1) in mDCs tended to more closely relate to ALT level than to viral load[55].

NK AND NKT CELLS

NK cells, the main innate immune cells, play indispensable roles in the clearance of HBV from hepatocytes. Although the numbers, subset distribution, and cytotoxic capacity of NK cells were retained, their activation and IFN-γ and tumor necrosis factor (TNF)-α production, particularly of the CD56(dim) subset, were strongly hampered in patients with CHB compared with healthy controls[56]. NK cells express several kinds of stimulatory and inhibitory receptors, which interact with their respective ligands results in functional activation and suppression[57]. Activation status and surface receptor expression patterns of NK cells may be altered in HBV infection[2]. Natural killer group 2D (NKG2D) is a well-characterized activating receptor expressed on NK cells, NKT cells, and CD8(+) cytotoxic T cells, which binds to a diverse group of ligands that resemble the MHC-class Ⅰ molecules. Accumulating evidence has shown that NKG2D-ligand interactions play a crucial role in the persistence of HBV infection and the development of liver injury and hepatocellular carcinoma. The expression of NKG2D ligands may be modulated post-trans-

criptionally by HBV[1]. Also, high serum HBV DNA loads upregulate the expression of inhibitory receptors such as NKG2A, but downregulate activating receptors, CD16, NKp30, NKG2D, and NKp46[56,58,59]. One study showed that the expression of NKp46 negatively correlated with the HBV DNA level and was much higher in inactive HBsAg carriers compared with active infection patients. NKp46 activation may restore NK cell cytolytic activity to HepG2 and HepG2.215 cell lines *in vitro*[59]. Furthermore, NK cell phenotype and functionality may partially be restored by viral load reduction through antiviral therapy, as shown by downregulated expression of NKG2A and improved IFN-γ production as a result of an increased ability of CD56(dim) NK cells[56]. And the recovered function of NK cells was strongly associated with HBsAg clearance[60]. Under the combination treatment of pegylated IFN-α-2a and adefovir, compared with nonresponders, responders had a remarkably lower expression of NKG2A on CD56(dim) NK cells and higher CD56 (bright) TNF-related apoptosis-inducing ligand expression and IFN-γ production at the end of the treatment. These results were not observed in HBeAg-positive patients who developed HBeAg seroconversion without HBsAg clearance[60]. The spontaneous reduction of HBV loads had similar results[61].

In addition, HBeAg may inhibit IFN-γ production by NK cells mediated by IL-18 in a dose-dependent manner[62]. HBV may specifically suppress pDC-induced IFN-γ production by NK cells without affecting their cytolytic ability through pDC-NK cell cross-talk[63]. Although NK cell IFN-γ production was impaired in response to TLR9 stimulation in CHB patients compared with controls, the upregulation of CD69 expression in response to TLR9 was maintained[64].

NKT cells are a unique subgroup of T-cells expressing both NK cell surface marker-CD56 and a T-cell receptor CD3 - which are stimulated by lipid antigens. NK and NKT are the two cell types that are promptly activated in the early phase of HBV infection, which probably contribute to controlling the HBV invasion and allowing timely induction of adaptive immune responses[65]. While, it has demonstrated that the frequency of hepatic NKT cells from HBV transgenic mice was low and the capability of producing IFN-γ was impaired[66]. Reports by Jiang *et al*[67] and Zhu *et al*[68] indicated that the frequency of peripheral invariant NKT (iNKT) cells is lower in patients with chronic HBV infection than in healthy subjects, and returns to normal levels during viral control with telbivudine. In patients treated with PEG-IFN-α, the ratio of peripheral blood NKT cells in T lymphocytes before, during, and after treatment significantly elevated in the significant-effect group compared with the effect and no-effect groups[69]. This implies that NKT cells modulate the innate immune response against HBV infection and play a major role in effective antiviral treatment.

The mechanism underlying the decrease in the number and function of circulating iNKT cells in patients with CHB remains unclear. It is believed that this reduction is at least partially due to trafficking to the liver[70] because iNKT cells express a high level of CC chemokine receptor 5 (CCR5) and CCR6[67] which enable iNKT cells migrate toward the liver. Other mechanism may involved in the high expression of inhibitory molecules PD-1 and Tim-3, and lower the expression of CD28[66,71]. In addition, HBV-induced lipid alterations also contributed to a change in NKT cell function[72].

IMMUNE TARGET OF SIGNALING PATHWAYS OF THE ANTIVIRAL RESPONSE AND CYTOKINES

Recognition of viral infections by PRRs, such as TLRs and RIG-Ⅰ/MDA5, activates signaling pathways and leads to the induction of inflammatory and antiviral cytokines, such as type Ⅰ IFN, that limit viral replication and initiation of adaptive immunity. The expression of TLR signaling molecules, such as MyD88, IL-1 receptor-associated kinase 1 (IRAK1), and IRAK4, significantly decreased in PBMCs from CHB patients compared with healthy controls[73,74].

HBV proteins, such as HBV Pol and HBx, could interfere with multiple sites of intracellular signaling pathways triggered by HBV infection, preventing IFN production and antiviral responses in hepatocytes[30,75]. HBV Pol can inhibit TANK-binding kinase 1 (TBK1)/IkappaB kinase-epsilon (IKKi), the effector kinases of IRF signaling. It can block IRF signaling activation mediated by TLR-3 or RIG-Ⅰ recognizing dsRNA in the endosomes or in the cytosol through interaction with DDX3, a transcriptional factor of the IFN-β promoter in human hepatoma cell lines[30,32,36]. HBV Pol mediates blockage of IFN-α signaling through suppressing IFN-α-induced signal transducers and activators of transcription 1 (STAT1) serine 727 phosphorylation and STAT1/2 nuclear accumulation[76]. Pol also affects STAT methylation through increasing protein phosphatase 2A (PP2A) expression, which inhibits protein arginine methyltransferase 1, the enzyme that catalyzes the methylation of STAT1[77]. This may be responsible for HBV resistance to PEG-IFN-α therapy[78]. However, HBV Pol does not interfere with STAT1 degradation and phosphorylation[79]. The cytosolic DNA sensor and key adaptor stimulator of *IFN* genes (STING) has been suggested to be critical in multiple foreign DNA-elicited innate immune signaling. Screening analysis demonstrated that the reverse transcriptase and the RNase H (RH) domains of HBV Pol were responsible for the inhibition of STING-stimulated IRF3 activation and IFN-β induction[80]. One study has demonstrated that HBV Pol preferentially suppresses TNF-α-, TLR3- or TLR4-induced NF-κB signaling by inhibiting the activity of IKK complex through disrupting the association of IKK/NF-κB essential modulator (NEMO) with Cdc37/Hsp90β in hepatoma cells[81]. Therefore, in addition to its inherent catalytic function, HBV Pol has multifunctional immunomodulatory effects. It may counteract the innate

Table 1 Innate immune cells, molecules and signaling pathways targeted by hepatitis B virus and hepatitis B virus proteins

HBV and HBV proteins	Innate immune cells, molecules and signaling pathways	Ref.
HBs	TLR, ISG, IRF3, IFN-β and NF-κB	[20]
	KCs, LSECs, IFN-γ, ISGs, MAPKs, TLR3	[21]
	JNK/MAPK, κBα	[24,25,87]
	mDCs, pDCs, TLR-9	[42,43]
HBe	Hepatocytes, KCs, PBMCs, and TLR2, ISG, IRF3, IFN-β and NF-κB	[20,22]
	TRAM, Mal, TLR2, and TIR:TIR	[28]
	NF-κB and IFN-β promoter, IFN-γ	[29,62]
	RIPK2	[83]
HBx	TRIF, RIG-I/MDA5, VISA, MAVS, NF-κB	[31-33,82]
	NEMO, TBK1, IKKi, and IRF3	[75]
HBV Pol	RIG-I, DDX3, NEMO-Cdc37/Hsp90β	[35,36,81]
	TBK1/IKKi, STAT1, PP2A, STING	[32,33,36,76,77,80]
HBV	NK, NKG2D, NKG2A, CD16, NKp30, and NKp46	[56,58,59]
	pDC–NK, NKT	[63,72]
	CTHRC1	[84]

HBs: Hepatitis B surface antigen; HBe: Hepatitis B e antigen; HBx: Hepatitis B x protein; HBV Pol: Hepatitis B polymerase; TLRs: Toll-like receptors; ISG: Interferon-stimulated genes; IFN: Interferon; NF-κB: Nuclear factor κB; KCs: Kupffer cells; LSECs: Liver sinusoidal endothelial cells; MAPKs: Mitogen-activated protein kinases; JNK: c-Jun N-terminal kinase; mDCs: Myeloid dendritic cells; pDCs: Plasmacytoid DCs; PBMCs: Periperal blood mononuclear cells; TRAM: TRIF-related adaptor molecule; TIR: Toll/interleukine-1 receptor; RIPK2: Receptor-interacting serine/threonine protein kinase 2; TRIF: TIR domain-containing adapter protein inducing IFN-β; RIG-Ⅰ: Retinoic acid inducible gene Ⅰ; IRF: Interferon-regulatory factors; MDA5: Melanoma differentiation associated gene 5; VISA: Virus-induced signaling adapter; MAVS: Mitochondrial antiviral signaling; TBK1: TANK-binding kinase 1; IKKi: KappaB kinase-epsilon; STAT1: Signal transducers and activators of transcription 1; PP2A: Protein phosphatase 2A; STING: Stimulator of IFN genes; NK: Natural killer; NKG2D: NK group 2D; NKG2A: NK group 2A; NKT: NK Tcell; CTHRC1: Collagen triple helix repeat containing 1; HBV: Hepatitis B virus; MAPK: Mitogen-activated protein kinase; NEMO: NF-κB essential modulator.

responses at different steps.

Similar to HBV Pol, HBx can target multiple points of signaling pathways negatively regulating type Ⅰ IFN production. In addition to RIG-Ⅰ, TNF receptor-associated factor 3, and TRIF, HBx also interacts with NEMO, TBK1, kinase-epsilon (IKKi), and IRF3[75]. HBx can also transactivate multiple transcription factors including NF-κB that regulates inflammatory-related genes. A recent report has suggested that HBx-evolutionarily conserved signaling intermediate in toll pathways interaction plays an important role in in IL-1β induction of NF-κB activation[82].

In addition to HBV Pol and HBx proteins, HBeAg may also modulate the intracellular signaling pathways. HBeAg may target receptor-interacting serine/threonine protein kinase 2 through inhibiting its expression and interacting with it[83] which may results in inactivation of NF-κB. Experiments indicate that collagen triple helix repeat containing 1 (CTHRC1) expressed in HBV-transfected cells facilitates HBV replication in cultured cells and BALB/c mice. On the other hand, HBV increases CTHRC1 expression, which downregulates the activity of type Ⅰ IFN, the transcription of ISGs, and the phosphorylation of STAT1/2[84].

However, some of the signaling pathways are important in restraining HBV replication. Tzeng *et al*[85] demonstrated that not IFN-α/β receptor, RIG-Ⅰ, MDA5, MyD88, NLR pyrin containing 3, caspase recruitment domain, and IL-1R but TNF-α is essential for HBV eradication. In the absence of TNF-α, or early treatment with the soluble blocker of TNF receptor in mice leads to HBV persistence[86]. This may explain the mechanism of HBV reactivation during TNF blockage agents therapy.

In contrast to HBeAg, research has reported that the treatment of human monocyte-derived DCs with HBsAg resulted in enhanced cell surface expression of CD80, CD83, CD86, and MHC-Ⅱ, and increased IL-12 p40, IL-12p70, and IL-10 production through decreasing inhibition of κBα concentrations and MAPK phosphorylation[87].

CONCLUSION

The suppression of various innate immune components targeted by HBV and HBV proteins may result in virus spread and subsequent inefficient adaptive immune responses, leading to HBV persistence. However, still controversies exist regarding the effects of HBV on the functionalities and phenotypes of innate immune cells, especially DCs. The conflicting results may be due to patient diversity, divergence of antigen sources, and inconsistent assay methods. Some of the findings derived from cell line and animal models remain to be defined for the human HBV infection. Furthermore, the knowledge of the exact mechanism of action of HBV and HBV proteins on some of the sites of the complicated innate signaling pathways is lacking. The updated findings of innate immune cells, molecules and signaling pathways targeted by HBV and HBV proteins are summarized in Table 1. The present study

provides evidence that multiple components of immune responses should be activated in combination with antiviral therapy to disrupt the tolerance to HBV for eliminating HBV infection.

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Basic Study

Anti-CD163-dexamethasone conjugate inhibits the acute phase response to lipopolysaccharide in rats

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Abstract

AIM: To study the effect of a new anti-CD163-dexamethasone conjugate targeting activated macrophages on the hepatic acute phase response in rats.

METHODS: Wistar rats were injected intravenous with either the CD163 targeted dexamethasone-conjugate (0.02 mg/kg) or free dexamethasone (0.02 or 1 mg/kg) 24 h prior to lipopolysaccharide (LPS) (2.5 mg/kg intraperitoneal). We measured plasma concentrations of

tumour necrosis factor-α (TNF-α) and interleukin 6 (IL-6) 2 h post-LPS and liver mRNAs and serum concentrations of the rat acute phase protein α-2-macroglobulin (α-2-M) 24 h after LPS. Also, plasma concentrations of alanine aminotransferase and bilirubin were measured at termination of the study. Spleen weight served as an indicator of systemic steroid effects.

RESULTS: The conjugate halved the α-2-M liver mRNA (3.3 ± 0.6 *vs* 6.8 ± 1.1, $P < 0.01$) and serum protein (201 ± 48 μg/mL *vs* 389 ± 67 μg/mL, $P = 0.04$) after LPS compared to low dose dexamethasone treated animals, while none of the free dexamethasone doses had an effect on liver mRNA or serum levels of α-2-M. Also, the conjugate reduced TNF-α (7208 ± 1977 pg/mL *vs* 21583 ± 7117 pg/mL, $P = 0.03$) and IL-6 (15685 ± 3779 pg/mL *vs* 25715 ± 4036 pg/mL, $P = 0.03$) compared to the low dose dexamethasone. The high dose dexamethasone dose decreased the spleen weight (421 ± 11 mg *vs* 465 ± 12 mg, $P < 0.05$) compared to controls, an effect not seen in any other group.

CONCLUSION: Low-dose anti-CD163-dexamethasone conjugate effectively decreased the hepatic acute phase response to LPS. This indicates an anti-inflammatory potential of the conjugate *in vivo*.

Key words: Acute phase response; Dexamethasone; Endotoxin; Hemoglobin scavenger receptor CD163; Cytokines; Inflammation; Rats

Core tip: We aimed to study the effect of a new anti-CD163-dexamethasone conjugate targeting activated macrophages on the hepatic acute phase response in rats. The central finding of the study was a reduction in liver mRNA and plasma levels of the acute phase protein α-2-macroglobulin, and plasma tumour necrosis factor-α and interleukin 6 by administration of the conjugate prior to a lipopolysaccharide-induced inflammatory response. This anti-acute phase effect exceeded that of the therapeutic dexamethasone dose and did not cause systemic adverse effects. Thus, the antibody conjugate may be a potential candidate in future anti-inflammatory macrophage-directed therapy, *e.g.*, in liver diseases with Kupffer cells activation.

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INTRODUCTION

In conditions with macrophage proliferation and activation, CD163, a haemoglobin-haptoglobin scavenger receptor expressed exclusively on monocytes and macrophages[1,2], is up-regulated[3,4]. Following toll-like receptor activation by inflammatory stimuli like lipopolysaccharide (LPS), receptor shedding to circulation as soluble CD163 (sCD163) is increased, and within hours upregulated on the cell surface[5]. As an example, hepatic macrophages (Kupffer cells) are activated and sCD163 is increased in patients with liver cirrhosis who chronically experience some degree of endotoxemia and acute phase response[6,7] and this may be involved in the development of the serious cirrhosis complications[6,8].

We have recently constructed a conjugate of CD163 antibody and the potent corticosteroid dexamethasone (anti-CD163mAb-dexa) specifically targeting dexamethasone to activated macrophages[9]. The conjugate reduces the LPS-stimulated cytokine release from activated macrophages *in vitro* and *in vivo* in rats and pigs[9,10]. The effect is obtained with very low concentration of dexamethasone, thereby minimizing steroid-induced systemic effects. A fifty-fold higher concentration of non-conjugated dexamethasone is needed to obtain the same anti-inflammatory response[9].

Exposure to LPS is a standard method to induce an acute phase response with a large increase in pro-inflammatory cytokines and hepatic synthesis and release of acute phase proteins[11,12]. While the conjugate reduces the LPS-mediated cytokine response in rats it remains unknown whether it also inhibits the hepatic acute phase protein synthesis response.

To approach this issue we measured the gene expression in liver tissue and serum concentrations of the prevailing acute phase protein α-2-macroglobulin (α-2-M) 24 h post-LPS exposure in rats. α-2-M is a hepatocyte-derived inhibitor of a wide range of proteinases that can be activated during inflammation[13]. Further, we compared plasma concentrations of tumour necrosis factor-α (TNF-α) and interleukin 6 (IL-6) 2 h post-LPS exposure. Spleen weight served as an indicator of systemic steroid effects.

MATERIALS AND METHODS

Animals

The animal protocol was designed to minimize pain or discomfort to the animals. Female Wistar rats (body weight 190-210 g; Taconic M and B, Ejby, Denmark) were housed at 21 ℃ ± 2 ℃ with a 12-h artificial light cycle. Two or three animals were housed in each cage, with free access to tap water and standard food (Altromin, Lage, Germany) and acclimatized for one week. Food intake and body weight were registered at the beginning and at the end of the experimental procedures. The study was performed in accordance with local and national guidelines for animal welfare and approved by the national Animal Ethics Committee, protocol No. 2010/561-1918.

Design

Forty animals were allocated in 5 groups of 8: One

Table 1 Weights, liver function tests, and cytokines.

	Controls	LPS	Anti-CD163-dexa plus LPS	High dexa plus LPS	Low dexa plus LPS
Body weight	199 ± 1	196 ± 2	207 ± 2[g]	204 ± 3	206 ± 3[g]
Weight loss	11 ± 1	14 ± 3	22 ± 2[a]	23 ± 2[a]	21 ± 1[a]
Spleen weight	465 ± 12	512 ± 31	492 ± 23	421 ± 11[a]	483 ± 23
ALT	42 ± 3	61 ± 16	57 ± 20	48 ± 9	77 ± 31
Bilirubin	3.0 ± 0.0	3.3 ± 0.3	3.1 ± 0.1	3.6 ± 0.4	4.0 ± 0.4
TNF-α	0 ± 0	26817 ± 9780[a]	7208 ± 1977[a,c]	16891 ± 4210[a]	21583 ± 7117[a]
IL-6	0 ± 0	23075 ± 6758[a]	15685 ± 3779[a,c,e]	32964 ± 8294[a]	25715 ± 4036[a]

Body weight (g), body weight loss (g), spleen weight (mg), plasma alanine aminotransferase (U/L), and bilirubin (μmol/L) in controls (n = 8) and in animals injected with LPS 24 h after vehicle (n = 8), anti-CD163mAb-dexa (n = 8), high dose (n = 8) and low dose (n = 8) dexamethasone at termination of study. Plasma TNF-α (pg/mL) and IL-6 (pg/mL) are measured 2 h after saline (controls) or LPS injection. [a]P < 0.05 *vs* controls; [c]P < 0.05 *vs* low dose free dexamethasone group; [e]P < 0.05 *vs* high dose free dexamethasone group; [g]P < 0.05 *vs* vehicle. ALT: Alanine aminotransferase; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; LPS: Lipopolysaccharide.

control group receiving only vehicle (PBS pH 7.4) intravenously and four groups injected intravenously with either vehicle, anti-CD163mAb-dexa (0.02 mg/kg dexamethasone), high dose free dexamethasone (1 mg/kg) (Sigma-Aldrich, Brøndby, Denmark), or low dose free dexamethasone (0.02 mg/kg). The high ("therapeutic") dose gives maximal steroid efficacy in other rat studies[14,15] and the low dose was the same as in the anti-CD163mAb-dexa. After 24 h, 0.5 mL of saline (controls) or LPS dissolved in 0.5 mL saline (2.5 mg/kg) (from *Ecsherichia coli* 0111:B4 obtained from Sigma-Aldrich, Brøndby, Denmark; product No. L2630) was injected intraperitoneally. Two hours later and following anaesthesia with inhalation of isofluran 2%-3% (Forene®, Abbott Laboratories, Gentofte, Denmark), a blood sample for determination of plasma TNF-α and IL-6 was drawn from a retrobulbary venous plexus using heparinised micropipettes. After an overnight 12-h fast the animals were anaesthetised with a subcutaneous injection of fentanyl/fluanisone (Hypnorm®, Jansen Pharma, Birkerød, Denmark) 0.5 mL/kg and midazolam (Dormicum®, La Roche, Basel, Schwitzerland) 2.5 mg/kg. All blood was collected for blood analyses and approximately 200 mg of liver tissue was snap-frozen in liquid N_2, and stored at -80 ℃. Finally, the spleen was weighed. In all animals we measured liver mRNA levels and serum concentrations of α-2-M and plasma concentrations of alanine aminotransferase and bilirubin at termination of the study.

Liver tissue

mRNA levels of α-2-M were determined by slot blot hybridization as previously described[16].

Blood analyses

The concentrations of α-2-M in serum were evaluated by rat ELISA (Immunology Consultants Laboratory, Newberg, OR, United States). The plasma concentrations of TNF-α and IL-6 were determined by immunoassay (R and D Systems, Minneapolis, MN, United States, both). Samples were analysed in duplicate and all assays had intra- and inter-assay coefficients of variance below 5% and 10%, respectively. Plasma concentrations of alanine aminotransferase and bilirubin were determined by standard clinical biochemical analytical methods.

Statistical analysis

Data were analysed using the Kruskal-Wallis One Way Analysis of Variance on Ranks; when significant, post-hoc tests were performed among groups by the Mann-Whitney rank sum test. Data are presented as the mean ± SEM. Differences were considered significant with P-values < 0.05. A statistical review of the study was performed by a biomedical statistician.

RESULTS

Body and spleen weight

LPS induced a body weight loss in all the intervention groups (P < 0.05) (Table 1) and there was no difference among these groups. The high dose dexamethasone dose decreased the spleen weight (P < 0.05), an effect not seen in any other group (Table 1).

Acute phase protein liver mRNA and serum levels

LPS increased the liver mRNA and serum levels of α-2-M several fold in all groups (P < 0.01) (Figure 1). Anti-CD163mAb-dexa approximately halved the α-2-M liver mRNA (P < 0.01) and serum response (P = 0.04) compared to low dose dexamethasone treated animals, while no free dexamethasone dose had any effect on liver mRNA or serum levels of α-2-M compared to vehicle (Figure 1).

TNF-α and IL-6

LPS markedly increased plasma TNF-α and IL-6 in all groups (P < 0.001). There was a trend for reduced TNF-α (P = 0.08) after anti-CD163mAb-dexa compared to vehicle and significantly so *vs* the low dose dexamethasone (P = 0.03). Also, the anti-CD163mAb-dexa decreased IL-6 compared to both dexamethasone doses (P < 0.05). None of the free dexamethasone doses had

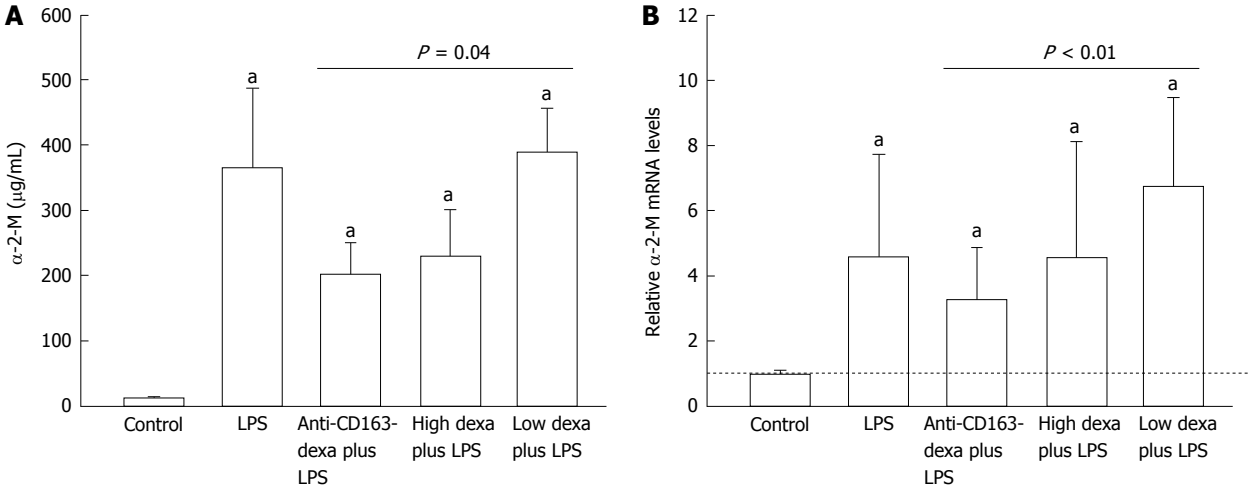

Figure 1 Relative levels of serum levels (A) and liver mRNA (B) of α-2-macroglobulin. Changes in serum levels (μg/mL) (A) and liver mRNA (% of controls) (B) of α-2-macroglobulin (α-2-M) in controls (n = 8) and in animals injected with LPS 24 h after vehicle (n = 8), anti-CD163mAb-dexa (n = 8), high dose (n = 8) and low dose (n = 8) dexamethasone. mRNA results from LPS-injected animals are presented as relative levels compared to control animals. Bars represent the mean and SEM. [a]P < 0.05 *vs* controls. LPS: Lipopolysaccharide; SEM: Standard error of mean.

an effect on TNF-α or IL-6 (Table 1).

Plasma-alanine transferase and bilirubin

LPS had no effect on these measures at termination of the study (Table 1).

DISCUSSION

The central finding of this study was the reduction in liver mRNA and plasma α-2-M, and plasma TNF-α and IL-6 by the administration of the anti-CD163-dexa conjugate prior to the LPS-induced inflammatory response. This anti-acute phase effect much exceeded that of the therapeutic dexamethasone dose and did not cause systemic adverse effects, as evidenced by reduced spleen weight in the group treated with high dose free dexamethasone. This study completes the chain of evidence that the conjugate not only suppresses the LPS elicited IL signaling but also the ultimate effect on synthesis and release of hepatic acute phase proteins that effectuate the acute phase response.

The increase in plasma α-2-M after LPS reflects *de novo* synthesis as almost no such protein is present under non-induced conditions[17] in contrast to conditions with ongoing low grade inflammation such as cirrhosis[18]. LPS as assumed caused a marked systemic acute phase response reflected in increased liver mRNA and plasma α-2-M, TNF-α, and IL-6. In contrast to the equal amount of free dexamethasone, the anti-CD163mAb-dexa efficiently suppressed this response. Still, however, the acute phase response to some extent serves to restore homeostasis and one needs to be aware that suppression of the response might not be entirely beneficial entailing a potential risk using the conjugate long term.

The anti-inflammatory effects of glucocorticoids are related to a decrease in lymphocyte expansion and cell survival and also a reduction in the expression of pro-inflammatory cytokines originating from macrophages[19]. However, as glucocorticoids bind to the ubiquitous intracellular glucocorticoid steroid receptor present in most cell types they also exert serious systemic metabolic side effects. Thus dexamethasone causes the spleen to undergo a corticosteroid-induced weight reduction due to lymphocyte depletion[20]. Accordingly, the high dose dexamethasone in our study decreased the spleen weight as compared with the other groups reflecting systemic non-macrophages effects. In contrast, the conjugate did not affect spleen weight and was still found to exert a potent anti-inflammatory effect.

In our animal model, the conjugate was given as a pre-emptive dose prior to the induction of the acute phase response as we aimed at establishing a proof-of-concept position of the conjugate's effects. We believe our findings support further studies on interference with on-going inflammation in relevant experimental models. Such studies are also essential for monitoring of long term effects of the conjugate.

In conclusion, the anti-CD163-dexa conjugate demonstrated potent effects in reducing the acute phase proteins without evident systemic side effects during an endotoxin-induced acute phase response in rats. The effect much exceeded that of a therapeutic dose of dexamethasone. Thus, the antibody conjugate may be a potential candidate in future anti-inflammatory macrophage-directed therapy, *e.g.*, in liver diseases with Kupffer cells activation[7].

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COMMENTS

Background

In conditions with macrophage proliferation and activation, CD163, a scavenger

receptor expressed exclusively on monocytes and macrophages, is up-regulated. As an example, hepatic macrophages (Kupffer cells) are activated and CD163 is increased in patients with liver cirrhosis who chronically experience some degree of endotoxemia and acute phase response.

Research frontiers

The authors have recently constructed a conjugate of CD163 antibody and the potent corticosteroid dexamethasone (anti-CD163mAb-dexa) specifically targeting dexamethasone to activated macrophages.

Innovations and breakthroughs

The anti-CD163-dexa conjugate exerts an anti-inflammatory effect, which is obtained with very low concentration of dexamethasone, thereby minimizing steroid-induced systemic effects.

Applications

The antibody conjugate may be a potential candidate in future anti-inflammatory macrophage-directed therapy, *e.g.*, in liver diseases with Kupffer cells activation.

Peer-review

This is an experimental report written by Thomsen *et al*, which indicates an efficacy of dexamethasone-conjugated anti-CD163 against lipopolysaccharide-induced acute inflammatory reaction. The well-designed study was carried out using firm methods.

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ORIGINAL ARTICLE

Clinical Trials Study

Therapeutic usability of two different fiducial gold markers for robotic stereotactic radiosurgery of liver malignancies: A pilot study

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Author contributions: Marsico M conceived the study, participated in the procedures for placement of the markers, wrote and reviewed the article; Gabbani T Participated in the procedures for placement of the markers, performed the statistical analysis, wrote and reviewed the article; Livi L and Galli A participated in the study design, coordinated and helped to draft the manuscript; Biagini MR participated in the study design, and in the procedures for placement of the markers and review of the article.

Institutional review board statement: The study was reviewed and approved by the Gastroenterology Unit, University of Florence review board.

Informed consent statement: All patients provided written informed consent for enrolment in the study, and inclusion in this article of information that could potentially lead to their identification.

Conflict-of-interest statement: The authors (Maria Marsico, MD, Tommaso Gabbani, MD, Andrea Galli, Professor, Maria Rosa Biagini, MD, Lorenzo Livi, Professor) have no conflicts to report.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at ma.marsico@libero.it. Participants gave informed consent for data sharing, however the data presented are anonymous and risk of identification is low.

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Abstract

AIM: To assess how the application of different types of markers affects the tracking accuracy of CyberKnife's.

METHODS: Fifteen patients were recruited and subjected to the ultrasound-guided placement of markers. Two different type of needles 25 gauge (G) and 17 G containing two different fiducial marker, gold notched flexible anchor wire 0.28 mm × 10 mm (25 G needle) and gold cylindrical grain 1 mm × 4 mm (17 G), were used. Seven days after the procedure, a CyberKnife planning computed tomography (CT) for the simulation of radiation treatment was performed on all patients.

A binary CT score was assigned to the fiducial markers visualization. Also, the CT number was calculated for each fiducial and the values compared with a specific threshold.

RESULTS: For each patient from 1 to 5, intra-hepatic markers were placed (one in 2 patients, three in 8 patients, four in 3 patients, and five in 2 patients). A total of 48 needles were used (thirty-two 17 G and sixteen 25 G) and 48 gold markers were placed (32 Grain shaped markers and 16 Gold Anchor). The result showed that the CT visualization of the grain markers was better than the anchor markers ($P = 5 \times 10^{-9}$). Furthermore, the grain markers were shown to present minor late complications ($P = 3 \times 10^{-6}$), and the best CT threshold number ($P = 0.0005$).

CONCLUSION: The study revealed that the Gold Anchor fiducial marker is correlated with a greater number of late minor complications and low visualization by the CT.

Key words: Robotic radiosurgery; Fiducial markers; Liver malignancies; CyberKnife; Radiation therapy; Stereotactic radiosurgery

Core tip: Robotic radiosurgery can employ different systems for the localization of the neoplastic targets to treat. The purpose of this study is to assess how the application of different types of markers affects the tracking accuracy of CyberKnife's. Fifteen patients have been recruited and analyzed for the study and two types of markers were used for the procedure. The computed tomography (CT) visualization of grain markers was better than anchor markers $P = 5 \times 10^{-9}$. Grain markers presented minor late complications of $P = 3 \times 10^{-6}$, and the best CT threshold number. The study revealed that the Gold Anchor fiducial marker is correlated with a greater number of late minor complication.

Marsico M, Gabbani T, Livi L, Biagini MR, Galli A. Therapeutic usability of two different fiducial gold markers for robotic stereotactic radiosurgery of liver malignancies: A pilot study. *World J Hepatol* 2016; 8(17): 731-738 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i17/731.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i17.731

INTRODUCTION

The stereotactic robotic radiosurgery is able to administer high-dose radiation that could reach any anatomic point with a sub-millimeter precision[1-4]. The high accuracy is achieved by the image-guidance system robotic technology and the dynamic tracking of targets, that remove the effect of breathing. The use of these techniques permits the CyberKnife system's to hit the lesion with high-dose radiation and to safeguard the surrounding critical organs which could suffer irreversible damage[5-13]. Robotic radiosurgery can employ different systems for the localization of the neoplastic targets to treat. In particular, for the treatment of the parenchymatous organ tumors, CyberKnife uses a localization system based on specific gold markers[14]. Various types of gold markers can be employed in relation to the characteristics of the lesion and the different technique of placement. In particular, the type of gold markers to use often depends on the choice of needles of different calibers and length. The choice of the needle is influenced by the type and site of the lesion to treat and its proximity to critical organs or vascular structures[15,16]. The physical characteristic (dimensions and length) of the gold markers strongly depends on the characteristics of the needle. The gold markers (Gold Anchor) contained in fine needle [25 gauge (G) and 22 G] must be smaller in dimension and longer than those contained in larger needles. Markers contained in fine needles, in order to reach an appropriate density for a normal computed tomography (CT) number and to be correctly recognized by the CyberKnife system, must assume a correct array in the parenchyma, when they are inserted. In fact, they have the advantage of being flexible and to curl up when they are pushed against the parenchyma tanks to the spindle and carried by the needle. Therefore, after their placement, the Gold Anchor reached some similar dimensions to those in grain and so, an appropriate density and a normal CT number. Therefore, if they are too crowded or shatter during their release, they do not achieve the proper density to have a normal CT number, and to be well recognized as a fiducial by the CyberKnife System. The markers (cylindrical markers) contained in larger gauge needles (17 G and 18 G) can not break and do not need to mass during their placement. Therefore they can not change their CT number (Figure 1)[17-21]. The placement of fiducial markers may be burdened by complications due to puncture or related to the gold markers. For instance, the major complications related to the gold markers could be the migration of fiducials from the positioning site and the physical alterations of the markers, like marker not deployed or shattered, that may occur during or after placement[22-26]. These complications determine the lack of fiducials recognition by CyberKnife and result to failure in targeting the lesions that prevented the execution of the treatment[27,28].

The aim of this prospective pilot study was to assess, how the use of two different types of gold fiducial markers: Grain type and Anchor type, affects the accuracy of tracking by the CyberKnife System, and consequently, the therapeutic efficacy of the treatment of primary or metastatic liver malignancy. We also aimed to identify which type of fiducial can ensure better viability of the SRR.

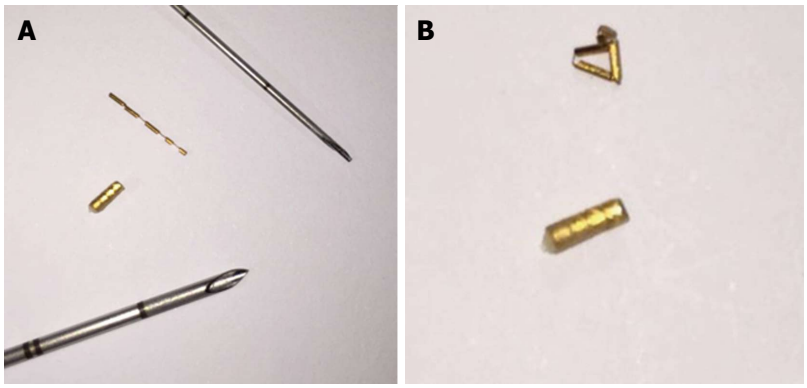

Figure 1 Types of gold fiducial markers. A: Twenty five gauge and 17 G needle and their gold markers: Grain cylindrical gold marker, 1 mm × 4 mm and flexible wire notched gold marker 0.28 mm × 10 mm, Gold Anchor Marker respectively; B: Grain cylindrical gold marker, 1 mm × 4 mm flexible wire notched gold marker 0.28 mm × 10 mm, Gold Anchor marker after massing.

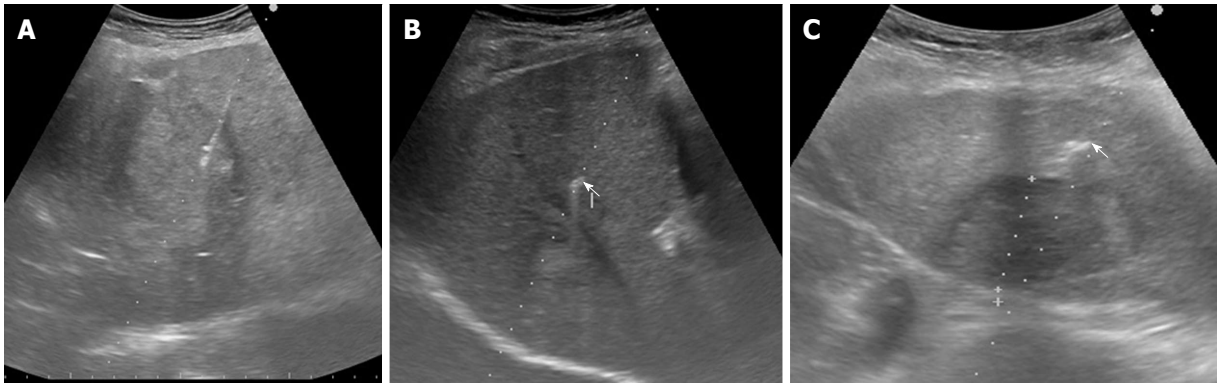

Figure 2 Ultrasonography-guided fiducial placement. The two different gold markers are sonographically undistinguishable. A: Needle delivering fiducial into a liver mass; B: Hyperechoic flexible wire notched gold marker, 0.28 mm × 10 mm, Gold Anchor marker (arrow) near a liver mass; C: Hyperechoic Grain cylindrical gold marker, 1 mm × 4 mm (arrow) near a liver mass.

MATERIALS AND METHODS

Fifteen consecutive patients, who were scheduled to receive robotic radiotherapy treatment for primary or metastatic liver malignancy, were recruited for percutaneous ultrasonography (US)-guided placement of intra-hepatic fiducial markers, from March 2014 to June 2014 (Figure 1). A written informed consent was obtained from the patients. Two different types of needles, 25 G and 17 G containing two different fiducial markers, gold notched flexible anchor wire of 0.28 mm × 10 mm (25 G needle) and gold cylindrical grain of 1 mm × 4 mm (17 G), were used. The needle type to use was selected according to the site of the lesion (deep or superficial liver lesion) and physical structure. The choice of the different fiducial markers depends mainly on the choice of the needle caliber. The number of fiducial markers to place was evaluated according to the acoustic window, the compliance of the patients and morphological characteristics of the lesions. The examination was performed by two expert ultrasonographers with the same echograph, ProSound Alfa7, (Hitachi-Aloka, Tokyo, Japan) with a 3.75-7.5 MHz hemispheric sound technology (HST) 91-30 Multi Frequency Convex Abdominal HST probe. Local anesthesia was achieved *via* the subcutaneous administration of 1% lidocaine. All the gold fiducial markers were placed with US-guidance through sub or intercostals access. After confirming that the needle tip had reached the target lesion, the fiducial marker was deployed, and then the needle was removed. We placed in each patient from 1 to 5 fiducial markers, and when at least two or more fiducials were placed, it was at a distance of about 1.5-2 cm apart, in a way to occupy the perpendicular edges of a cube containing the tumor inside. The Gold Anchor markers were always placed with the same technique to take advantage of their mass effect. Fiducial positioning was confirmed with ultrasound image. A marker was usually seen as a hyperechoic structure. The two different fiducial markers used were sonographycally undistinguishable (Figure 2). Technical success was defined when the implantation enables adequate treatment planning and CT simulation. Fiducial migration was defined as seed dislodgement outside the volume of the original injection site that is unusable for guiding stereotactic body radiation therapy (SBRT) as determined by planning CT. Clinical success was defined as the completion of SBRT. Seven days after the procedure, a CyberKnife planning CT for the simulation of radiation treatment was performed on all patients.

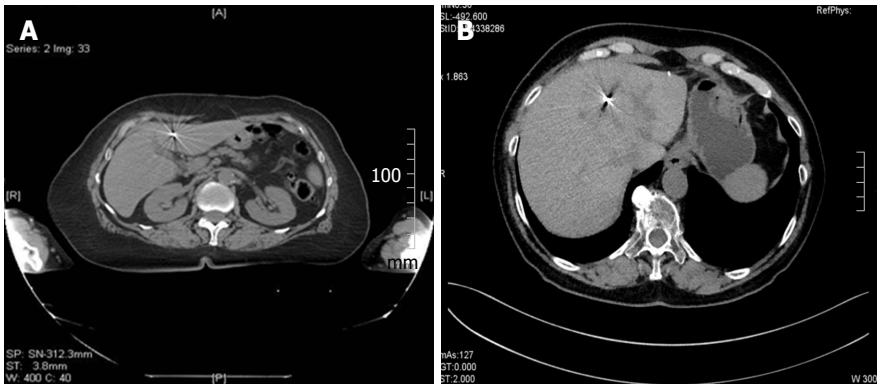

Figure 3 Well visualized fiducial markers. A: CT displaying of Grain cylindrical gold marker, 1 mm × 4 mm. This marker exhibits good contrast on X-ray images with typical "star effects"; B: CT displaying types of flexible wire notched gold marker, 0.28 mm × 10 mm, Gold Anchor marker. This marker exhibits good contrast on X-ray images demonstrating less artifacts. CT: Computed tomography.

A binary CT score for the fiducial markers visualization was assigned (not visualized or poorly visualized = 0; well visualized = 1) (Figure 3). In the case of CT score of zero (0) which prevented treatment, we organized a series of multidisciplinary meetings (with regards to the procedure, the physician and the radiation oncologist responsible for the radiosurgery treatment) to achieve the correct radiological visualization of the fiducial marker. Moreover, for the execution of treatment with CyberKnife, it is necessary that each fiducial reaches a CT number above a specific threshold (CT number threshold). The CT number of the fiducial is assigned in an automated manner by the CyberKnife machine (Figure 4). Database construction and data analysis were performed using Office Excel 2007, XLSTAT 2016 (microsoft) and SPSS for Windows (SPSS Inc., Chicago, United States). We examined the data with the use of appropriate parametric and non-parametric statistical tests (Student's *t*-test two-tailed and a χ^2 test according to Fischer considering $P < 0.05$ as significant). A Lilliefors (Kolmogorov-Smirnov) test for normality has been previously performed. Statistical analysis was performed by Tommaso Gabbani, MD, and reviewed by Principal Investigator, Maria Marsico, MD.

RESULTS

Fifteen consecutive patients (men: 9, women: 6, mean age: 72.9 years old, range: SD ± 7.9) who had already undergone percutaneous ultrasound-guided fiducial marker implantations for CyberKnife therapy were employed for this study. Eleven patients (8 males) presented liver metastasis from a note primary neoplasm (2 right colon carcinoma, 2 sigmoid carcinoma, 2 rectum carcinoma, 1 gastric carcinoma, 1 lung carcinoma, 1 ovaric carcinoma and 2 pancreatic carcinoma). Four patients (2 males) showed liver primary malignancy [2 hepatocellular carcinoma (HCC), 1 cholangio-carcinoma, 1 hepatic cholangio-carcinoma]. Among 11 patients who presented liver metastasis, 9 patients had previously undergone radical surgery of primary neoplasm. Among these 9 patients, 4 had submitted to adjuvant therapy, 2 to metastasectomy and adjuvant therapy, 2 to neo-adjuvant and adjuvant therapy and 1 to metastatectomy without chemotherapy. Moreover among these 9 patients, 7 developed new liver metastasis during or at the end of the treatment, while 2 patients presented a metastatic recurrence. Only 2 patients of the 11 affected by liver metastasis undergone treatment by chemotherapy and not surgery, one performed a palliative chemotherapy and the other performed an effective chemotherapy with failure of treatment. In the group of patients with primary hepatic neoplasm, patients affected by HCC and hepato-cholangiocarcinoma were treated by chemoembolization, while another one affected by cholangiocarcinoma undergone chemotherapy. Patients treated with chemoembolization showed relapse of neoplasm, while the patient treated with chemotherapy showed no response to the treatment. In the group of patients with hepatic metastasis, 8 of them have a single nodule, 2 of them have two nodules, and 1 has three nodules so the total of liver lesions treated was 15. These 15 liver lesions presented a maximum diameter between 2 to 4 cm. In the group of patients with primary liver lesions, 3 patients showed a single nodule and another one presented two nodules so the total primary lesion treated was 5. Four of these measured a maximum diameter from 2 to 4 cm and only one measured a maximum diameter over 4 cm. 2 patients showed a moderate ascites at the moment of the procedure. The 20 liver lesions were localized into the Ⅶ liver segment (n = 7), Ⅵ liver segment (n = 3), Ⅷ segment (n = 3), Ⅴ segment (n = 1), Ⅳ segment (n = 1), Ⅲ segment (n = 1), between Ⅴ-Ⅵ segment (n = 1), between Ⅴ-Ⅵ-Ⅶ segments (n = 1) and between Ⅵ-Ⅶ segments (n = 2). Five lesions were localized close to vascular structures and 2 lesions close to critical organs. Considering the closeness of the critical organs or vascular structures and the patients' compliance, 8 patients undergone a combined placement of the two types of gold markers. Two patients presented severe compliance problems (panic attack), so they received only anchor markers (placed with fine needles). Five patients received only cylindrical grain markers. For each

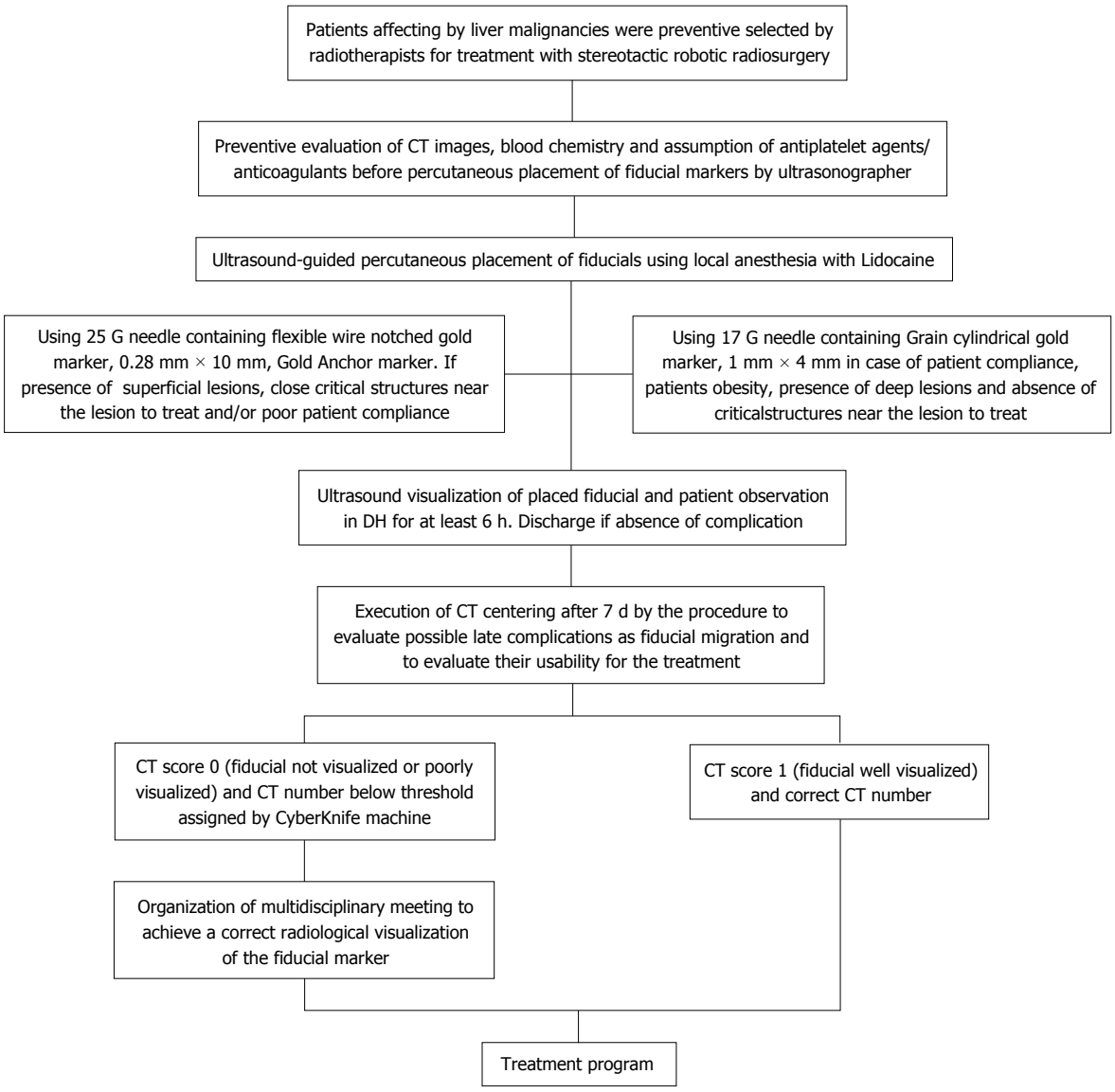

Figure 4 Flow chart of the study conducted tank to a prospective collection of data (compliance, demographic and clinic characteristics of the patients, liver lesions characteristics, type of needle and markers used, ultrasonographic and computed tomography visualization of fiducial markers, usability of the markers and immediate and late complications) and a retrospective statistical analysis. CT: Computed tomography.

patient about 1 to 5 intra-hepatic markers were placed (one in 2 patients, three in 8 patients, four in 3 patients, and five in 2 patients). A total of 48 needles were used (thirty-two 17 G and sixteen 25 G) and 48 gold markers were placed (32 Grain shaped markers and 16 Gold Anchors). In 47 cases, the gold markers were placed through subcostal access and only in a single case with an inter-costal access. Every patient received a local anesthesia with lidocaine. All fiducials placement were sonographically confirmed right after the procedure. No patient presented any major complication related to the procedure.

After the placement of markers, 14 patients underwent the planning simulation CT scan to allow fiducials to settle. One patient did not perform the CT because of a complication related to the primary tumor (hepatic failure). Removing the latter patient who was excluded from the treatment for causes not correlated to the fiducial placement, the technical and clinical success rate was 100%. The CT scan revealed that 14 markers (11 Gold Anchors and 3 Grain shaped markers) showed late complications. Few markers showed more than one complications at the same time for a total of 27 complications. Shattered markers (n = 2; 2 Gold Anchors), extra-hepatic migration (n = 4; 1 Gold Anchor and 3 Grain markers), extra-hepatic migration and marker not visualized (n = 1; 1 Gold Anchor), intra-hepatic migration (n = 5; 5 Gold Anchors), not massed markers (n = 5; 5 Gold Anchor). The Gold Anchor marker presented more frequent late minor complications (68.75% *vs* 9.375%, $P = 3 \times 10^{-6}$). Moreover, 38 markers were visualized with CT score = 1 and 10 markers with CT score = 0, the markers visualized with CT score = 0 were all Gold Anchors and we demonstrated that the CT subjective visualization of Grain shaped markers was significantly higher than the CT subjective visualization for Gold

Anchor (100% *vs* 37.5%, $P = 5 \times 10^{-9}$). For 5 patient it was necessary to organize multidisciplinary meetings to identify the correct intra-hepatic localization of the markers visualized with CT score = 0. Finally, 5 markers showed a CT number below the threshold (5 Gold Anchors). The 5 markers with the CT number below the threshold were not recognized by the CyberKnife system and so were not used for the treatment (one marker not recognized for 5 patients). Forty-three markers (32 Grain shaped markers and 11 Gold Anchors) with regular CT number were recognized by CyberKnife system and were used for the treatment. The clinical success achieved was 89.6%. We demonstrated that the Gold Anchor marker is associated with a threshold below the CT number (31.25% *vs* 0%, $P = 0.0005$) that is not suitable for treatment. A total of 14 patients underwent radiosurgery treatment, only one patient was excluded because of a complication related to his primary tumor.

DISCUSSION

The CyberKnife Robotic Radiosurgery System is a non-surgical option for patients who have inoperable or surgically complex tumors or who may be looking for an alternative to surgery. It is an option in the case where no response and/or relapse is observed after chemotherapy and standard radiotherapy[29-31]. In our study, we compared the therapeutic usability of the two different gold fiducial markers for robotic radiosurgery treatment of primary and metastatic liver malignancies. We used the two different gold markers according to the necessity to either use 17 or 25 G needle, depending on the patient's compliance, patient physical structure and the proximity of critical or vascular structures. This pilot trial demonstrate that the Anchor marker (0.28 mm × 10 mm) is correlated with a greater number of late minor complications that results from a frequent association with a CT number below threshold and a low subjective CT visualization, resulting in a delay or a difficulty in starting the treatment. In our opinion, the use of the Gold Anchor marker should be limited to use of the 25 G needle and in combination with the other types of markers. Only few studies have compared the use of different fiducial in the terms of efficacy and complications[32,33]. Our study differs from others because it compares the two different types of gold fiducial markers in terms of usability for CyberKnife treatment. In our study, we identified some of the factors related to the type of fiducials (the Gold Anchor) that may prevent the treatment with CyberKnife. The identification and knowledge of these factors allows us to limit the use of Gold Anchor marker type, to specific cases and preferably, in combination with the other marker types, in order to reduce the tracking problems of CyberKnife. Infact, CyberKnife tracking problems are causes of increasing costs and delay in treatment execution. Contrary to what is shown in our study, other trials have demonstrated the advantage of Gold Anchor fiducial than the other types of fiducial markers for the treatment with CyberKnife. Nevertheless, in these other studies, inserting of the fiducial markers was executed by endoscopic ultrasonography technique to treat tumors of the pancreas and lung. Therefore, it is our opinion that the different techniques for positioning, and the different localization of the lesions may be the basis of the different results obtained in the study. Furthermore, we must consider the variable offered by the needle. The percutaneous placement of Gold Anchor (0.28 mm × 10 mm) occurred with the 25 G needle that originally contained the gold marker. In the cases of endoscopic ultrasound guided placement of fiducial gold markers (in particular, for treatment of pancreas lesion), the 25 G needle originally containing the marker, serves only as a carrier to put the fiducials inside the other needles (22 G or 19 G) usually used for the fiducial placement. The use of needles with a greater caliber (22 G and 19 G) and less flexibility than the 25 G needle, may facilitate the placement of Gold Anchor limiting the complications.

COMMENTS

Background

The treatment of liver malignancies has evolved over the years. Although surgery is the current standard treatment for localized surgically operable lesions. Alternative treatment approaches for unresectable liver metastasis and primary liver cancer include: Chemoembolization, radiofrequency ablation, cryotherapy, and the oral multikinase inhibitor sorafenib, chemotherapy and standard radiotherapy. The CyberKnife Robotic Radiosurgery System is a non-surgical option for patients who have inoperable or surgically complex tumors or who may be looking for an alternative to surgery. It also provides an option in the case where no response and/or relapse is observed after standard treatment.

Research frontiers

Many points still remain unclear in literature to ameliorate the treatment by CyberKnife and a lot of them seem to correlate with the type of fiducial to be use, the technique of placement and the number of fiducial to use. Nowadays, there are many different types of gold fiducial markers with different dimensions, lengths and physical characteristics. Therefore, many other studies of fiducial comparison, like the authors', should be conducted. This is necessary to identify the basis of compliance, demographic and clinical characteristics of the patients and liver lesions characteristics, the best type of fiducial and needle to use. Percutaneous fiducial marker placement could be under computed tomography (CT) fluoroscopic guidance or ultrasonographic (US) guidance. In this study, fiducial placement was entirely conducted under US guidance demonstrating a great safety and efficacy. Cost-effectiveness studies should also be conducted to compare the CT and the US percutaneous fiducial placement to identify the best method in the terms of cost-effectiveness. Nowadays, there is yet no consensus in literatures on the exact number of fiducials necessary to effectively perform the treatment with CyberKnife. Many studies define the technical success as the ability to place more of a fiducial near the tumor target before the treatment; other studies have resulted in higher clinical success placing a unique fiducial marker for patient. In this study, the authors also demonstrated a high clinical success from using one to five fiducial for each patient, in relation to which fiducials were really recognized and used by the CyberKnife system (fiducial with correct relegated CT number). Therefore, many studies should be conducted regarding the different analysis of tracking accuracy resulting from the use of a different number of fiducial for treatment. This is important to establish the best number of fiducials to use in terms of cost effectiveness.

Innovations and breakthroughs

The study differs from others because it compares the two different types of gold fiducial markers in terms of usability for CyberKnife treatment of liver

malignacies. The study also differs for describing other possible complications related to Gold Anchor - their wrong stacking and their break after the placement. In this study, the authors identified some factors related to the type of fiducial, (the Gold Anchor) that may prevent the treatment with CyberKnife. The identification and knowledge of these factors allows them to limit the use of Gold Anchor marker type, to specific cases and preferably in combination with other marker types, in order to reduce tracking problems of CyberKnife. In fact, CyberKnife tracking problems are causes of increasing costs and delay in treatment execution. Contrary to what is shown in the study, other trials have demonstrated the advantage of Gold Anchor fiducial over the other types of fiducial markers for the treatment with CyberKnife. Nevertheless, in these other studies, the inserting of fiducial markers was executed by endoscopic ultrasonography technique for treating tumors of the pancreas and lung. Therefore, it is the authors' opinion that the different techniques of positioning, and the different localization of the lesions and the different needles (generally of higher caliber) used for the placement of Gold Anchor may be the basis of the different results obtained in this study.

Applications

In the authors' opinion, the use of Gold Anchor marker type has to be limited to specific cases; in order to reduce tracking problems of CyberKnife treatment which is the major cause of increasing costs and delay in treatment execution. Therefore, the authors suggest that the use of the Gold Anchor marker should be limited to the necessity to use the 25 G needle and in combination with the other type of markers. In particular, the 25 G needle should be used in the case of low patient compliance, absence of obesity and in the presence of superficial lesions at critical structure near the liver lesions.

Terminology

Stereotactic robotic radio surgery: Ability to dispense high doses of focused radiation in a minor number of fractions respect to the standard treatment (2-5 *vs* 30-40). Ability to reach any point with anatomical precision and extreme sub-millimeter accuracy tanks to a target localization computerized system offered by CyberKnife system; CyberKnife: Robot with a complete autonomy characteristic with more than 1500 dispensing positions of X-ray; Variable diameter collimator; Synchrony Respiratory Tracking System to preserve the near organs from toxicity; Fiducial gold markers: Markers exploited by CyberKnife for the target localization in the treatment of parenchymatous organs lesions. This marker is made from gold, which makes it biocompatible and ensures it exhibits good contrast on X-ray images; CT number: A normalized value of the calculated X-ray absorption coefficient of a pixel (picture element) in a computed tomogram, expressed in Hounsfield units, where the CT number of air is -1000 and that of water is 0.

Peer-review

In this work, the authors reported a comparison study of two different types of fiducial markers for robotic radiosurgery. In this study, 15 patients have been recruited, in which 48 gold markers were placed (32 Grain shaped markers and 16 Gold Anchor). All these patients except one were scanned with CT for visualization and identification of these markers. The data of these patients were analyzed and reported in this work. The work intended to address an interesting clinical issue.

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ORIGINAL ARTICLE

Prospective Study

Circulating insulin-like growth factor-binding protein 3 as prognostic biomarker in liver cirrhosis

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Abstract

AIM: To investigate the prognostic significance of insulin-like growth factor-binding protein 3 (IGFBP-3) in patients with cirrhosis.

METHODS: Prospective study that included two cohorts: outpatients with stable cirrhosis (n = 138) and patients hospitalized for acute decompensation (n = 189). Development of complications, mortality or liver transplantation was assessed by periodical phone calls and during outpatient visits. The cohort of stable cirrhosis also underwent clinical and laboratory evaluation yearly (2013 and 2014) in predefined study visits. In patients with stable cirrhosis, IGFBP-3 levels were measured at baseline (2012) and at second re-evaluation (2014). In hospitalized subjects, IGFBP-3 levels were measured in serum samples collected in the first and in the third day after admission and stored at -80 ℃. IGFBP-3 levels

were measured by immunochemiluminescence.

RESULTS: IGFBP-3 levels were lower in hospitalized patients as compared to outpatients (0.94 mcg/mL *vs* 1.69 mcg/mL, $P < 0.001$) and increased after liver transplantation (3.81 mcg/mL *vs* 1.33 mcg/mL, $P = 0.008$). During the follow-up of the stable cohort, 17 patients died and 11 received liver transplantation. Bivariate analysis showed that death or transplant was associated with lower IGFBP-3 levels (1.44 mcg/mL *vs* 1.74 mcg/mL, $P = 0.027$). The Kaplan-Meier transplant-free survival probability was 88.6% in patients with IGFBP-3 $\geqslant$ 1.67 mcg/mL and 72.1% for those with IGFBP3 < 1.67 mcg/mL ($P = 0.015$). In the hospitalized cohort, 30-d mortality was 24.3% and was independently associated with creatinine, INR, SpO_2/FiO_2 ratio and IGFBP-3 levels in the logistic regression. The 90-d transplant-free survival probability was 80.4% in patients with IGFBP-3 $\geqslant$ 0.86 mcg/mL and 56.1% for those with IGFBP3 < 0.86 mcg/mL ($P < 0.001$).

CONCLUSION: Lower IGFBP-3 levels were associated with worse outcomes in patients with cirrhosis, and might represent a promising prognostic tool that can be incorporated in clinical practice.

Key words: Liver cirrhosis; Acute decompensation; Insulin-like growth factor binding protein 3; Acute-on-chronic liver failure; Prognosis

Core tip: Insulin-like growth factor-binding protein 3 (IGFBP-3) levels are decreased in cirrhosis and seem to correlate with the intensity of hepatic dysfunction, but its prognostic significance is uncertain. In this prospective cohort study, IGFBP-3 levels correlated with variables associated with the intensity of liver dysfunction in both outpatients with stable cirrhosis and in subjects hospitalized for acute decompensation. IGFBP-3 levels increased significantly after discharge and after liver transplantation. Lower IGFBP-3 levels were associated with poor outcomes in both outpatients with stable cirrhosis and in those hospitalized for acute decompensation, suggesting that it can be used in clinical practice as a prognostic biomarker in cirrhosis.

Correa CG, Colombo BS, Ronsoni MF, Soares e Silva PE, Fayad L, Silva TE, Wildner LM, Bazzo ML, Dantas-Correa EB, Narciso-Schiavon JL, Schiavon LL. Circulating insulin-like growth factor-binding protein 3 as prognostic biomarker in liver cirrhosis. *World J Hepatol* 2016; 8(17): 739-748 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i17/739.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i17.739

INTRODUCTION

Cirrhosis is a late stage progressive hepatic fibrosis characterized by distortion of hepatic architecture and the formation of regenerative nodules. Although considered potentially reversible in some clinical scenarios, in its advanced stages, liver cirrhosis is associated with high mortality and the only treatment option may be liver transplantation[1]. Patients with cirrhosis are susceptible to a variety of complications such as variceal bleeding, ascites, spontaneous bacterial peritonitis (SBP), hepatic encephalopathy, hepatocellular carcinoma, hepatorenal syndrome, hepatopulmonary syndrome and portal vein thrombosis[1]. The prognosis of cirrhosis is highly variable because it is influenced by a number of factors including etiology, severity, presence of complications, and comorbidities. Mortality rate increases significantly once decompensation occurs[2].

Changes in the growth hormone-insulin-like growth factor (GH-IGF) axis occur as a result of liver disease and has been reported in cirrhosis[3,4]. GH and IGF-Ⅰ have important anabolic effects on the metabolism of proteins, carbohydrates and lipids[5]. GH is a peptide hormone released from anterior pituitary that stimulates growth, cell reproduction and regenerations while exerting metabolic effects on bone, cartilage, fat, muscles, heart and the immune system[6-8].

In the liver, GH activation of GH receptors induces *IGF-Ⅰ* gene transcription, and the subsequent synthesis and release of IGF-Ⅰ to plasma[9]. However, the actions of IGF-Ⅰ are tightly controlled by the binding of IGF to binding proteins (IGFBP-1 to -6). The IGFBPs carry IGFs in the serum and regulate their activity and bioavailability[10]. IGFBP-3 is the major binding protein of IGF and carries 80%-90% of circulating IGF-Ⅰ. The IGFBP-3 is predominantly produced in the liver and synthesized in Kupffer cells. It forms a 150 kDa ternary complex with IGFs and the acid-labile subunit[7,9].

Low serum concentrations in both IGF-Ⅰ and IGFBP-3 have been reported in patients with cirrhosis *vs* healthy controls. This likely reflects decreased hepatic synthesis function[5,11-13]. In fact, some studies reported that IGFBP-3 serum levels are abnormally low in patients with liver cirrhosis and correlated with several variables associated with the intensity of liver dysfunction[5,7]. Even though these reports suggest the clinical utility of monitoring circulating IGFBP-3 in cirrhosis, there is little data on the prognostic significance of this biomarker. The aim of this study was to investigate the relationship between serum IGFBP-3 levels and prognosis in both outpatients with stable cirrhosis and in patients hospitalized for acute decompensation (AD).

MATERIALS AND METHODS

Patients

This prospective study included two cohorts of adult patients ($\geqslant$ 18 years of age) with liver cirrhosis in the University Hospital of the Federal University of Santa Catarina. The diagnosis of cirrhosis was established either histologically (when available) or by combination of clinical, imaging and laboratory findings in patients

with evidence of portal hypertension. The first cohort was comprised of patients with stable cirrhosis in the outpatient clinic. In this case, patients in the following situations were excluded: Diagnosis of hepatocellular carcinoma; interferon-based therapy over the last 30 d; or refusal or inability of the patient to understand the terms of the informed consent.

The second cohort included patients admitted to the emergency room due to AD of liver cirrhosis. In this group, the following exclusion criteria were adopted: Hospitalization for elective procedures; admissions not related to complications of liver cirrhosis; and hepatocellular carcinoma outside Milan criteria. Exclusion criteria for both groups also included: Pregnancy; chronic renal failure requiring hemodialysis; severe heart disease; severe chronic pulmonary disease; and active extrahepatic cancer. The study protocol complies with ethical principles of the Declaration of Helsinki and was approved by the Ethics Committee on Human Research of the Federal University of Santa Catarina.

Methods

This initial cohort was initially evaluated from June to October 2012. The development of complications, mortality, or liver transplantation was assessed by periodic phone calls and during outpatient visits. Patients also underwent clinical and laboratory evaluation yearly (2013 and 2014) in predefined study visits. The second cohort included subjects hospitalized for AD between January 2011 and November 2013. AD was defined by acute development of hepatic encephalopathy, large ascites, gastrointestinal bleeding, bacterial infection or any combination of these. Patients were evaluated within 24 h of admission by one of the researchers involved in the study. They were followed during their hospital stay and 30- and 90-d mortality was evaluated by phone call in case of hospital discharge. In case of more than one hospital admission during the study period-only the most recent hospitalization was considered.

The following clinical variables were collected for all patients: Age, gender, etiology of cirrhosis, history of previous decompensation, current complications of cirrhosis, active alcoholism and regular propranolol and omeprazole use. All subjects underwent laboratory evaluation including total leukocytes, serum sodium, creatinine, international normalized ratio (INR), albumin, C-reactive protein (CRP), total bilirubin and IGFBP-3. In patients hospitalized for AD of cirrhosis, blood samples were obtained on the first and third day after admission.

Active alcoholism was defined as an average overall consumption of 21 or more drinks per week for men and 14 or more drinks per week for women during the 4 wk before enrollment (one standard drink is equal to 12 g absolute alcohol)[14].

Hospitalized individuals with a suspected infection at admission received a clinical examination to confirm this diagnosis and to establish the primary source of infection. Diagnosis of infection was made according to the criteria of Center for Disease Control[15]. A diagnostic paracentesis was performed in all patients with ascites at admission. SBP was diagnosed when the neutrophil count in the ascitic fluid was $\geqslant$ 250 neutrophils/mm^3 in the absence of an intra-abdominal source of infection regardless of negative culture[16]. All patients with SBP received ceftriaxone plus weight-based intravenous albumin in the first and third day after diagnosis. Hepatic encephalopathy was graded according to West-Haven criteria[17]. If this was present, a precipitant event was actively investigated and lactulose was initiated, and the dose was adjusted as needed. All subjects with acute variceal bleeding received intravenous octreotide, an antibiotic (either oral quinolone or intravenous ceftriaxone), and underwent urgent therapeutic endoscopy after stabilization.

The severity of liver disease was estimated using the Child-Pugh classification system[18] and model for end-stage liver disease (MELD)[19] calculated based on laboratory tests performed at admission in the case of hospitalized patients. Acute-on-chronic liver failure (ACLF) was defined as proposed by the EASL-CLIF Consortium[20].

IGFBP-3 serum levels

In patients with stable cirrhosis, IGFBP-3 levels were measured at baseline (2012) and at second re-evaluation (2014). In hospitalized subjects, IGFBP-3 levels were measured in serum samples collected in the first and in the third day after admission and stored at -80 ℃ until use. The IGFBP-3 levels were measured by immunochemiluminescence (Immulite® 2000, Diagnostic Products Corp., Los Angeles, CA, United States). The reported analytical sensitivity of this assay is 0.50 mcg/mL.

Statistical analysis

The normality of variable distribution was determined using the Kolmogorov-Smirnov test. The correlation between numerical variables was evaluated using Spearman's correlation coefficient. Continuous variables were compared using the Student's *t*-test in case of a normal distribution or Mann-Whitney test in the remaining cases. Categorical variables were evaluated with a χ^2 test or Fisher's exact test, as appropriate. Multiple logistic regression analysis (forward stepwise regression) was used to investigate factors independently associated with death or liver transplantation during follow-up period. The best cutoffs of IGFBP-3 for predicting mortality, in both cohorts, were chosen based on the receiver operating characteristics (ROC) curves. Survival curves were calculated using the Kaplan-Meier method and survival differences between groups were compared using a log-rank test. Wilcoxon signed rank-test was used for comparing IGFBP-3 at two times. All tests were performed using SPSS software, version 22.0 (SPSS, Chicago, IL, United States). A *P* value of less than 0.05 was considered statistically

Table 1 Demographic, clinical and biochemical features of included patients *n* (%)

	Stable cirrhosis (*n* = 138)	Acute decompensation (*n* = 189)
Age; yr, mean ± SD	53.62 ± 12.52	53.58 ± 11.56
Caucasians	128 (92.8)	129 (68.6)
Male gender	97 (70.3)	138 (73.0)
Etiology of cirrhosis		
Alcohol	42 (30.4)	68 (36.0)
Hepatitis C	50 (36.2)	78 (41.3)
Hepatitis B	6 (4.3)	8 (4.2)
Cryptogenic	14 (10.1)	15 (7.9)
Other	26 (18.8)	20 (10.6)
Previous decompensation	104 (75.4)	120 (63.5)
Active alcoholism	5 (3.6)	68 (36.0)
Propranolol	87(63.0)	74 (40.2)
PPI	69 (50.0)	43 (23.4)
Complication at evaluation		
Ascites	28 (20.3)	92 (48.7)
Hepatic encephalopathy	14 (10.1)	112 (59.3)
Gastrointestinal bleeding	0	99 (52.4)
Bacterial infection	0	50 (26.6)
ACLF	0	45 (23.8)
Laboratory data		
Leucocyte count ($\times 10^9$), median	4.90	7.20
Sodium (meq/L), median	138	135
Creatinine (mg/dL), median	0.90	1.1
INR, median	1.20	1.41
Albumin (g/dL), mean ± SD	3.44 ± 0.46	2.35 ± 0.69
CRP (mg/L), median	3.5	10.05
Total bilirubin (mg/dL), median	1.00	2.10
IGFBP-3 (mcg/mL), median	1.69	0.94
Child-Pugh classification		
A	92 (66.7)	23 (12.2)
B	43 (31.2)	91 (48.1)
C	3 (2.2)	75 (39.7)
MELD score, mean ± SD	9.84 ± 2.28	16.32 ± 6.53

PPI: Proton-pump inhibitors; ACFL: Acute-on-chronic liver failure; INR: International normalized ratio; CRP: C-reactive protein; IGFBP-3: Insulin-like growth factor-binding protein 3; MELD: Model for end-stage liver disease.

significant.

RESULTS

Characteristics of included patients

The study included 138 patients with stable cirrhosis and 189 subjects hospitalized for AD of cirrhosis. Table 1 lists the characteristics of the included patients. In the cohort of stable cirrhosis, the mean age was 53.6 ± 12.5 years, 92.8% were Caucasians with a predominance of men (70.3%). A previous history of cirrhosis decompensation was observed in 75.4% of the sample and only 3.6% of subjects reported active alcoholism during the past month. Regular propranolol and PPI use was 63.0% and 50.0% of the patients, respectively. The mean MELD score was 9.84 ± 2.28 and 66.7% of subjects were Child-Pugh A.

In the group hospitalized for AD, the mean age was 53.6 ± 11.6 years, 68.6% were Caucasians and 73.0% were males. Previous decompensation was reported by 63.5% of the sample, and active alcoholism was present in 36.0% of the sample. Upon admission, upper gastrointestinal bleeding was observed in 52.4% of cases, ascites in 48.7%, hepatic encephalopathy in 59.3%, bacterial infections in 26.6% and ACLF in 23.8%. In hospitalized patients, propranolol and PPI use prior to admission was reported in 40.2% and 23.4% of the patients, respectively. The mean MELD score was 16.32 ± 6.53 and 39.7% of subjects were Child-Pugh C. Patients hospitalized for AD of cirrhosis exhibited significantly lower median IGFBP-3 *vs* outpatients (0.94 mcg/mL *vs* 1.69 mcg/mL, $P < 0.001$).

IGFBP-3 in outpatients with stable cirrhosis

In patients with stable cirrhosis, IGFBP-3 levels were positively correlated with total leukocytes ($r = 0.215$, $P = 0.011$) and albumin levels ($r = 0.579$, $P < 0.001$). A negative correlation was observed between IGFBP-3 levels and INR ($r = -0.412$, $P < 0.001$), total bilirubin ($r = -0.329$, $P < 0.001$), CRP ($r = -0.265$, $P = 0.002$) and MELD ($r = -0.327$, $P < 0.001$). No significant correlations were observed between IGFBP-3 and other studied variables.

Significantly lower IGFBP-3 median levels were observed in Child-Pugh B/C patients (1.38 mcg/mL *vs* 1.88 mcg/mL, $P < 0.001$), in those with previous decompensation of cirrhosis (1.58 mcg/mL *vs* 2.19 mcg/mL, $P = 0.012$), hospitalization secondary to complications of cirrhosis (1.57 mcg/mL *vs* 1.95 mcg/mL, $P = 0.052$), and those with a history of ascites (1.53 mcg/mL *vs* 1.85 mcg/mL, $P = 0.024$). Previous variceal bleeding or hepatic encephalopathy as well as an etiology of cirrhosis had no impact on IGFBP-3 levels ($P > 0.05$).

The median follow-up of patients with stable cirrhosis was 20 mo. During the study period, 17 patients died and 11 received liver transplantation. Bivariate analysis showed that progression to death or liver transplantation was associated with previous ascites (70.4% *vs* 42.3%, $P = 0.009$), history of hospitalization for complications of cirrhosis (88.9% *vs* 65.8%, $P = 0.018$), and Child-Pugh B/C (70.4% *vs* 24.3%, $P < 0.001$) (Table 2). In addition, those with unfavorable outcome showed a higher median total bilirubin (1.80 mg/dL *vs* 0.90 mg/dL, $P < 0.001$), INR (1.27 *vs* 1.16, $P < 0.001$), CRP (4.51 mg/L *vs* 3.50 mg/L, $P = 0.014$), MELD score (11.98 ± 2.24 *vs* 9.32 ± 1.97, $P < 0.001$) and lower sodium (136.00 mEq/L *vs* 138.00 mEq/L, $P = 0.022$), albumin (3.11 ± 0.39 g/dL *vs* 3.52 ± 0.44 g/dL, $P < 0.001$) and IGFBP-3 values (1.44 mcg/mL *vs* 1.74 mcg/mL, $P = 0.027$). A stepwise forward logistic regression analysis was performed including the following variables with $P < 0.05$ in bivariate analysis: Previous ascites, history of hospitalization for complications of cirrhosis, albumin, INR, total bilirubin, CRP, sodium, and IGFBP-3. Multivariate analysis showed that only albumin (OR = 0.183, 95%CI: 0.053-0.631, $P = 0.007$) and total bilirubin (OR = 2.482, 95%CI:

Table 2 Factors associated with mortality or liver transplantations among patients with stable cirrhosis *n* (%)

	Survivors (n = 111)	Death/liver transplantation (n = 27)	P value
Age (yr), mean ± SD	54.33 ± 12.11	50.67 ± 13.91	0.173
Male gender	79 (71.2)	18 (66.7)	0.646
Etiology of cirrhosis			
Alcohol	34 (30.6)	8 (29.6)	0.919
Hepatitis C	42 (37.8)	8 (29.6)	0.426
Hepatitis B	4 (3.6)	2 (7.4)	0.334
Cryptogenic	10 (9.0)	4 (14.8)	0.475
Other	21 (18.9)	5 (18.5)	0.962
Previous hospitalization	73 (65.8)	24 (88.9)	0.018
Active alcoholism	3 (2.7)	2 (7.4)	0.252
Propranolol	67 (60.4)	20 (74.1)	0.185
PPI	54 (48.6)	15 (55.6)	0.520
Complication at evaluation			
Ascites	19 (17.1)	9 (33.3)	0.060
Hepatic encephalopathy	8 (7.2)	6 (22.2)	0.032
Previous ascites	47 (42.3)	19 (70.4)	0.009
Laboratory data			
Leucocyte count ($\times 10^9$), median	4.93	4.83	0.548
Sodium (meq/L), median	138.00	136.00	0.022
Creatinine (mg/dL), median	0.9	0.8	0.480
INR, median	1.16	1.27	< 0.001
Albumin (g/dL), mean ± SD	3.52 ± 0.44	3.11 ± 0.39	< 0.001
CRP (mg/L), median	3.5	4.51	0.014
Total bilirubin (mg/dL), median	0.9	1.8	< 0.001
IGFBP-3 (mcg/mL), median	1.74	1.44	0.027
Child-Pugh B/C	27 (24.3)	19 (70.4)	< 0.001
MELD score, mean ± SD	9.32 ± 1.97	11.98 ± 2.24	< 0.001

PPI: Proton-pump inhibitors; INR: International normalized ratio; CRP: C-reactive protein; IGFBP-3: Insulin-like growth factor-binding protein 3; MELD: Model for end-stage liver disease.

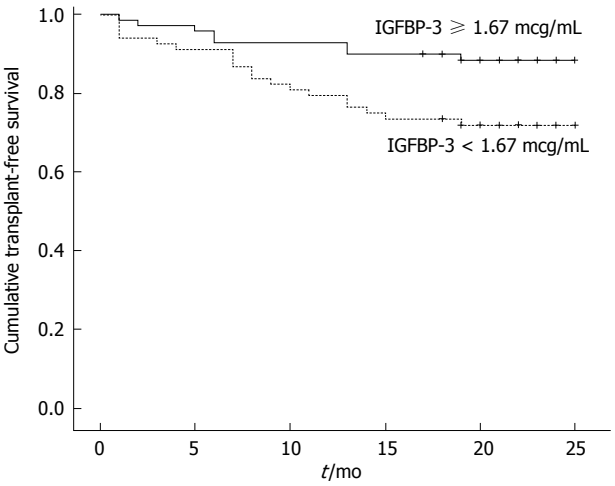

Figure 1 Kaplan-Meier transplant-free survival of 138 outpatients with cirrhosis stratified according to insulin-like growth factor-binding protein 3 cut-off level of 1.67 mcg/mL. Survival probability after a median follow-up of 20 mo was 88.6% for patients with IGFBP-3 ⩾ 1.67 mcg/mL and 72.1% for those with IGFBP-3 < 1.67 mcg/mL (P = 0.015). IGFBP-3: Insulin-like growth factor-binding protein 3.

1.358-4.538, P = 0.003) were independently associated with death or liver transplantation during follow-up. However, at the end of follow-up, Kaplan-Meier survival probability was 88.6% for patients with IGFBP-3 ⩾ 1.67 mcg/mL and 72.1% for those with IGFBP-3 < 1.67 mcg/mL. The survival was significantly shorter for those with lower IGFBP-3 values (20.24 mo, 95%CI: 18.32-22.17) as compared to the remaining subjects (23.06 mo, 95%CI: 21.72-24.40) (P = 0.015) (Figure 1).

Twenty-seven patients developed variceal bleeding during follow-up. Those patients exhibited similar baseline IGFBP-3 levels when compared to those who did not develop this complication (1.74 mcg/mL *vs* 1.69 mcg/mL, P = 0.478).

One-hundred and nine patients underwent laboratory evaluation in 2014, and 12 subjects refused blood collection. Of those who did not receive liver transplantation, IGFBP-3 significantly decreased at the second assessment (1.67 mcg/mL *vs* 1.74 mcg/mL, P = 0.013). However, in patients who underwent liver transplantation, a significant increase in IGFBP-3 was observed (3.81 mcg/mL *vs* 1.33 mcg/mL, P = 0.008). Actually, IGFBP-3 increased in all nine patients, and its levels were restored to normal after transplantation in five subjects.

Prognostic significance of IGFBP-3 in patients hospitalized for AD of cirrhosis

In hospitalized patients, IGFBP-3 levels were positively correlated with sodium (r = 0.173, P = 0.018) and albumin levels (r = 0.422, P < 0.001). A negative correlation was observed between IGFBP-3 levels and

Table 3 Factors associated with 30-d mortality among patients hospitalized for acute decompensation of cirrhosis *n* (%)

	Survivors (*n* = 143)	Deaths (*n* = 46)	*P* value
Age (yr), mean ± SD	53.61 ± 11.54	53.49 ± 11.75	0.952
Male gender	101 (70.6)	37 (80.4)	0.192
Etiology of cirrhosis			
Alcohol	47 (32.9)	21 (49.7)	0.116
Hepatitis C	61 (42.7)	17 (37.0)	0.495
Hepatitis B	6 (4.2)	2 (4.3)	1.000
Cryptogenic	14 (9.8)	1 (2.2)	0.123
Other	15 (10.5)	5 (10.9)	1.000
Previous decompensation	88 (61.5)	32 (69.6)	0.325
Active alcoholism	45 (31.5)	23 (50.0)	0.023
Complication at evaluation			
Ascites	57 (39.9)	35 (76.1)	< 0.001
Hepatic encephalopathy	73 (51.0)	39 (84.8)	< 0.001
Gastrointestinal bleeding	81 (56.6)	18 (39.1)	0.039
Bacterial infection	28 (19.6)	22 (48.9)	< 0.001
ACLF	16 (11.2)	29 (63.0)	< 0.001
Laboratory data			
Leucocyte count (× 10^9), median	6.59	8.46	0.005
Sodium (meq/L), median	136.00	134.00	0.011
Creatinine (mg/dL), median	1.00	1.80	< 0.001
INR, median	1.38	1.60	< 0.001
Albumin (g/dL), mean ± SD	2.47 ± 0.70	1.98 ± 0.51	< 0.001
CRP (mg/L), median	8.40	24.8	0.002
Total bilirubin (mg/dL), median	1.51	3.10	< 0.001
IGFBP-3 (mcg/mL), median	1.05	0.63	< 0.001
Child-Pugh C	41 (28.7)	34 (73.9)	< 0.001
MELD score, mean ± SD	14.35 ± 5.00	22.44 ± 6.95	< 0.001
Vital signs			
MAP (mmHg), mean ± SD	85.11 ± (13.95)	80.33 ± (15.27)	0.053
Heart rate (BPM), mean ± SD	81.27 ± 19.19	88.66 ± 16.40	0.022
SpO_2/FiO_2 ratio, median	461.9	442.86	< 0.001

INR: International normalized ratio; CRP: C-reactive protein; IGFBP-3: Insulin-like growth factor-binding protein 3; MELD: Model for end-stage liver disease; MAP: Mean arterial pressure; SpO_2/FiO_2: Oxygen saturation to fraction of inspired oxygen ratio; ACLF: Acute-on-chronic liver failure.

INR (r = -0.437, P < 0.001), total bilirubin (r = -0.278, P < 0.001), CRP (r = -0.365, P < 0.001), MELD (r = -0.373, P < 0.001), and CLIF-SOFA (r = -0.410, P < 0.001). Significantly lower levels of IGFBP-3 were observed in Child-Pugh C patients (0.73 mcg/mL *vs* 1.13 mcg/mL, P < 0.001), in those with ascites (0.76 mcg/mL *vs* 1.22 mcg/mL, P < 0.001), hepatic encephalopathy (0.85 mcg/mL *vs* 1.12 mcg/mL, P = 0.001), ACLF (0.78 mcg/mL *vs* 1.02 mcg/mL, P = 0.007) and bacterial infection at admission (0.66 mcg/mL *vs* 1.11 mcg/mL, P < 0.001). The etiology of cirrhosis had no impact on IGFBP-3 levels (P > 0.05).

Overall 30-d mortality was 24.3%, and it was associated with active alcoholism in bivariate analysis (Table 3) (50.0% *vs* 31.5%, P = 0.023) as well as ascites (76.1% *vs* 39.9%, P < 0.001), hepatic encephalopathy (84.8% *vs* 51.0%, P < 0.001), bacterial infection (48.9% *vs* 19.6%, P < 0.001), Child-Pugh C (73.9% *vs* 28.7%, P < 0.001), ACLF at admission (63.0% *vs* 11.2%, P < 0.001), lower median SpO_2/FiO_2 ratio (442.86 *vs* 461.90, P < 0.001), and higher MELD score (22.44 ± 6.95 *vs* 14.35 ± 5.00, P < 0.001). The 30-d mortality was also related to higher median leucocyte count (8.46 × 10^9/L *vs* 6.59 × 10^9/L, P = 0.005), creatinine (1.80 mg/dL *vs* 1.00 mg/dL, P < 0.001), INR (1.60 *vs* 1.38, P < 0.001), CRP (24.80 mg/L *vs* 8.40 mg/L, P = 0.002), total bilirubin (3.10 mg/dL *vs* 1.51 mg/dL, P < 0.001) as well as lower mean albumin (1.98 ± 0.51 g/dL *vs* 2.47 ± 0.70 g/dL, P < 0.001), lower median sodium (134.00 mEq/L *vs* 136.00 mEq/L, P = 0.011), and IGFBP-3 levels (0.63 mcg/mL *vs* 1.05 mcg/mL, P < 0.001).

A stepwise forward logistic regression analysis was performed including the following variables with P < 0.010 in the bivariate analysis: Ascites, hepatic encephalopathy, infection at admission, SpO_2/FiO_2 ratio, leukocyte count, creatinine, INR, albumin, CRP, total bilirubin, and IGFBP-3. In this regression analysis, the 30-day mortality was independently associated with creatinine (OR = 5.331, 95%CI: 2.563-11.090, P < 0.001), INR (OR = 5.830, 95%CI: 1.492-22.785, P = 0.011), SpO_2/FiO_2 ratio (OR = 0.985, 95%CI: 0.975-0.995, P = 0.004), and IGFBP-3 (OR = 0.332, 95%CI: 0.120-0.915, P = 0.033).

Fifty-four patients died and three subjects underwent liver transplantation during 90 d of follow-up. The Kaplan-Meier survival probability at 90-d was 80.4% in patients with IGFBP-3 ⩾ 0.86 mcg/mL and 56.1% for subjects with IGFBP-3 < 0.86 mcg/mL (P < 0.001) (Figure 2A). For the prediction of 90-d mortality, the IGFBP-3 at a cutoff of 0.86 mcg/mL showed a sensitivity of 63% and a specificity of 65%. The negative predictive value was 80% with a positive predictive value of only 44%. Figure 2A exhibited Kaplan-Meier curves for mortality during the follow-up period according to the presence of ACLF and IGFBP-3 categories. The Kaplan-Meier survival probability at 90-d was 89.5% in patients without ACLF and IGFBP-3 ⩾ 0.86 mcg/mL. It was 70.7% for those with IGFBP-3 < 0.86 mcg/mL only, 42.9% for those with ACLF only, and 20.8% for patients with both ACLF and IGFBP-3 < 0.86 mcg/mL (P < 0.001, long-rank test). Similarly, the 90-d survival was 90.8% in patients with Child-Pugh A/B and with IGFBP-3 ⩾ 0.86 mcg/mL, 78.9% for those with IGFBP-3 < 0.86 mcg/mL only, 54.8% for those with Child-Pugh C only, and 36.4% for Child-Pugh C patients with IGFBP-3 < 0.86 mcg/mL (P < 0.001) (Figure 2B).

The MELD score was dichotomized as ⩾ 17 and < 17 based on ROC curve. The 90-d Kaplan-Meier survival probability was 90.7% in patients with MELD < 17 and IGFBP-3 ⩾ 0.86 mcg/mL, 80.5% for those with MELD < 17 and IGFBP-3 < 0.86 mcg/mL, 56.3% for those with MELD ⩾ 17 and IGFBP-3 ⩾ 0.86 mcg/mL, and 31.7% for patients with both MELD ⩾ 17 and IGFBP-3 < 0.86 mcg/mL (P < 0.001) (Figure 2C).

The IGFBP-3 levels were available for 163 patients at the third day of hospitalization and showed a significant decline *vs* admission values (0.76 mcg/mL *vs* 0.94 mcg/mL, P < 0.001). However, neither the magnitude

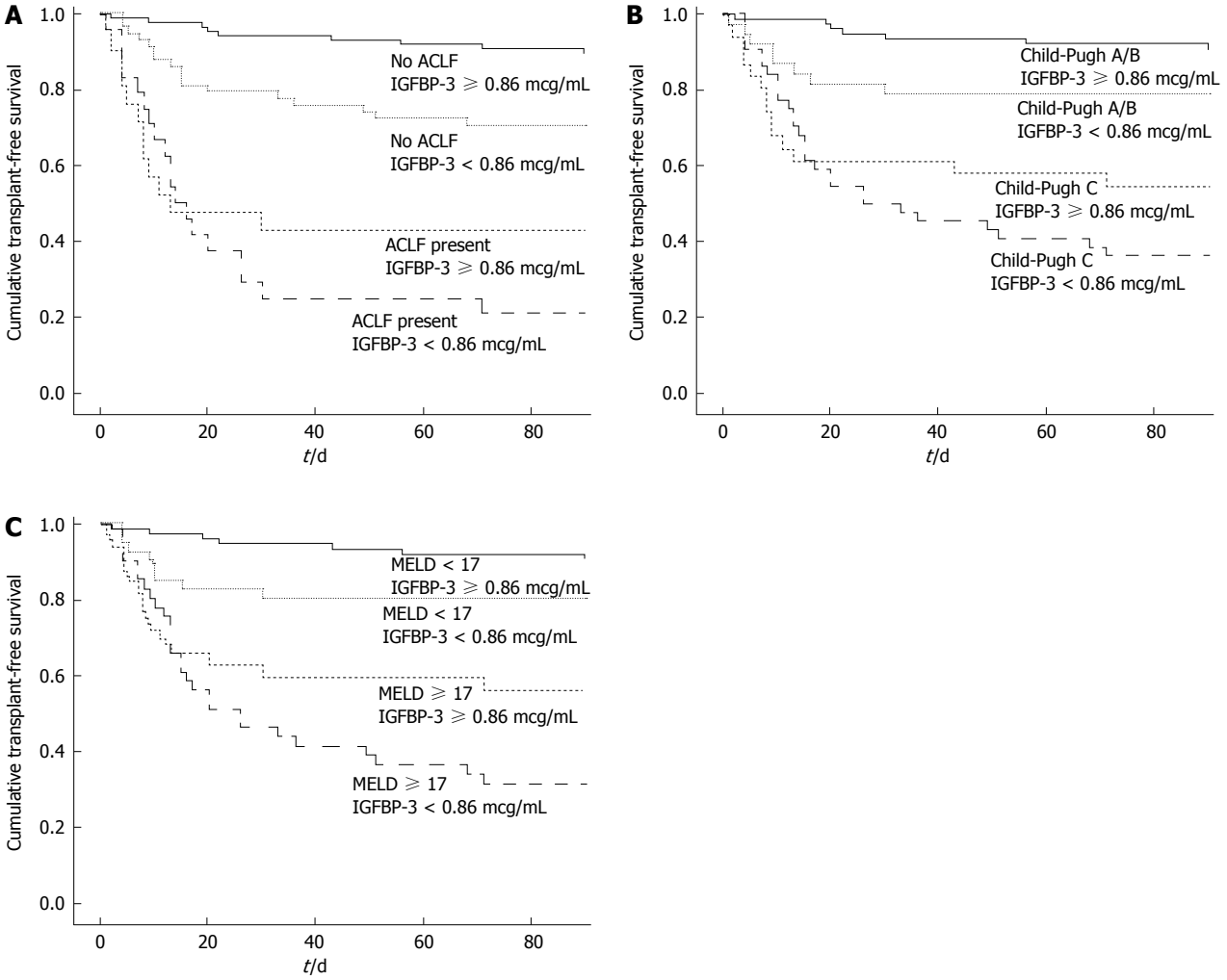

Figure 2 Cumulative 90-d transplant-free survival of hospitalized patients with cirrhosis according to insulin-like growth factor-binding protein 3, acute-on-chronic liver failure, Child-Pugh and model for end-stage liver disease. The 90-d survival was 89.5% in patients without ACLF and with IGFBP-3 ⩾ 0.86 mcg/mL, 70.7% for those with IGFBP-3 < 0.86 mcg/mL only, 42.9% for those with ACLF only and 20.8% for patients with both ACLF and IGFBP-3 < 0.86 mcg/mL (A: $P < 0.001$). Similarly, survival probability was 90.8% in patients Child-Pugh A/B and with IGFBP-3 ⩾ 0.86 mcg/mL, 78.9% for those with IGFBP-3 < 0.86 mcg/mL only, 54.8% for those Child-Pugh C only and 36.4% for Child-Pugh C patients with IGFBP-3 < 0.86 mcg/mL (B: $P < 0.001$). The 90-d Kaplan-Meier survival probability was 90.7% in patients with MELD < 17 and IGFBP-3 ⩾ 0.86 mcg/mL, 80.5% for those with MELD < 17 and IGFBP-3 < 0.86 mcg/mL, 56.3% for those with MELD ⩾ 17 and IGFBP-3 ⩾ 0.86 mcg/mL and 31.7% for patients with both MELD ⩾ 17 and IGFBP-3 < 0.86 mcg/mL (C: $P < 0.001$). ACLF: Acute-on-chronic liver failure; IGFBP-3: Insulin-like growth factor-binding protein 3; MELD: Model for end-stage liver disease.

nor the occurrence of IGFBP-3 decline was related to the severity of cirrhosis or bad prognosis (data not shown). In addition, IGFBP-3 measured at the third day showed similar performance to admission levels when used at its best cutoff for prediction of 90-d mortality (0.68 mcg/mL). These metrics offered a sensitivity of 64%, specificity of 65%, negative predictive value of 83% and positive predictive value of 41%.

IGFBP-3 levels after hospital discharge

The 30 patients evaluated during hospitalization underwent laboratory analysis within a median 105 days after discharge. These were compared at two time points to investigate the impact of AD on IGFBP-3 levels. *Vs* inpatient assessment, significantly higher median IGFBP-3 levels were observed with outpatient evaluation (1.51 mcg/mL *vs* 1.07 mcg/mL, $P < 0.001$). Likewise, an increase in IGFBP-3 levels at outpatient evaluation were observed in 24 out of 30 patients included in this analysis (80%).

DISCUSSION

Even though the course of cirrhosis varies according to several factors, the need for prognostic markers and scoring systems is critical to manage individuals facing different therapeutic options[21]. It was previously shown that IGFBP-3 levels in cirrhosis are related to the severity of liver dysfunction. This marker undergoes only slight influence from other factors not related to liver synthesis capacity. Therefore, IGFBP-3 is a potential and underexplored prognostic biomarker in liver cirrhosis.

Here, the IGFBP-3 levels correlated with several variables directly or indirectly associated with the intensity of liver dysfunction in both stable and decom-

pensated patients. These findings agree with previous studies, which demonstrated an association between lower IGFBP-3 levels and the severity of liver disease[5,7,10,21-24]. In a recent study including patients with AD of cirrhosis, we showed that patients with more severe liver dysfunction exhibited lower IGFBP-3 levels - these levels were not influenced by other parameters such as gender, etiology of cirrhosis and comorbidities[25]. In addition, this study showed that IGFBP-3 increased significantly after liver transplantation as well as after hospital discharge. These findings reinforce the impact of hepatic synthetic function on IGFBP-3 levels and support its utility as a potential biomarker for assessment of liver function.

In patients with stable cirrhosis, death or liver transplantation during follow-up was associated with lower IGFBP-3 levels in bivariate analysis. In addition, transplant-free survival was significantly shorter in subjects with IGFBP3 < 1.67 mcg/mL. Data about the prognostic significance of IGFBP-3 in cirrhosis are scarce. IGFBP-3 was evaluated in 354 patients with alcohol-induced liver disease from a large multicenter trial of the effect of malotilate on survival[26]. The mean follow-up period was 569 d and low IGFBP-3 levels were associated with poor prognosis, especially at a cutoff of 1.35 mcg/mL[26]. The difference between this cutoff and that in the present study probably reflects methodological issues or disparities in the severity of the disease across the cohorts. It is important to note that the European study included both patients with and without cirrhosis-no detailed analysis of only those with cirrhosis was provided[26]. In our data, IGFBP-3 was not associated with death or liver transplantation in the logistic regression analysis in stable cirrhosis. This can be explained by the relatively low number of events in this cohort. Nevertheless, these results indicate the potential of IGFBP-3 as a prognostic marker in stable cirrhosis.

In patients hospitalized for AD of cirrhosis, lower IGFBP-3 levels were associated with short-term mortality in both bivariate and multivariate analysis. There is no data about IGFBP-3 prognostic value in this setting. It is possible that suppressed IGFBP-3 reflects the acute deterioration of hepatic function as suggested by its lower levels in hospitalized cirrhosis *vs* outpatients. The Kaplan-Meier survival probability at 90 d was significantly worse in patients with IGFBP3 < 0.86 mcg/mL *vs* control subjects (56.1% *vs* 80.4%, $P < 0.001$). This cutoff is markedly lower than the limits suggested by Møller *et al*[26] as well as in our study for stable cirrhosis. This likely reflects the severe deterioration of hepatic function in AD of cirrhosis.

The significance of IGFBP-3 in AD of cirrhosis was evaluated according to two of the most important prognostic parameters in this setting: The presence of ACLF and Child-Pugh Classification. The ACLF definition used here was based on a modified version of the SOFA score (CLIF-SOFA) and was first proposed by the EAS-CLIF Consortium in a large multicenter trial and subsequently validated[20,27]. Patients who presented with higher IGFBP-3 and without ACLF showed good prognosis (90-d survival approximately 90%). However, even in the absence of ACLF, low IGFBP-3 was associated with worse prognosis and a 90-d survival of 70.7%. Similarly, in the presence of ACLF, higher IGFBP-3 was associated with 90-d survival of 42.9% *vs* 20.8% for those with both ACLF and low IGFBP-3. These results indicate that combining IGFBP-3 and ACLF definition provided a well-defined four-level stratification for short-term prognosis in patients with cirrhosis hospitalized for AD. Similar results were observed for Child-Pugh Classification and MELD score although with a less clear stratification than that observed by using ACLF.

This study does have some limitations. The relatively small number of events in the stable cohort may have influenced the results - especially concerning regression analysis. In fact, regarding stable patients with cirrhosis there is still a need for validation of our results in larger cohorts with a longer follow-up before this biomarker is incorporated into clinical practice. Another limitation that we should highlight is the fact that we included a very heterogeneous population in distinct clinical scenarios and no specific treatment guidelines were created for the purpose of this study. Therefore, variations in the approach to specific cases are expected. In addition, the underlying liver disease status (if active or not) was not investigated. However, this issue is common in almost all studies investigating biomarkers in clinical settings. In addition, patients were evaluated according to standardized charts developed specifically for the purpose of the study and were followed both in outpatient clinic and in the ward by the same medical team. This minimized the impact of non-standardized approach.

In conclusion, in patients with cirrhosis IGFBP-3 levels correlated with several variables associated with severity of liver disease and improved significantly after discharge and after liver transplantation, indicating the impact of impaired hepatic function on its levels. Lower IGFBP-3 levels were associated with worse long-term prognosis in outpatients with stable cirrhosis and worse short-term prognosis in those hospitalized for AD. These findings suggest that measurement of circulating IGFBP-3 is of prognostic relevance and can be incorporated into clinical practice to improve the care of patients with liver cirrhosis.

COMMENTS

Background

Insulin-like growth factor-binding protein 3 (IGFBP-3) is the major binding protein of insulin-like growth factor (IGF) system, carrying 80%-90% of circulating IGF-Ⅰ. IGFBP-3 has predominantly hepatic production and decreased IGFBP-3 serum levels have been reported in patients with cirrhosis and seem to correlate with hepatic dysfunction intensity. Although preliminary reports indicate a clinical applicability for the assessment of circulating IGFBP-3 in cirrhosis, there are very few data on prognostic significance of this biomarker

in this context.

Research frontiers

Defining the prognosis of patients with cirrhosis is of great relevance in order to select appropriate candidates for distinct therapeutic approaches, such as liver transplantation.

Innovations and breakthroughs

In the present study, IGFBP-3 levels correlated with several variables associated with hepatic dysfunction. Lower IGFBP-3 levels were associated with worse long-term prognosis in outpatients with stable cirrhosis and worse short-term prognosis in those hospitalized for acute decompensation.

Applications

These data suggested that IGFBP-3 levels can be used solely or in combination with other models (including Child-Pugh, model for end-stage liver disease and acute-on-chronic liver failure definition) to evaluate the prognosis of patients with liver cirrhosis.

Peer-review

This prospective study investigated the prognostic significance of IGFBP-3 in patients with cirrhosis. This is an interesting study, and this manuscript could provide useful information to readers.

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