Both normal stem cells and CSCs largely share surface marker phenotype and molecular machinery concerning self-renewal and differentiation. These phenotypic characteristics of CSCs have been well documented in hematological malignancies [8,9], but little is shown in solid tumors including hepatocellular carcinoma (HCC). Furthermore, it is likely that CSCs are closely associated with not only carcinogenesis but also the recurrence and metastasis of tumors [10]. HCC is one of the most common malignancies worldwide and frequently shows strong resistance to traditional anticancer therapies such as chemotherapy and radiotherapy [11,12]. However, there is not sufficient evidence on CSCs in primary HCC. Thus, both the characterization and an understanding of the CSC system in liver are of paramount importance to elucidate mechanisms underlying hepatocarcinogenesis and to establish novel therapeutic approaches.

In this review, we will summarize the recent progress in CSC research in HCC and the molecular machinery underlying hepatocarcinogenesis. We also provide a perspective on therapeutic approaches against HCC from the CSC standpoint.

2. Normal hepatic stem cells

Normal adult hepatocytes are ordinarily in the quiescent G_0 state and mitotically inactive, although the liver regenerates quickly after acute injury and volume loss [13]. The regeneration is accomplished by the simple duplication of mature hepatocytes without the activation of stem/progenitor cells [14]. The presence of hepatic stem cells has been in doubt for some time. However, recent studies have successfully identified and characterized hepatic stem cells not only in fetal livers but also in adult livers (Table 1) [15,16].

2.1. Stem/progenitor cells in fetal livers

In the developing liver, both fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) signals promote the commitment of ventral endoderm to the liver bud (Fig. 1) [17]. During liver bud growth, bipotent progenitors termed hepatoblasts proliferate and differentiate into hepatocytes and cholangiocytes under the control of various sets of transcription factors. Hepatoblasts could be identified as RT1A1$^-$OX18^{low}ICAM-1$^+$, Liv2$^+$, E-cadherin$^+$, Dlk1$^+$ or c-Met$^+$CD49f$^{+/low}$c-Kit$^-$CD45$^-$TER119$^-$ cells in the developing rodent liver utilizing cell sorting technology and clonal colony assays [18–23]. Interestingly, a portion of c-Met$^+$CD49f$^{+/low}$c-Kit$^-$CD45$^-$TER119$^-$ cells lack the expression of albumin, a specific marker of hepatocyte differentiation. These albumin$^-$ cells give rise to albumin$^+$ progenitor cells in response to hepatocyte growth factor (HGF) and differentiate into hepatocytes and cholangiocytes [24]. They also retain the potential to differentiate into non-hepatic lineages, pancreatic aciner cells and intestinal cells [23,24]. These findings indicate that c-Met$^+$CD49f$^{+/low}$c-Kit$^-$CD45$^-$TER119$^-$ cells include a small number of pluripotent precursors of hepatoblasts or hepatic stem cells.

In an investigation of human fetal liver, Schmelzer et al. found that epithelial cell adhesion molecule (EpCAM/CD326)$^+$ cells can be divided into two subtypes [25]; (a) hepatoblasts positive for intercellular adhesion molecule-1 (ICAM-1), alpha-fetoprotein (AFP), albumin, cytokeratin 19 (CK19) and CD133, and (b) human hepatic stem cells (hHpSCs), accounting for less than 5% of EpCAM$^+$ cells, positive for CK19, CD133, neural cell adhesion molecule (NCAM) and claudin 3, but not for ICAM-1, AFP and albumin. These populations behaved differently in colony assays and hHpSCs are assumed to be pluripotent precursors of hepatoblasts (Fig. 1).

2.2. Stem/progenitor cells in adult livers and oval cells

Although the identification of adult stem cells in normal liver has been a challenge for some time, recent studies have shown that EpCAM$^+$ liver cells purified from adult livers possess considerably similar biological characteristics to those from fetal livers and function as hepatic stem cells [25]. Given that some EpCAM$^+$ cells are also positive for CD133 and NCAM, it might be possible to further enrich

Table 1
Cell surface markers for hepatic stem/progenitor cells.

Surface markers	Source	Frequency (%)	Reference
Fetal liver			
RT1A1$^-$OX18^{low}ICAM-1$^+$	Rat (ED13)		[18]
Liv2$^+$	Mouse (ED10.5–12.5)	20–60	[19]
E-cadherin$^+$	Mouse (ED 12.5)		[20]
Dlk-1$^+$	Mouse (ED 14.5)	Nearly 10	[21]
Dlk-1$^+$	Rat (ED 14)	5.7 ± 0.9	[22]
c-Met$^+$CD49f$^{+/low}$c-Kit$^-$CD45$^-$TER119$^-$	Mouse (ED 13.5)	1.90 ± 0.33	[23]
EPCAM$^+$	Human (16–20 wk fetus)	12.1 ± 2.3	[25]
Adult liver			
EpCAM$^+$	Human (pediatric)	2.1 ± 1.6	[25]
	(adult)	1.3 ± 1.0	
Injured liver			
CD133$^+$ CD45$^-$	Mouse (DDC diet)	<0.05	[27]
Thy-1$^+$	Rat (AAF/PH or AAF/CC14)		[28]
Sca-1$^+$CD34$^+$CD45$^+$	Mouse (DDC diet)		[29]
EpCAM$^+$	Rat (AAF/PH)		[32]

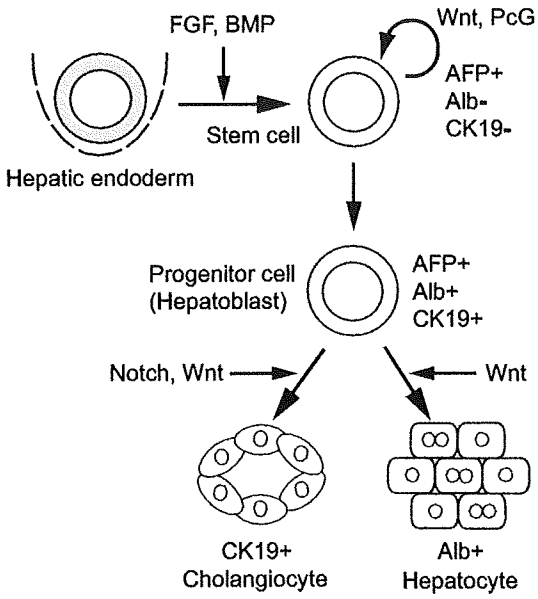

Fig. 1. Hepatogenesis and the regulatory machinery involved. In the developing liver, both fibroblast growth factor (FGF) signal from the cardiac mesoderm and bone morphogenetic protein (BMP) signal from the septum transversum mesenchyme cells promote the commitment of ventral endoderm to the liver bud. In the liver bud, hepatic stem cells generate hepatoblasts, bipotent progenitor cells which subsequently give rise to hepatocytes and cholangiocytes.

the hepatic stem cell population. Prospective assays using these cells would be beneficial to promote investigations of both normal stem cells and CSCs in liver.

Oval cells are defined as small cells with an oval nucleus and scanty cytoplasm and are considered to be progenitor cells with the ability to differentiate into hepatocytes and cholangiocytes [26,27]. They emerge from the periportal region in regenerating liver where the proliferation of mature hepatocytes is impaired. On the basis of phenotypic characteristics in common with hematopoietic stem cells (HSCs), such as the expression of cell surface markers, CD34, c-Kit, Sca-1, and Thy1, oval cells have been considered to originate from bone marrow [28,29]. However, several reports have provided evidence that hepatic oval cells originate from the liver, not from bone marrow [30,31]. Recently, Yovchev et al. reported that $EpCAM^+$ oval cells express not only epithelial markers such as AFP, CK19 and OV-1, but also mesenchymal markers such as vimentin, glypican1 and BMP7 [32]. These oval cells show decreased expression of CD34, c-Kit and NCAM. The origin of oval cells is still controversial. This could be partially due to the heterogeneity of oval cells.

Kuwahara et al. focused on the functional definition of stem/progenitor cells of hepatic origin using label-retaining cell (LRC) assays, widely utilized for the detection of somatic stem cells in many tissues and organs [33]. They conducted bromodeoxyuridine (BrdU) LRC assays in a mouse model of N-acetyl-p-aminophenol (APAP)-induced liver injury and successfully identified LRCs in the canal of Hering (proximal biliary tree), intralobular bile ducts, periductal mononuclear cells that lack hepatocytic and biliary markers, and peribiliary hepatocytes, suggesting that the liver has a multi-tiered, flexible system of regeneration rather than a single stem/progenitor cell location [33]. These findings clearly indicate the heterogeneity of hepatic stem/progenitor cells including oval cells and the need to clarify the extent to which each subset contributes to regeneration of the liver.

3. Cancer stem cells in liver

Dysregulated self-renewal capability, following oncogenic mutations, is one of the key events in the early stages of carcinogenesis [34]. It is believed that CSCs usually arise from normal stem/progenitor cells with enhanced or acquired self-renewal capability (Fig. 2). However, it remains unclear whether this hypothesis can be applied to hepatocarcinogenesis. Many attempts have been made to detect a small subset of cancer cells with the characteristics of CSCs in HCC (Table 2).

Side population (SP) cell sorting is useful for detecting CSCs in various cancer cell lines [35]. The SP phenotype is determined by the ability to efflux the dye Hoechst 33342 through an adenosine triphosphate (ATP)-binding cassette (ABC) membrane transporter [36]. Only 0.25–0.80% of Huh7 and PLC/PRF/5 HCC cells exhibits the SP phenotype. Of note, tumor initiating capacity detected in xenotransplantation assays using immunodeficient mice has been strictly confined to SP cells [37]. One thousand SP cells was enough to generate tumors in xenotransplantation, while at least 1×10^6 unsorted HCC cells were required for tumor formation, suggesting that TICs are enriched by SP cell sorting at least 1000-fold. Considering that the frequency of HCC SP cells was less than 1%, the minority cell population detected as SP cells, but not non-SP cells, might possess tumorigenic potential in these HCC cells. However, several paradoxes have been reported to this technique. C6 glioma cells contain approximately 0.4% SP cells. In serum-free medium, C6 SP cells, but not non-SP cells are responsible for the *in vivo* tumorigenesis [38]. On the other hand,

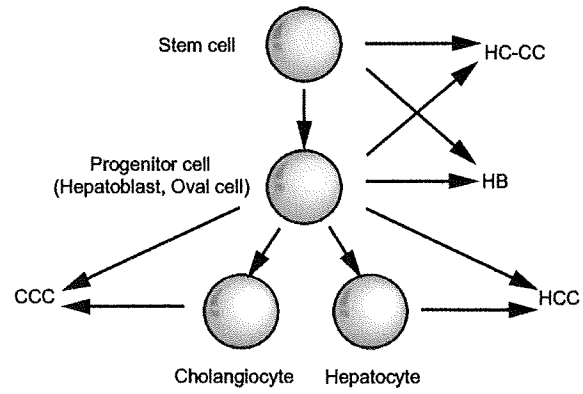

Fig. 2. Proposed model for the cellular origin of liver malignancies. Liver tumors develop from more heterogeneous cells as expected. Combined hepatocellular and cholangiocellular carcinoma (HC–CC) and hepatoblastoma (HB) are thought to be derived from primitive hepatoblasts and pluripotent stem cells. It remains unclear whether hepatocellular carcinoma (HCC) and cholangiocellular carcinoma (CCC) originate from mature cells or stem/progenitor cells.

Table 2
Cell surface markers for CSCs in HCC.

Surface markers	Frequency (HCC cell lines analyzed)	Minimal number of cells initiating tumors (cells)	Reference
Cell line			
Side population	0.25–0.80% (Huh7, PLC/PRF/5)	1×10^3	[37]
$CD133^+$	0.1–2.0% (SMMC7721)	1×10^2	[42]
$CD133^+$	8–90% (HepG2, Huh7, PLC8024, Hep3B)	1×10^3	[43]
$OV6^+$	0.2–3.0% (Huh7, SMMC7721, Hep3B, PLC, HepG2)	5×10^3	[46]
$EpCAM^+$	58.1–99.2% (Huh1, Huh7, Hep3B)	2×10^2	[47]
$CD90^+CD44^+$	0.02–2.53% (HepG2, Hep3B, PLC, Huh7, MHCC97L, MHCC97H)	5×10^2	[48]
Primary tumor			
$CD90^+CD44^+$	0.74–4.14%	2.5×10^3	[48]

most C6 cells cultured in serum-containing medium, including both SP and non-SP cells exhibit tumor initiating capacity *in vivo*, raising the possibility that C6 cells do not follow cancer stem cell theory and that non-SP cells easily suffer Hoechst toxicity and fail to grow under serum-free conditions [39,40]. The similar findings that exclude SP cell sorting technique from the major defining markers of cancer stem cells are also reported in other cancer cell types [41]. Thus, we should be more careful when applying SP cell sorting to cancer stem cell analysis including culture conditions and Hoechst cytotoxicity.

HCC cells positive for CD133, a potential cell surface marker for CSCs in a number of tumors, have been reported to exhibit greater tumorigenicity than the corresponding $CD133^-$ cells in HCC cell lines [42,43]. In mouse models of HCC, *methionine adenosyltransferase I, alpha (Mat1a)* knockout mice and *Pten* knockout mice, tumorigenic capacity was mainly detected in $CD133^+$ oval cells [44,45]. OV6 and EpCAM are also reported as specific surface markers for CSCs in HCC cell lines [46,47].

In contrast to HCC cell lines, HCC cells from surgical specimens hardly engrafted in immunodeficient mice. Recently, $CD90^+CD44^+$ cells were reported to engraft in the livers of severe combined immunodeficient (SCID)/Beige mice and behave as CSCs [48]. However, the engraftment of these cells was very inefficient and no obvious tumor masses developed. To prove that CSCs exist in human primary HCC, technical improvements to obtain better engraftment in xenotransplantation are needed. The usage of more immunodeficient mice and longer observation periods would be the approach to try first as reported [49].

4. Cell origin of liver malignancies

The cell origin of HCC has long been debated, but whether HCC originates from mature hepatocytes or stem/progenitor cells remains unclear. HCC usually develops against a setting of chronic liver injury due to chronic infection of hepatitis viruses [11]. Most well-differentiated HCCs in the early stages are detected as a small lesion with a normal level of serum AFP. Subsequently, they increase in diameter and become moderately to poorly differentiated cancerous tissues producing AFP. These findings imply that HCC might develop and progress during the de-differentiation of mature hepatocytes. On the other hand, the concept of blocked ontogeny, that maturation arrest in stem/progenitor cells contributes to cancer development, is also accepted in hepatocarcinogenesis [50]. It is evident that transformed oval cells could be a cellular origin of liver tumors [51,52]. Additionally, the activation of oval cells has been observed in not only various rodent models of carcinogenesis but also in human chronic liver disease, HCC and CCC [53,54]. Combined hepatocellular and cholangiocellular carcinoma (HC–CC) is a rare but distinct type of liver malignancy. Histological analyses revealed the proliferation of an oval-cell-like subpopulation to varying degrees except in the HCC and CC components, which indicates the role of stem/progenitor cells as the cellular origin of the tumor [55]. Consistent with this, it has been also reported that fetal liver-derived hepatic stem/progenitor cells transduced with *Bmi1* or mutant *β-catenin* acquired enhanced self-renewal capability and tumorigenicity to initiate HC–CC [56]. These observations imply that dysregulated propagation of hepatic stem/progenitor cells is an important early step in hepatocarcinogenesis. Similarly, the implantation of *p53*-null oval cells resulted in the development of HC–CC in recipient mice [57]. Therefore, HC–CC might be derived from hepatic stem/progenitor cells at least in some instances (Fig. 2).

Interestingly, it was reported that HCC could be divided into distinct subtypes sharing gene expression features with subsets of cells in differing stages of differentiation and the HCC subtypes with a similar gene expression profile to hepatic stem/progenitor cells had a poor prognosis [58,59]. Thus, HCC might develop from more heterogeneous cells during differentiation (Fig. 2).

5. Molecular machinery operating in normal and cancer stem cells in liver

Cancer-related signaling pathways, such as the Wnt, Shh, Notch and PI3K/AKT/mTOR pathways, play an important role in the maintenance or augmentation of the self-renewal capability of CSCs as well as normal stem cells (Fig. 3) [60,61]. Interestingly, however, the dependency on these signals differs somewhat between normal stem cells and CSCs [62]. Understanding the molecular mechanisms operating in normal stem cells and CSCs is essential to innovate novel therapeutic approaches.

5.1. Polycomb-group (PcG) gene products

PcG proteins are transcriptional repressors that function by modulating chromatin structure. They form chromatin-associated multiprotein complexes, polycomb repressive complex (PRC) 1 and PRC2 [63]. Bmi1, one of

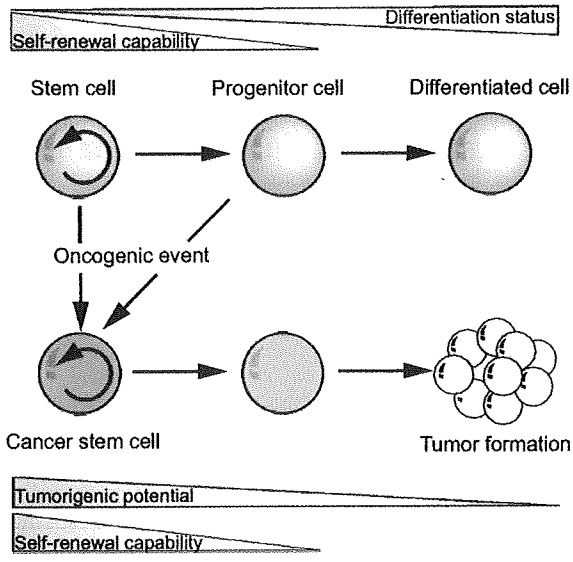

Fig. 3. Organization of cancer stem cell systems. Normal stem cells with self-renewal capability generate progenitor cells and subsequently various terminally differentiated cells. Oncogenic mutations in normal stem/progenitor cells or even in differentiated cells enhance or endow the self-renewal capability. Consequently, these cells function as cancer stem cells and contribute to the formation of bulk tumors.

the components of PRC1, regulates the cell cycle, apoptosis and senescence by repressing the *Ink4a/Arf* tumor suppressor gene locus [64]. Bmi1 is essential for the maintenance of the self-renewal capability of somatic stem cells including hepatic stem/progenitor cells [58]. On the other hand, overexpression of *Bmi1* in hepatic stem/progenitor cells augments their self-renewal capability and induces tumor development in mice [58]. Consistent with these findings, BMI1 is overexpressed in a number of tumors [65]. Of note is that it is preferentially expressed in $CD44^+$ CSCs in head and neck tumors [66] and in tumor-initiating SP cells in HCC cell lines [67]. Expression levels of BMI1 in HCC cell lines are faithfully correlated with the proportion of SP cell fraction and tumor-initiating capacity in mice [67]. Furthermore, levels of BMI1 expression in HCC were well correlated with the progression and prognosis of the disease [68]. These findings suggest that Bmi1 regulates self-renewal of both normal stem cells and CSCs by repressing the transcription of negative regulator genes for stem cell maintenance such as *Ink4a* and *Arf* [63] and by doing so, acts against oncogene-induced senescence, which is of substantial importance to the elimination of transforming cells that potentially develop into CSCs [69].

The important role of Ezh2, one of the components of PRC2, has been also recognized recently. EZH2 is also overexpressed in a variety of cancers including HCC [65]. Elucidation of its role in normal stem cells and CSCs requires further analysis.

5.2. Wnt/β-catenin signaling

Wnt/β-catenin signaling is a general regulator of self-renewal in a wide range of stem cell systems and closely associated with carcinogenesis [70]. It has been demonstrated that murine hepatic stem/progenitor cells transduced with mutant *β-catenin* acquired excessive self-renewal capability and tumorigenicity in a similar fashion to *Bmi1* [58]. In addition, Yang et al. reported that Wnt/β-catenin signaling is activated in both rodent oval cells and $OV6^+$ tumorigenic HCC cells [46]. These findings indicate that Wnt/β-catenin signaling is involved in the development and maintenance of CSCs.

Hepatoblastoma (HB) is a pediatric liver tumor. Because it shows various morphological patterns including epithelial and mesenchymal lines of differentiation, HB is considered to be derived from developmentally primitive pluripotent stem cells in some instances. Aberrant activation of Wnt/β-catenin signaling due to deletions or mutations of *β-catenin*, *adenomatous polyposis coli* (*APC*), and *Axin* is frequently observed in HBs [71]. It is reported that HBs could be divided into two subclasses, namely immature and differentiated subtypes, based on their genetic features [72]. Of interest, transcriptional programs driven by the activated Wnt/β-catenin signaling differ considerably between the two subtypes, and additional Myc activation plays an important role in the conversion of differentiated tumors into immature ones. This highlights the important role of dysregulated Wnt/β-catenin signaling in the transformation of stem/progenitor cells.

5.3. Transforming growth factor beta (TGF-β) signaling

The TGF-β/Smad signaling pathway is involved in the self-renewal and differentiation of stem cells and carcinogenesis in a variety of tissues and organs [73]. Tang et al. reported that hepatic stem/progenitor cells express TGF-β signaling-related proteins, TGF-β receptor type II (TBRII) and embryonic liver fodrin (ELF), in post-transplant human liver tissues [74]. In addition, they assumed that the activated IL-6/Stat3 pathway concomitant with the impaired TGF-β signaling in these cells is relevant to the hepatocarcinogenesis in $elf^{+/-}$ mice, which spontaneously develop HCC. This implicates the importance of TGF-β and IL-6 signaling in the CSC population in HCC.

6. Therapeutic resistance of cancer stem cells in liver

Therapeutic resistance to standard chemotherapy and radiotherapy has been attributed to CSCs in a wide spectrum of cancers [75]. Many different types of cancer cells show overexpression of ABC transporters and drug resistance genes [76]. High drug efflux capacity through ABC transporters is one of the most striking characteristics of SP cells, a rare subset of CSCs in various cancer cell lines. Consistent with this, HCC SP cells were reported to exhibit resistance to anti-cancer agents such as doxorubicin [77]. In a human acute myelogenous leukemia (AML) xenotransplantation model, leukemic stem cells (LSCs) engrafted in the bone marrow niche, where they stayed in a quiescent G_0 state. Surprisingly, up to 70% of CSCs survive cell cycle-dependent cytotoxic treatment, while LSC progenies are effectively eradicated [78]. Given the large population of HCC SP cells in G_0 phase [79], it is conceivable that CSCs in HCC also show resistance to cell cycle-specific agents.

Bao et al reported that CSCs in glioma possess an efficient DNA repair system through the activation of Chk1 and Chk2 checkpoint kinases and show resistance to irradiation therapy [80]. Although the cyclin-dependent kinase inhibitor $p21^{Waf1}$ could function as a tumor suppressor, a recent study showed that activation of $p21^{Waf1}$ is critical for DNA repair to maintain LSCs by preventing the accumulation of DNA-damage [81]. $p21^{Waf1}$ is preferentially expressed in HCC tissues rather than surrounding non-tumor tissues [82]. It is possible that well-developed DNA repair machinery operates in HCC CSCs and confers resistance to radiation therapy.

7. Therapeutic approaches for liver cancer stem cells

Although the investigation of treatments targeting CSCs in HCC has just started, strategies reported for CSCs of other tumors may offer hints for novel therapeutic approaches in HCC.

7.1. Molecular target therapy

The inhibition of CSC-specific pathways is one promising therapeutic approach. For example, LSCs of chronic myelogenous leukemia (CML) reside in the bone marrow niche in a quiescent G_0 state and are resistant to chemotherapy and targeted therapies. Nuclear protein promyelocytic leukemia protein (PML) is essential for keeping LSCs in a quiescent state. Pharmacological inhibition of PML has been shown to change LSCs sensitive to conventional and targeted therapies by recruiting them into the cell cycle [83]. Ma et al. documented that activation of the Akt/PKB and Bcl-2 pathway contributes to the chemoresistance observed in $CD133^+$ HCC cells [84]. It is noteworthy that treatment with an Akt1 inhibitor sensitized $CD133^+$ HCC cells to conventional anti-cancer drugs such as 5-FU. Aldehyde dehydrogenase (ALDH), a detoxifying enzyme which eliminates toxic byproducts of reactive oxygen species (ROS), is a marker of both normal stem cells and CSCs. It was found that ALDH is highly expressed and confers chemoresistance to alkylating agents such as cyclophosphamide in LSCs and breast CSCs [85,86]. Given that the majority of $CD133^+$ cells in HCC cell lines also show strong ALDH enzymatic activity [87], ALDH inhibitors might be effective for the eradication of CSCs in HCC.

7.2. Differentiation therapy

It is presumed that the differentiation of CSCs ultimately results in the suppression of carcinogenesis, because the tumorigenicity of CSCs is largely determined by their own self-renewal capability. It has been documented that BMPs promote the differentiation of glioma stem cells and reduce their tumorigenic potential [88]. In transgenic mice in which the expression of c-Myc is conditionally regulatable, c-Myc expression induced multiple HCCs. Upon the inactivation of Myc, HCC cells lost neoplastic properties and differentiated into hepatocytes and cholangiocytes [89]. The mice showed a decrease in tumor volume and prolonged survival. Hepatocyte nuclear factor (HNF) 4α is a central transcription factor essential for hepatogenesis [90]. A recent report showed that the gene transfer of *HNF4α* reduced a population of tumorigenic $CD90^+$ and $CD133^+$ cells purified from HCC cell lines by inducing differentiation of these subpopulations [91]. Interferon therapy is effective for not only eradicating the hepatitis viruses but also preventing the development of HCC regardless of the virological response. Interferon alpha treatment accelerated hepatocytic and biliary differentiation in oval cell lines [92]. Thus, interferon could be applied to the treatment of HCC for targeting CSCs.

7.3. Antibody therapy

Monoclonal antibody therapy is considered an important therapeutic modality for cancer. Rituximab (Anti-CD20) has already proven effective against lymphoid malignancies [93]. Although CD44, a receptor for hyaluronic acid and osteopontin, is widely expressed in both HSCs and LSCs, a monoclonal antibody specific to CD44 had a favorable effect on eradicating LSCs without affecting normal HSCs in a xenograft mouse model of human AML [94]. The administration of the CD44 antibody diminished the capacity of LSCs to home to the supportive microenvironment and promoted the terminal differentiation of LSCs *in vivo*. Given that $CD44^+$ cells function as CSCs in a variety of solid tumors including HCC [6,48], anti-CD44 antibody therapy might be a promising CSC-specific treatment in HCC.

Brain tumor stem cells reside in close proximity to blood vessels, called "vascular niches", where they receive signals that allow them to self-renew and to generate transit-amplifying cells [95]. Anti-angiogenic approaches such as the administration of anti-vascular endothelial growth factor (VEGF) monoclonal antibody disrupt vascular niches and dramatically reduce the number of CSCs. On losing the niches, CSCs cannot self-renew and differentiate into transit-amplifying cells. It is well-known that moderately to poorly differentiated HCCs are abundant in tumor vessels. Anti-angiogenic agents such as bevacizumab have already entered clinical trials for HCC and shown efficacy in some instances [96]. However, the CSC niche in HCC remains elusive. Identification of the microenvironment supportive for HCC progression is definitively needed.

8. Perspective

CSC research for HCC is somewhat behind that for other solid tumors. The major problem is that we are not sure of their existence in primary HCC. As described above, HCC cells from surgical specimens hardly engraft in conventional immunodeficient mice. This technical problem prevents us from obtaining an overall view of the CSC system in primary HCC. However, recent advances in xenotransplantation, including the co-injection of Matrigel (basement membrane matrix), usage of more immunodeficient nonobese diabetic/severe combined immunodeficiency (NOD/SCID)/interleukin-2 receptor (IL-2R) γ^{null} (NOG) mice in xenotransplantation, and longer observation periods have considerably improved the engraftment of human cancer cells. By using these approaches, approximately 25% of

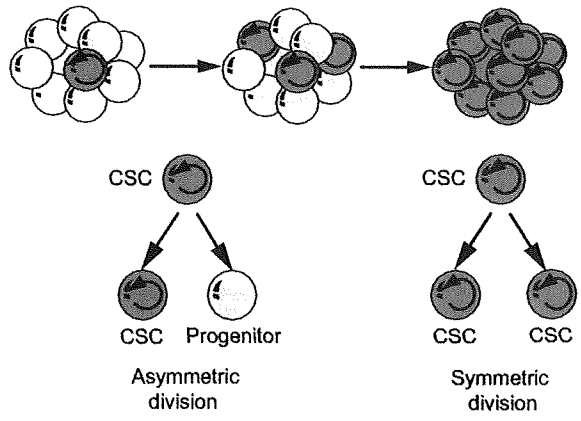

Fig. 4. Hierarchical diversity in cancer cells. Not all types of tumors have a cancer stem cell (CSC) system and CSCs do not necessarily represent a minor subpopulation of cancer cells. Asymmetrical self-renewal division of CSCs is crucial for the maintenance of a hierarchical organization in the tumor. On the contrary, predominant symmetrical self-renewal division of CSCs ultimately produces a highly homogenous population in terms of tumorigenicity as shown in melanoma cells [49].

unselected melanoma cells have been substantiated to possess tumorigenic potential [49]. Moreover, as few as 10 cells purified from murine lymphoma and AML were sufficient for the development of original hematological malignancies in syngenic transplantation assays [97]. These findings pointed out the possibility that the frequency of CSCs was underestimated in xenotransplant experiments because of a microenvironment unable to support the engraftment of donor cells. At the same time, these findings indicated that not all types of cancers fit a CSC model (Fig. 4).

Does HCC follow the CSC theory? This is a very important question. Without answering it, we cannot solve other issues, including the origin of HCC, niche for HCC, and mechanism of chemoresistance observed in HCC. Further efforts to identify and characterize HCC CSCs using improved xenotransplantation systems would provide a whole picture of the cellular organization of HCC.

9. Conflicts of interest

None declared.

Acknowledgements

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Pre-treatment hemodynamic features involved with long-term survival of cirrhotic patients after embolization of gastric fundal varices

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ABSTRACT

Purpose: To clarify the pre-treatment hemodynamic features involved in the long-term survival of cirrhotic patients with gastric fundal varices (FV) after balloon-occluded retrograde transvenous obliteration (B-RTO).

Materials and methods: Eighty-one cirrhotic patients with medium- or large-grade FV treated by B-RTO were enrolled in this retrospective study. Pre-treatment flow volume ratio between gastric vein and portal trunk (GP-R) was obtained by Doppler ultrasound.

Results: The cumulative survival rate was 90% at 1 year, 74.8% at 3 years, 57.2% at 5 years, and 45.8% at 7 years without recurrence in a median period of 1148.5 days The survival was poorer in patients with HCC (47% at 3 years, 9.4% at 5 years, $p < 0.0001$) than without (89.2% at 3 years, 81.9% at 5 years, 67.5% at 7 years), in patients with Child B/C (57.7% at 3 years, 42.1% at 5 years, 28.1% at 7 years, $p = 0.0016$) than with Child A (91.8% at 3 years, 71.5% at 5 years, 62.1% at 7 years), and in patients with GP-R $\geq$ 1.0 (58.9% at 3 years, $p = 0.0485$) than with GP-R < 1.0 (76.3% at 3 years, 62% at 5 years, 49.6% at 7 years). Multivariate analysis identified the presence of HCC (hazard ratio, 12.486; 95% CI, 4.08–38.216; $p < 0.0001$), Child B/C (hazard ratio, 3.41; 95% CI, 1.594–7.15; $p = 0.0051$) and GP-R $\geq$ 1.0 (hazard ratio, 2.701; 95% CI, 1.07–6.15; $p = 0.0221$) as independent factors for poor prognosis.

Conclusion: GP-R $\geq$ 1.0 on Doppler ultrasound before B-RTO may be a predictive indicator for poor prognosis in cirrhotic patients with FV after B-RTO, in addition to the presence of HCC and severe liver damage.

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1. Introduction

Portal hypertension causes gastroesophageal varices, which present a certain bleeding risk [1]. Although lower bleeding rates of gastric varices compared to esophageal varices (EV) have been reported, gastric variceal bleeding sometimes results in serious consequences during the clinical course [2,3]. As variceal bleeding is still one of the major causes of mortality in cirrhotic patients, certain treatment methods using endoscopy, surgery or interventional techniques have been introduced and applied for gastric varices [4,5].

Balloon-occluded retrograde transvenous obliteration (B-RTO) provides an efficient therapeutic effect for gastric fundal varices (FV) with less recurrence [6–8]. Previous reports have shown satisfactory prognosis after B-RTO, with 3- and 5-year survival rates over 70% and 50%, respectively, and hepatocellular carcinoma (HCC) and/or liver function were reported to be prognostic factors associated with survival after the treatment [9–11].

As B-RTO embolizes large collateral vessels, portal hemodynamic changes may occur in the manner of an opposite effect to a decompressive treatment like transjugular intrahepatic portosystemic shunt. In fact, Akahane et al. reported a significant increase in portal venous pressure (PVP) as a logical consequence after B-RTO, with an increase in portal blood flow [12]. Although portal hemodynamics before B-RTO might predict the clinical course after B-RTO, the relationship between pre-treatment hemodynamics and post-treatment prognosis has not been fully investigated. The aim of this study was to clarify the pre-treatment clinical parameters including hemodynamic conditions involved in the long-term survival of cirrhotic patients with FV after B-RTO.

2. Patients and methods

2.1. Patients

Between March 1998 and August 2007, 98 cirrhotic patients underwent B-RTO for medium- or large-grade FV in Chiba University Hospital. The application criteria for B-RTO in our department

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were as follows: (i) patients with medium- or large-grade FV on endoscopy, (ii) patients with a bleeding history from FV (secondary prophylaxis), (iii) Child's A or B grade patients with hopeful prophylaxis treatment for FV (primary prophylaxis), (iv) patients with gastrorenal shunt and/or inferior phrenic vein for the drainage route of FV on ultrasound (US), computed tomography (CT) or percutaneous transhepatic portography images. Among the 98 patients, eligible subjects for this study were selected according to the following criteria: (i) patients with FV completely embolized on contrast-enhanced CT after B-RTO, (ii) patients followed up on an outpatient basis for 1 year or more, (iii) patients received a medical check-up with blood tests at least twice a year, and had US examination and endoscopy at least once a year. Complete embolization of FV was obtained in 94 of the 98 patients, and the clinical course of 13 patients became unknown within 1 year because they stopped visiting to the hospital. Therefore, data on 81 cirrhotic patients (age: 63.5 ± 9.4, 42–80 years; male 47, female 34) with medium- or large-grade FV treated by B-RTO were analyzed in this retrospective study. The cause of cirrhosis was viral in 57 patients (HCV 49, HBV 8), alcohol abuse in 12, primary biliary cirrhosis in two, autoimmune hepatitis in one, and cryptogenic in nine. The severity of liver function (Child-Pugh score) was A in 39, B in 39, and C in three, and laboratory findings just before B-RTO were as follows: total bilirubin 1.5 ± 1.7 (0.2–15.4 mg/dl), serum albumin 3.2 ± 0.5 (2–4.5 g/dl), prothrombin time 67.6 ± 14.7 (18–107%).

At the time of B-RTO, 18 patients had concomitant HCC, of which the number of nodules was one in seven patients, two in four patients, three in three patients and more than three in four patients. The diameter of the nodules ranged 10–45 mm (mean 24.8 ± 12.3). One patient had portal vein tumor thrombosis in the portal trunk, and none had distant metastasis. They received treatments for HCC after B-RTO — surgical treatment in one, percutaneous ethanol injection (PEI) in three, transcatheter arterial chemoembolization (TACE) in eight, and TACE followed by PEI in six. Meanwhile, nine patients had a treatment history of HCC, which was controlled by non-surgical treatments in eight (PEI in four, radiofrequency ablation in three, TACE in one) and surgical treatment in one. Therefore, 54 of the 81 patients had neither concomitant HCC nor treatment history of HCC.

Informed written consent was obtained from all patients, and the investigation was carried out in accordance with the Helsinki Declaration. This retrospective study was judged as having an appropriate design for publication by the ethics committee of our hospital.

2.2. Endoscopy

Endoscopic findings of FV and EV were classified according to the General Rules for Recording Endoscopic Findings set by the Japan Research Society for Portal Hypertension [13]; F1 (straight), F2 (winding), and F3 (nodule-beaded), corresponding to the grades of small, medium and large, respectively. The grades of FV were F2 in 42 and F3 in 39; 37 of the FV patients were accompanied by EV, F1 in 22 and F2 in 15. According the FV classification by Sarin et al, there were 44 patients with IGV1 and 37 patients with GOV2 [1]. Forty-four of the 81 FV patients were bleeders, 32 confirmed by endoscopy and 12 by clinical symptoms of hematemesis or melena; the other 37 were non-bleeders with no history of hematemesis or melena. Twenty-four of the 44 bleeder patients underwent emergent endoscopic treatment for hemostasis of FV bleeding, sclerotherapy in 20, band ligation in two, and clipping in two. Prophylactic treatment (band ligation followed by sclerotherapy with absolute ethanol) for EV was done in two patients with medium-grade EV, one before B-RTO and one after B-RTO. All endoscopic procedures were performed by H.M. and S.K. Follow-up endoscopy was performed approximately every 6–12 months after B-RTO, and aggravation of EV was defined when the grade of EV worsened.

2.3. US examination

The US system used in the present study was SSA-270A, SSA-390A and SSA-770A (Toshiba, Tokyo, Japan) with a 3.75-MHz convex or sector probe. Mean flow volume (ml/min) of the portal trunk and gastric vein before B-RTO was measured by pulsed Doppler method in all patients under a fasting state of over 6 h, with the sampling width corresponding to the maximum diameter of the vessel and at an angle less than 60° between the US beam and the vessel. Demonstration of gastric vein was done as previously reported [14,15], using a middle longitudinal or oblique scan for the left gastric vein (LGV), and a left intercostal scan for the short gastric vein (SGV). In cases with multiple gastric veins, that with the highest flow volume showing hepatofugal flow direction was chosen for Doppler measurement result. All US examinations were performed by H.M., and 22 of the 81 patients also received second Doppler measurements by H.O. or H.Y. The US operators had more than 8 years of experience with US. Blood flow measurement was performed two or more times in each patient, and the average value was obtained for each vessel. With the use of these data, the flow volume ratio of gastric vein to portal trunk (GP-R) was calculated in each patient. When the blood flow in the portal trunk had to and fro appearance, the data were not used for GP-R. The results obtained by second US examination were used only for the measurement of inter-observer variability.

2.4. B-RTO

The application of B-RTO represented secondary prophylaxis for 44 bleeders and primary prophylaxis for 37 non-bleeders. B-RTO was conducted by H.M., H.O., S.K. and H.Y. by standard technique using a balloon catheter (Selecon balloon catheter; 5-French, 9 mm, 13 mm; 6-French, 20 mm, Clinical Supply, Gifu, Japan) as previously reported [6–8]. The outflow route of FV for balloon catheter insertion was the gastrorenal shunt in 77 patients, inferior phrenic vein in one, and both in three. The sclerosing agent which contained equal amounts of 10% ethanolamine oleate (Oldamin, Mochida Pharmaceutical, Tokyo, Japan) and iopamidole 300 (Iopamiron 300; Schering, Osaka, Japan) was administered via the catheter to fill both the gastric varices and inflow vessels under balloon occlusion from 1 h to overnight. Haptoglobin (200 ml; Midori Jyuji, Osaka, Japan) was administered continuously to prevent hemolysis. The embolization effect in FV was evaluated on contrast-enhanced CT within 1 week after B-RTO in all patients.

2.5. Statistical analysis

All results were expressed as mean ± standard deviation (SD) or percentage. The survival time of the patients was based on the date of death, liver transplantation or final date confirmed to be alive during the study period. The Kaplan–Meier method was used to calculate survival probabilities for each clinical background, and the difference was compared with log-rank test. The analysis for survival was done by Cox's proportional hazards models with univariate and multivariate using step-wise method. The results were reported as hazard ratios with 95% confidence intervals (CI). Inter-observer variability was calculated with coefficient of variation, calculated by dividing the standard deviation by mean and multiplying by 100. p-Value less than 0.05 was considered statistically significant in all analyses. Statistical analysis was performed using the Dr. SPSS package (version 11.0J for Windows; SPSS Inc., Chicago, IL, USA).

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3. Results

3.1. Clinical course, prognosis and causes of death after B-RTO

The median follow-up period after B-RTO was 1148.5 days, the mean being 1292.6 ± 930.7, 60–3645 days. Although there were only minor short-term complications, fever (over 37.5 °C, 56%), hemoglobinuria (74%) and pain (38%), all cases recovered within 3 days. Aggravation of EV was found in 57 patients (72.2%) and four patients (5.1%) bled from EV after B-RTO among 79 patients who did not receive prophylactic treatment for EV. Neither recurrence nor rebleeding of FV was encountered in this study (Fig. 1). The cumulative overall survival rate was 90% at 1 year, 74.8% at 3 years, 57.2% at 5 years, and 45.8% at 7 years. Thirty-one patients (38.3%) died, with the causes of death being HCC in 13, hepatic failure in 13, other malignancies in three, and sudden death of unknown cause in two. The 3 Child's C grade bleeders were anxious to receive B-RTO, and their survival was 60, 214 and 455 days, respectively, with the cause of death being hepatic failure, metastatic liver tumor from breast cancer and HCC rupture.

3.2. Portal hemodynamics on Doppler US before B-RTO

Blood flow direction in the portal trunk was hepatopetal in 73 patients, to and fro in five patients, and the other three had poor visualization. The main gastric vein was the short gastric vein in 53 (65.4%) and the left gastric vein in 19 (23.5%), showing hepatofugal flow direction on sonograms, and US could not demonstrate gastric veins in nine patients (11.1%). The presence of the gastric veins in 72 patients was confirmed on retrograde

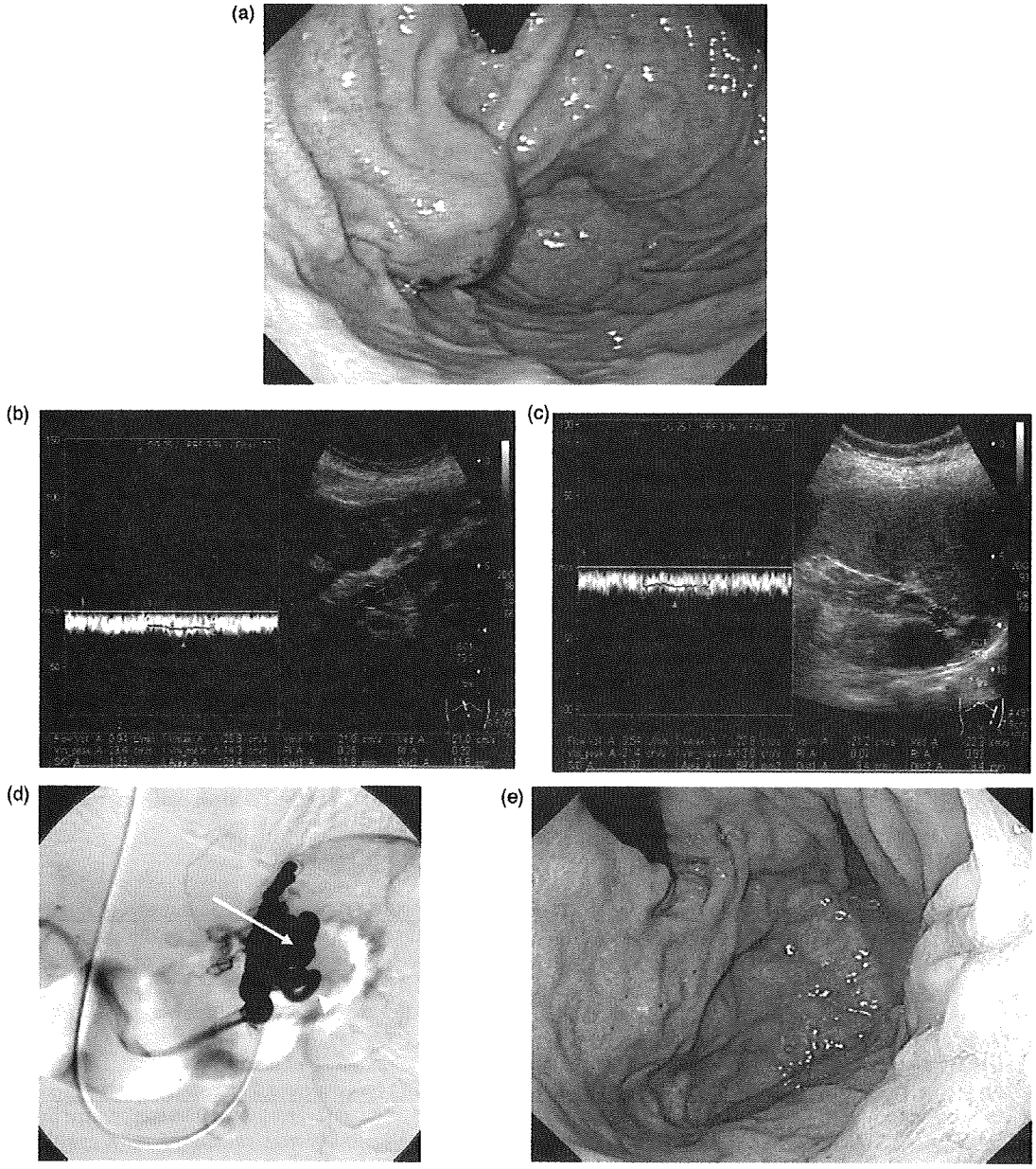

Fig. 1. 61-year-old female, cirrhotic patient (HCV positive), non-bleeder (prophylactic treatment). (a) Endoscopic finding before B-RTO. Large-grade FV was demonstrated at the fundus of the stomach. (b) Doppler US finding. The mean flow volume of the portal trunk was 940 ml/min before B-RTO. (c) Doppler US finding. Doppler US showed development of the short gastric vein with a mean flow volume of 540 ml/min before B-RTO. GP-R of this patient was 0.57 (540/940). (d) Retrograde venography at the time of B-RTO. FV (arrow) and short gastric vein (arrowhead) were demonstrated on this image. (e) Endoscopic finding after B-RTO. FV disappeared completely after B-RTO, and this case was alive 690 days after B-RTO.

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Table 1
Univariate analysis of potential risk factors for survival in patients with gastric fundal varices.

	HR[a]	95% CI[b]	*p*-Value
Gender (F)	0.828	0.389–1.760	0.623
Age	1.007	0.964–1.052	0.7395
HCC[c]	8.907	3.606–22.002	<0.0001
Application[d]	0.698	0.332–1.468	0.3436
Grade of FV[e]	1.334	0.628–2.836	0.4533
EIS	0.719	0.324–1.594	0.4164
EV (before)	0.697	0.329–1.476	0.3461
EV (after)	0.515	0.301–1.449	0.2952
Child B, C	3.476	1.524–7.930	0.0031
PV[f]	0.999	0.998–1.0	1.747
Gastric vein[f]	1.001	0.999–1.002	0.3974
GP-R[g]	2.794	1.064–7.812	0.0487

EV (before): presence of esophageal varices before B-RTO. EV (after): aggravated or bleeding esophageal varices after B-RTO.
[a] Hazard ratio.
[b] Confidence interval.
[c] Concomitant or treatment history.
[d] Secondary prophylaxis.
[e] Medium-grade FV.
[f] Mean flow volume.
[g] Flow volume ratio of gastric vein to portal trunk ≥1.0.

venograms, though 10 of the 53 gastric veins judged to be SGV by US appeared as posterior gastric veins by retrograde venography. The mean flow volume in the portal trunk with hepatopetal flow was 669.8 ± 344.2 ml/min (n = 73) and that in the gastric vein was 326.5 ± 365.3 ml/min (n = 72). Average GP-R was 0.48 ± 0.58 (n = 70), and 11 patients (15.7%) had GP-R ≥ 1.0. The inter-observer variability was 10.7 ± 5.4% for Doppler measurement results.

3.3. Survival rate and clinical background

Univariate analysis showed that concomitant/history of HCC (hazard ratio, 8.907; 95% CI, 3.606–22.002; $p < 0.0001$), liver function of Child B/C (hazard ratio, 3.476; 95% CI, 1.524–7.930; $p = 0.0031$) and GP-R ≥ 1.0 (hazard ratio 2.794; 95% CI, 1.064–7.812; $p = 0.0487$) were significant factors for poor prognosis (Table 1). Concomitant HCC (hazard ratio, 11.822; 95% CI, 4.15–33.69; $p < 0.0001$) and history of HCC (hazard ratio, 6.951; 95% CI, 2.41–20.02; $p = 0.0003$) were also significant factors for poor prognosis, and the cumulative survival rate in Child A cases with neither concomitant HCC nor history of HCC was 92.3% at 5 years and 83.9% at 7 years. The cumulative survival rate was poorer in patients with concomitant HCC or a history of HCC (47% at 3 years, 9.4% at 5 years, $p < 0.0001$) than in patients without (89.2% at 3 years, 81.9% at 5 years, 67.5% at 7 years), in patients with Child B or C (57.7% at 3 years, 42.1% at 5 years, 28.1% at 7 years, $p = 0.0016$) than patients with Child A (91.8% at 3 years, 71.5% at 5 years, 62.1% at 7 years), and in patients with GP-R ≥ 1.0 (58.9% at 3 years, $p = 0.0485$) than in patients with GP-R < 1.0 (76.3% at 3 years, 62% at 5 years, 49.6% at 7 years; Fig. 2). Multivariate analysis identified the presence of HCC (hazard ratio, 12.486; 95% CI, 4.08–38.216; $p < 0.0001$, liver function of Child B or C (hazard ratio, 3.41; 95% CI, 1.594–7.15; $p = 0.0051$), and GP-R ≥ 1.0 (hazard ratio, 2.701; 95% CI, 1.07–6.15; $p = 0.0221$) as independent factors for poor prognosis (Table 2).

4. Discussion

The therapeutic effects of B-RTO for FV could be concisely summarized as an efficient embolization effect and less recurrence [7,8]. All 81 patients treated by B-RTO in our study remained free of FV recurrence during the clinical course of 1292.6 ± 930.7 (60–3645) days, showing a satisfactory therapeutic effect similar to some previous reports [9–11].

Table 2
Multivariate analysis of potential risk factors for survival in patients with gastric fundal varices.

	HR[a]	95% CI[b]	*p*-Value
HCC[c]	12.5	4.08–38.22	<0.0001
Child B, C	3.41	1.594–7.15	0.0051
GP-R[d]	2.701	1.07–6.15	0.02

[a] Hazard ratio.
[b] Confidence interval.
[c] Concomitant or treatment history..
[d] Flow volume ratio of gastric vein to portal trunk ≥1.0.

As for the longitudinal clinical course aspect, the survival rate after B-RTO of our study was almost the same as that of the previous reports — 76% at 3 years and 54% at 5 years by Ninoi et al. [9], 68% at 5 years by Hiraga et al. [10], and 76% at 3 years, 61% at 5 years and 47% at 8 years by Chikamori et al. [11], and the backgrounds of their patients were also quite similar. These results may suggest that the successful B-RTO procedure provides a stable long-term therapeutic effect irrespective of the facility. Meanwhile, inevitable concern may arise regarding the relationship between the load on portal hemodynamics after B-RTO and survival, because B-RTO embolizes large collateral vessels with a large amount of blood flow [16,17]. To assess the severity of the portal hemodynamics on FV, we proposed here the ratio of mean blood flow volume between gastric vein and portal trunk (GP-R) as an important predictive factor for poor prognosis of cirrhotic patients with FV after B-RTO, though approximately 10% inter-observer variability should be taken into account. In this regard, however, it should be emphasized that this result does not necessarily limit the application of B-RTO for FV, because only 15% of the patients had GP-R equal to or more than 1.0, and patients with GP-R less than 1.0 showed acceptable long-term survival: 76.3% at 3 years, 62% at 5 years, and 49.6% at 7 years. In contrast, embolization of FV in patients with GP-R equal to or more than 1.0 might result in excessive congestion in the portal venous system. In fact, Chikamori et al. reported that application of partial splenic embolization (PSE) was effective for preventing the hemodynamic congestion after B-RTO [18]. However, as PSE is invasive and requires additional radiation exposure, the use of Doppler US before B-RTO might be effective for selecting appropriate candidates that require PSE.

Doppler US could demonstrate neither portal trunk in three patients (3.7%) nor gastric vein in nine patients (11.1%) in our study. As it is reported that microbubble contrast agents have improved the blood flow detection by US [19], application of contrast-enhanced US might increase the detection rate of portal vein and gastric veins. However, presence of microbubble in the vessel influences the measurement results of flow velocity and/or flow volume by pulsed Doppler method [20]. This dilemma remains to be solved in the future.

Significant non-hemodynamic factors regarding prognosis after B-RTO, HCC and liver function reserve were noted in our study, while the cumulative survival rate in Child A cases with neither concomitant HCC nor history of HCC was quite good; 92.3% at 5 years and 83.9% at 7 years. In particular, HCC was the most influential factor, similar to that of previous reports, which showed survival rates at 1, 3 and 5 years after B-RTO to be 83%, 60%, and 18% by Ninoi et al. [9], and 100%, 64%, and 21% by Chikamori et al. [11] in HCC patients. The results of those two reports were somewhat better than ours, the reason possibly related to a different progression of HCC. Concerning this point, the definition of the patients with HCC differed between their studies and ours, as we adopted patients with concomitant HCC or a history of HCC in this study. Based on this, however, we had the interesting result that the treatment history of HCC as well as concomitant HCC was a significant factor for poor prognosis after B-RTO.

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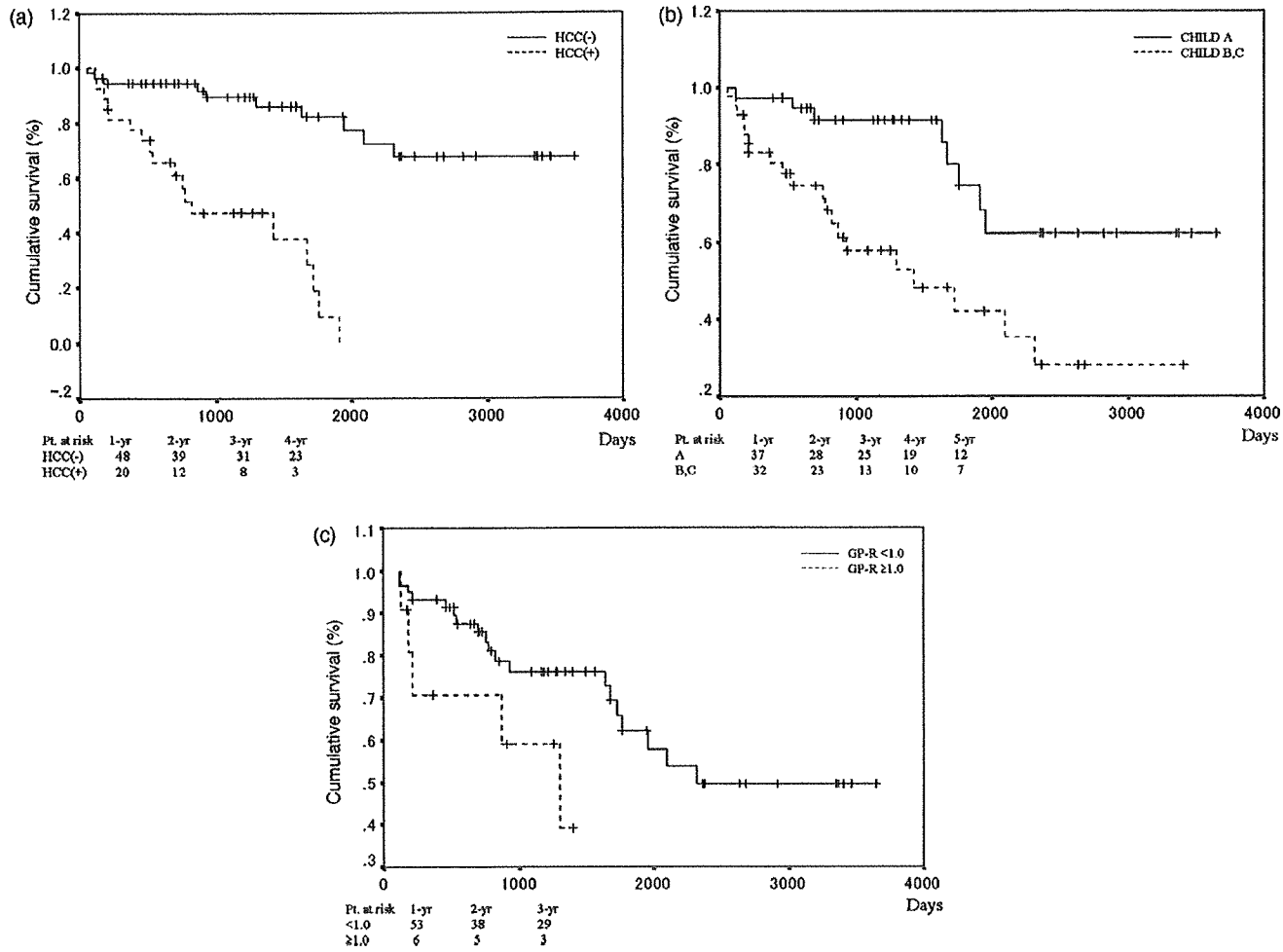

Fig. 2. Cumulative survival rate of cirrhotic patients with FV after B-RTO. (a) Cumulative survival rate was poorer in patients with concomitant HCC or history of HCC (47% at 3 years, 9.4% at 5 years, $p < 0.0001$) than in patients without (89.2% at 3 years, 81.9% at 5 years, 67.5% at 7 years). (b) Cumulative survival rate was poorer in patients with Child B or C (57.7% at 3 years, 42.1% at 5 years, 28.1% at 7 years, $p = 0.0016$) than in patients with Child A (91.8% at 3 years, 71.5% at 5 years, 62.1% at 7 years). (c) Cumulative survival rate was poorer in patients with GP-R ≥ 1.0 (58.9% at 3 years, $p = 0.0485$) than in patients with GP-R < 1.0 (76.3% at 3 years, 62% at 5 years, 49.6% at 7 years).

This needs to be considered in the management of patients after B-RTO.

All B-RTO procedures were safely performed in our study, in spite of some minor short-term complications. However, aggravation of EV was found over 70% patients and 5.1% bled from EV after B-RTO probably due to the increase of portal venous pressure after B-RTO. Although aggravated or bleeding EV was not a significant factor for the prognosis in our study, this may be a careful adverse event of this treatment. Application and optimal timing of prophylactic treatment for EV need to be standardized in the B-RTO cases.

Endoscopic injection therapy such as cyanoacrylate or alcohol injection is effective for attaining hemostasis in bleeding FV. However, such treatment alone does not always provide sufficient long-term protection against FV bleeding, and previous reports have shown a cumulative rebleeding rate from 18% to 33% per year after cyanoacrylate injection [21,22]. Although band ligation is another method for treating bleeding FV and is easy to perform [23,24], the rebleeding rate was reportedly significantly higher with ligation than with endoscopic obturation [25,26]. Thus, the application of endoscopic treatment alone as a curative treatment for FV is controversial, and subsequent additional treatment such as B-RTO may be required to acquire the long-term effect.

A model to predict survival in patients with an end-stage liver disease (MELD) scoring system is known as a predictive factor for prognosis of end-stage patients with liver cirrhosis [27–31]. However, the parameter of portal hypertension is not included in the calculation formula for this scoring. Ripoll et al. reported that inclusion of hepatic vein pressure gradient (HVPG) in MELD score variables for survival would help differentiate between the different types of patients with the same MELD score, because a more accurate prediction of survival was achieved when HVPG was included, with the result of 3% increase in death risk caused by a 1-mm Hg increase in HVPG [32]. Our result may also support the importance of portal hemodynamics to estimate the prognosis in patients with portal hypertension. In respect to this point, a major limitation of our study was the lack of information concerning PVP or HVPG, which may be closely related to the pathophysiology of the patient with portal hypertension. The relationship between these pressure data and prognosis after B-RTO is expected to be resolved in the near future. However, pressure measurement is more or less an invasive procedure that also requires radiation exposure. Although Doppler US could not demonstrate the pressure itself, the pre-treatment measurement of blood flow may allow a non-invasive assessment of the prognosis after B-RTO.

A second limitation may be that the blood flow measurement was done after the endoscopic treatments prior to B-RTO in 24 of the 44 bleeders, as they might have affected the portal hemodynamics. However, such cases are inevitable in clinical practice, and the relationship between portal hemodynamics just before B-RTO

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and survival may be assessed in spite of the presence or absence of preceding treatments. The third was that approximately half of the patients received prophylactic B-RTO because of the patients' strong desire, though there was no randomized controlled trial regarding the efficacy of B-RTO for primary prophylaxis of FV bleeding. Our results would not justify the usage of B-RTO for primary prophylaxis for FV, and the application criteria of this technique needs to be established in further studies. Furthermore, as our study did not include control subject, that is untreated FV patients with HCC, poor liver function and/or GP-R equal to or more than 1.0 which were poor prognostic factor after B-RTO, significance of the application of B-RTO for the patients with these factors should also be investigated as a next challenge.

5. Conclusions

B-RTO provided a long-term therapeutic effect without recurrence for FV in cirrhotic patients. Poor prognosis after B-RTO was related to the development of HCC and severity of liver disease, and measurement of GP-R by Doppler US may be of value to obtain some indication regarding prognosis.

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Hepatitis A virus (HAV) proteinase 3C inhibits HAV IRES-dependent translation and cleaves the polypyrimidine tract-binding protein

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SUMMARY. Hepatitis A virus (HAV) infection is still an important issue worldwide. A distinct set of viruses encode proteins that enhance viral cap-independent translation initiation driven by an internal ribosome entry site (IRES) and suppress cap-dependent host translation. Unlike cytolytic picornaviruses, replication of HAV does not cause host cell shut off, and it has been questioned whether HAV proteins interfere with its own and/or host translation. HAV proteins were coexpressed in Huh-7 cells with reporter genes whose translation was initiated by either cap-dependent or cap-independent mechanisms. Among the proteins tested, HAV proteinase 3C suppressed viral IRES-dependent translation. Furthermore, 3C cleaved the polypyrimidine tract-binding protein (PTB) whose interaction with the HAV IRES had been demonstrated previously. The combined results suggest that 3C-mediated cleavage of PTB might be involved in down-regulation of viral translation to give way to subsequent viral genome replication.

Keywords: 3C protease, hepatitis A virus, IRES, PTB, translation.

INTRODUCTION

The messenger-sense RNA genome of hepatitis A virus (HAV) is about 7500 nucleotides in length and contains a single large open-reading frame (ORF) encoding a polyprotein with the capsid proteins representing the amino-terminal third and the remainder comprising a series of nonstructural proteins required for viral RNA replication: 2B, 2C, 3A, 3B, $3C^{pro}$ (cysteine proteinase responsible for most post-translational cleavage events within the polyprotein) and $3D^{pol}$ (RNA-dependent RNA polymerase, see Fig. 1a, top panel) [1]. In a regulated cascade, the viral polyprotein is cleaved by $3C^{pro}$ into intermediate and mature products that fulfill distinct functions in the viral life cycle. At both ends of the picornaviral genome, the ORF is flanked by highly structured nontranslated regions (5′NTR and 3′NTR). The down-stream part of the 5′NTR presents an internal ribosome entry site (IRES) that allows translation by a cap-independent mechanism [1–3]. Several IRES trans-acting factors (ITAF) have been identified as mediating IRES binding to the ribosome [4]. Whereas glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and La autoantigen suppress HAV IRES activity, the poly(C)-binding protein (PCBP) and the polypyrimidine tract-binding protein (PTB) were found to enhance HAV translation [3,5–9]. PTB, a 57-kDa protein, is a member of the heterogeneous nuclear ribonucleoprotein family that shuttles between the nucleus and cytoplasm [10]. While experimental data have demonstrated PTB binding to polypyrimidine tracts (UCUUU or UCUUC) in picornaviral IRES, the exact cellular functions of PTB are as yet incompletely defined [3,10,11].

Proteolytic cleavage of host proteins is a common mechanism executed by picornaviruses to shut off host cell protein synthesis and to regulate viral protein and RNA synthesis. These two synthetic processes are central in the viral life cycle and mutually exclusive on the same RNA template. As HAV does not shut off host protein synthesis, it seems that HAV cap-independent translation constantly

Abbreviations: GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; HAV, Hepatitis A virus; IRES, internal ribosome entry site; ITAF, IRES trans-acting factors; ORF, open-reading frame; PABP, poly(A)-binding protein; PCBP, poly(C)-binding protein; PTB, polypyrimidine tract-binding protein.

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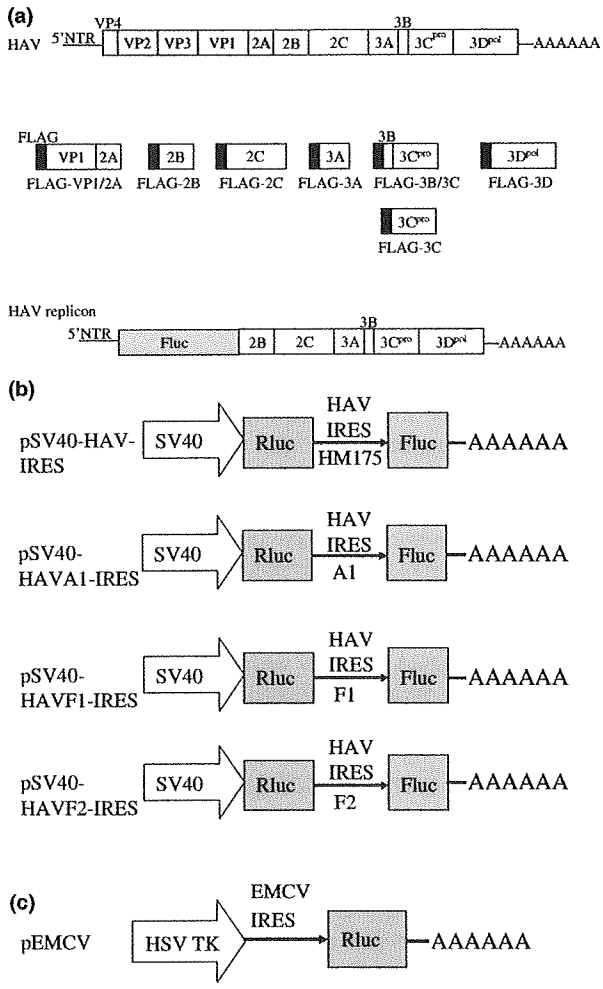

Fig. 1 Schematic representation of the hepatitis A virus (HAV) constructs used in this study. (a) Structures of the HAV genome (upper panel), FLAG-tagged HAV proteins (middle panel; ref. 17) and HAV replicon pT7-18f-luciferase (lower panel; ref. 13). AAAAAA, poly A tail. (b) Structure of the bicistronic plasmids used. pSV40-HAV-internal ribosome entry site (IRES) encodes the Renilla luciferase, the IRES of HAV strain HM175 and the firefly luciferase (Fluc) under the control of the simian virus 40 promoter (SV40) (ref. 16). pSV40-HAVA1-IRES, pSV40-HAVF1-IRES and pSV40-HAVF2-IRES encode IRES elements derived from an acute hepatitis and two fulminant hepatitis cases, respectively. (c) Structure of plasmid pEMCV.

competes with cap-dependent translation of host proteins [1,12]. In this study, we show that HAV $3C^{pro}$ cleaved PTB and suppressed cap-independent translation initiation. The data indicate that the viral proteinase might play an important role in the regulation of HAV IRES-mediated cap-independent translation by targeting noncanonical translation factors.

MATERIALS AND METHODS

Cell lines

Huh-7, a human hepatoma cell line, and its stably transformed derivative Huh-T7 that expresses the T7 RNA polymerase [3] were grown in Dulbecco's modified Eagle medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated foetal bovine serum with or without G418 sulfate (400 μg/mL; Promega, Madison, WI, USA), in addition to penicillin and streptomycin.

Plasmids

pT7-18f-luciferase (LUC), a replication-competent HAV replicon, containing an open-reading frame with the firefly luciferase (Fluc) flanked by the first four amino acids of the HAV polyprotein and by 12 C-terminal amino acids of VP1, followed by the P2 and P3 domains of the HAV polyprotein (HAV strain HM175 18f, GenBank Accession No. M59808), and pT7-18f-LUCmut, a replication-deficient replicon, were described previously [13] (Fig. 1a).

The constructs encoding the simian virus 40 (SV40) promoter-driven *Renilla reniformis* luciferase (Rluc), the IRES derived from the cell culture adapted HAV strain HM175 [14], and Fluc, named pSV40-HAV-IRES, was prepared as described previously [2,15] (Fig. 1b). To investigate the specific effect exerted by the HAV IRES sequences, bicistronic reporter constructs (pSV40-HAVA1-IRES, pSV40-HAVF1-IRES and pSV40-HAVF2-IRES; Kanda *et al.*, manuscript in preparation) were prepared, which included the IRES of clinical specimens. Construction of HAV protein expression plasmids was described previously [16]. Briefly, seven regions of the HAV genome were amplified by reverse transcription-polymerase chain reaction (PCR) with HAV region-specific primers [16]. These regions were HAV VP1-2A, 2B, 2C, 3A, 3BC, 3C and 3D. The amplified products were cloned into pCXN2 (kindly provided by Prof. J. Miyazaki, Osaka University, Japan), a mammalian expression vector with a beta-actin-based CAG promoter and a SV40 origin [17], to generate pCXN2-FLAG-VP1-2A, 2B, 2C, 3A, 3BC, 3C, 3D expressing FLAG-tagged proteins [16] (Fig. 1a). To control for the target specificity, pEMCV, which contains the encephalomyocarditis virus (EMCV) IRES upstream of Rluc, was generated (Fig. 1c). Transient expression of $3C^{pro}$ using vaccinia virus, pGEM-3C, and pEXT7-HAV3C was described before [12].

Transfection and protein analyses

Approximately 60% confluent Huh-7 cells, grown in 6-well culture plates, were transfected with 0.3 μg of the LUC reporter plasmid and 0.1 μg of each HAV protein-expressing plasmid using Effectene transfection reagent (Qiagen, Tokyo, Japan). Forty-eight hours after transfection, cell

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extracts were prepared, and a LUC assay kit (Toyo Ink, Tokyo, Japan) was used according to the manufacturer's instructions. LUC activity was measured in relative light units with a luminometer (AB-2200-R; ATTO, Tokyo, Japan). The assays were adjusted to protein amount and were conducted, on average, in duplicate [18]. To determine cleavage of the host proteins PTB and poly(A)-binding protein (PABP), extracts of transfected cells were analysed for viral antigen and host proteins, as described previously [12]. Viral proteins were identified using anti-FLAG and anti-HAV 3C antibodies. PTB was recognized by the monoclonal antibody BB7 [19].

RESULTS

HAV proteinases 3BC and 3C suppress IRES-dependent translation

Translation of the HAV polyprotein is initiated cap-independently and is driven by an IRES. As a first approach to assess the role of HAV proteins, we examined cap-independent and cap-dependent translation using the bicistronic reporter constructs depicted in Fig. 1. pSV40-HAV-IRES, which contains the IRES of HAV strain HM175 (Fig. 1b), was transfected into Huh-7 together with various expression vectors encoding FLAG-tagged HAV protein (Fig. 1a). The expression of these proteins was confirmed by Western blotting with anti-FLAG antibodies (data not shown and ref. 16). Compared to the control (pCXN2) and to the other HAV proteins tested (VP1-2A, 2B, 2C, 3A and 3D), expression of HAV 3BC or 3C specifically inhibited cap-independent translation initiated by the HAV IRES as determined by the Fluc activity (Fig. 2).

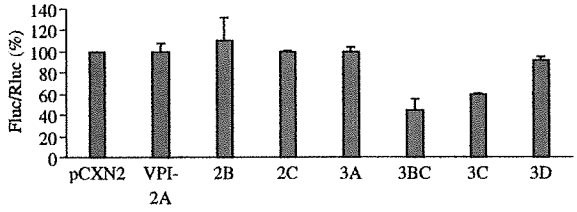

Fig. 2 Effects of hepatitis A virus (HAV) proteins on HAV internal ribosome entry site (IRES)-dependent translation. Relative luciferase activities are indicated (IRES/Cap; firefly luciferase/*Renilla reniformis* luciferase, %). Luciferase activities were determined in three independent experiments. Error bars represent standard errors of the mean.

To corroborate the observed suppression of HAV IRES-independent translation, we next examined the effect of $3C^{pro}$ on translation, which was dependent on HAV IRES elements derived from clinical isolates; IRES A1 was taken from an acute self-limited hepatitis (pSV40-HAVA1-IRES), and F1 and F2 were derived from fulminant HAV infections (pSV40-HAVF1-IRES and pSV40-HAVF2-IRES) (Fig. 3a–c). After coexpression of pSV40-HAVA1-IRES, pSV40-HAVF1-IRES and pSV40-HAVF2-IRES with 3BC or $3C^{pro}$, the Fluc activity was specifically suppressed when compared to the control (pCXN2, Fig. 3a–c). The results confirm our findings shown in Fig. 2 and demonstrate that HAV proteinases 3BC and $3C^{pro}$ suppress HAV IRES-dependent translation. For yet unknown reasons, the negative effect of 3BC was generally more pronounced than that exerted by $3C^{pro}$. However, as $3C^{pro}$ is the prevailing and stable form of the viral proteinase, only this form was used in the subsequent studies.

Translation of the viral polyprotein is the first metabolic step in the viral life cycle and a prerequisite for viral RNA synthesis. It can be assumed that a negative effect on

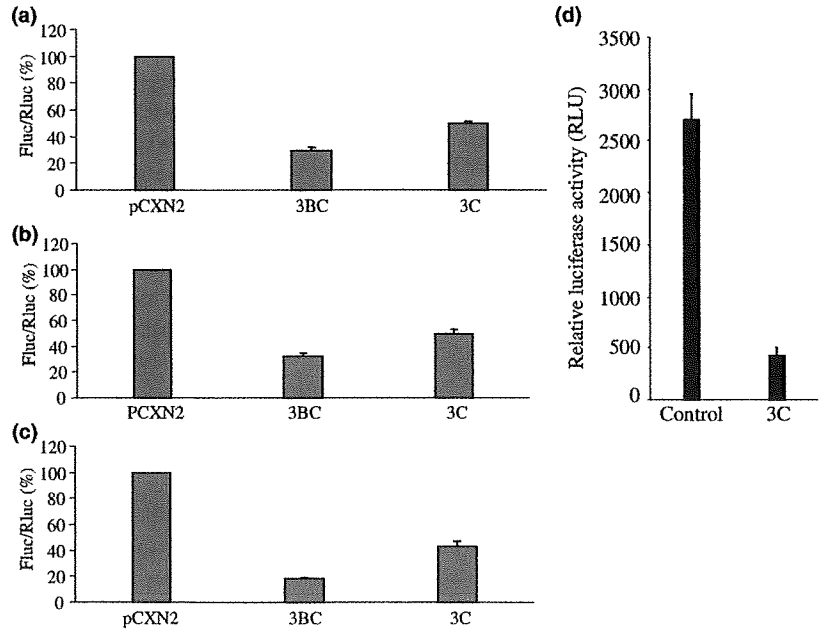

Fig. 3 Effects of hepatitis A virus (HAV) 3BC or $3C^{pro}$ on HAV internal ribosome entry site (IRES)-dependent translation (a–c) and on expression of the HAV replicon [pT7-18f-luciferase (LUC)] (d). HAV was derived from clinical isolates: (a) acute hepatitis; (b) and (c) two forms of fulminant hepatitis. Relative luciferase activities are indicated (IRES/Cap; firefly luciferase/*Renilla reniformis* luciferase, %) (a–c). LUC activities are presented as an average of three independent experiments. Error bars represent standard errors of the mean. RLU, relative light units.

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translation might indirectly cause a reduction in viral genome production. HAV replication was efficiently studied using the viral replicon (see Fig. 1a, lower panel) with Fluc as reporter gene in place of the viral structural proteins [13]. The reporter gene activity is directly proportional to viral RNA synthesis. To investigate whether 3C-mediated suppression of translation affects genome replication, we cotransfected HAV replicon RNA with the 3C-expression or control vector into Huh-T7 cells (Fig. 3d). HAV replicon replication was monitored by reporter assay 72 h post-transfection. Compared to the replication-deficient replicon (pT7-18f-LUCmut), the reporter activity at this point was derived from newly synthesized viral genomes and therefore represents viral genome synthesis [13]. Compared to the control, HAV replication was significantly suppressed in the presence of excess 3C, indicating that 3C-mediated inhibition of translation restrained HAV genome replication in human hepatoma cells.

To assess the specificity of the inhibitory effect exerted by HAV proteinase $3C^{pro}$, translation initiated at the EMCV IRES was compared with the HAV IRES. For this, HAV $3C^{pro}$ was coexpressed with pEMCV (Fig. 1c), and the Rluc activity of the cell extracts collected 48 h post-transfection was determined. Compared to the HAV IRES tested in parallel experiments, the EMCV IRES activity was similar in the presence and absence of coexpressed HAV $3C^{pro}$ [118 ± 29 (%)]. Combined and in light of the results described in the following, these findings suggest that an essential ITAF was cleaved by HAV $3C^{pro}$. As shown in the following, HAV $3C^{pro}$ partially cleaved PTB, whose active role in picornaviral IRES-dependent translation has been demonstrated previously [6,8]. As EMCV IRES translation was unaffected by HAV $3C^{pro}$, PTB is not an essential ITAF for this IRES, confirming an earlier report [20]. Intriguingly, an excess of PTB even suppressed EMCV IRES-driven translation [21]. The abundance and distribution of PTB varies significantly among cell types [6,8]. Large amounts of PTB were found in the cytoplasmic fraction of Huh-7 cells that were used in our studies. Based on these observations, the results reported here suggest that HAV $3C^{pro}$ reduced the cytoplasmic levels of intact PTB to such a degree that only the activity of the HAV IRES was affected, but not that of the EMCV IRES.

$3C^{pro}$ cleaves PTB

It has been reported that the HAV IRES is associated with La autoantigen, GAPDH, PTB, PABP and PCBP [6,8,12–14]. The latter two proteins were cleaved by HAV $3C^{pro}$ [12,13]. Furthermore, it was shown that PTB is cleaved by polioviral $3C^{pro}$ and that PTB fragments inhibit polioviral IRES-dependent translation [22]. To assess whether the observed suppression of HAV IRES translation might be because of 3C-mediated cleavage of PTB, we tested the levels of endogenous PTB after transient expression of $3C^{pro}$ in Huh-7 cells. As GAPDH was found to suppress HAV IRES translation and to antagonize the enhancing effect of PTB [8], GAPDH levels were tested in parallel. As control for the proteolytic activity of $3C^{pro}$ *in vivo*, cleavage of the poly(A)-binding protein was also analysed. Recombinant $3C^{pro}$ was identified by immunoblot with anti-3C (Fig. 4, left panel) [12,13], and PABP was partially cleaved as demonstrated earlier (Fig. 4, middle panel). Whereas the levels of GAPDH were unchanged (data not shown), a PTB cleavage product of approximately 45 kDa and a slightly faster migrating polypeptide were clearly detectable when HAV $3C^{pro}$ was expressed (Fig. 4, right panel). The extent of host protein cleavage significantly depended on the amount of 3C expressed (compare lanes 1 and 3). Specific PTB cleavage was also observed when the extracts used in Fig. 2 were tested (not shown). Moreover, PTB of Huh-7 cells, the rabbit

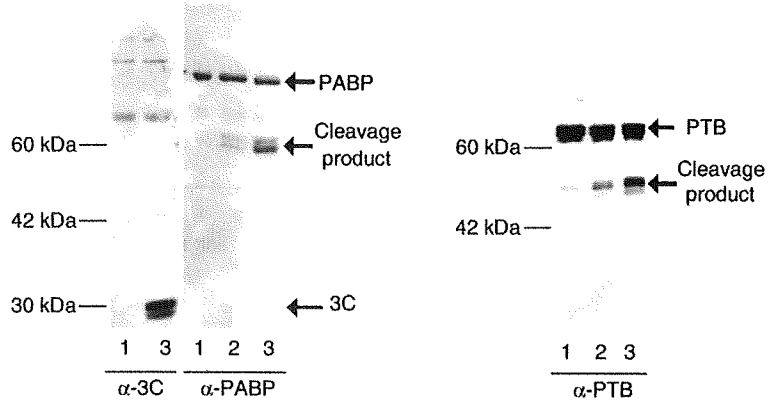

Fig. 4 Hepatitis A virus (HAV) $3C^{pro}$ cleaves the polypyrimidine tract-binding protein (PTB). Huh-7 cells were transfected with pGEM (lanes 1), pGEM-3C (lanes 2) and pEXT7-HAV3C (lanes 3) and infected with vaccinia virus T7. Cell lysates were collected 24 h post-transfection and subjected to immunoblot using anti-3C, anti-poly(A)-binding protein and anti- PTB. As less $3C^{pro}$ was produced by pGEM-3C in comparison with pEXT7-HAV3C, cleavage of host proteins was more pronounced in lanes 3 when compared to lanes2.

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reticulocyte lysate, and recombinant PTB produced in *E. coli* was substrate to cleavage-mediated *in vitro* by purified recombinant HAV $3C^{pro}$ (data not shown). Combined with its well-documented translation enhancing effect and binding specificity to stem-loop IIIa of the HAV IRES [6–8], the results strongly suggest that the inhibitory effect of HAV $3C^{pro}$ on HAV IRES translation is because of proteolytic cleavage of PTB.

DISCUSSION

The expression level of the viral proteinase was found to substantially affect the detection of PTB cleavage products (see Fig. 4). Neither in HAV-infected cells nor in cells expressing the HAV replicon were PTB cleavage fragments detectable (not shown). A similar discrepancy was observed for PCBP, another ITAF that is essential for picornaviral translation and the molecular switching to RNA replication [9,23,24]. Whereas PCBP cleavage by recombinant HAV $3C^{pro}$ was clearly shown, PCBP-processing products were not apparent in extracts of HAV-infected cells [9]. Combined, our findings on HAV-3C-mediated cleavage of PCBP and PTB suggest that because of the protracted replication of HAV, very low quantities of $3C^{pro}$ are present in infected cells and cleavage of these host proteins is not discernible. This is in clear contrast to poliovirus whose highly efficient replication resulted in obvious cleavage of both PCBP and PTB [22,24].

The functional domains of PTB are four RNA recognition motifs that all bind short pyrimidine-rich sequences. By binding to different sites on the same RNA molecule, PTB can lead to distinctive RNA restructuring. Such conformational changes are thought to be critical in enabling the ribosomal recruitment in IRES-driven translation initiation. Our constructs do not include the 1–138 nt region of 5′NTR, in which a pyrimidine-rich-tract exists. PTB interacts with stem-loop IIIa of the HAV IRES that contains short polypyrimidine tracts [7]. These binding sites can be bridged by a single PTB molecule, which is an arrangement that favours a role for PTB as an RNA chaperone. It is likely that PTB stabilizes or alters the IRES structure to enable the recruitment of the ribosome and to position it correctly at the start codon.

For poliovirus, direct evidence was provided that PTB cleavage products inhibited IRES-dependent translation [22]. As outlined by the authors, it is possible that PTB fragments may interfere with the binding of intact PTB to poliovirus IRES or that cleaved PTB may no longer function as translational activator that facilitates the recruitment of translational machinery to the IRES element. Although not directly assessed here, it is assumed that suppression of HAV IRES translation is induced by similar mechanism(s). Moreover, in poliovirus-infected Hela cells, PTB cleavage fragments are redistributed to the cytoplasm [22]. As abundant quantities of PTB are present in the cytoplasm of Huh-7 cells used in our study [8], PTB redistribution might not be essential for the effect of PTB cleavage on HAV translation. Yet it is attractive to speculate that the PTB fragment(s) might have altered RNA-binding specificity. For poliovirus IRES translation, an attractive model was put forward for the participation of PTB and PCBP in the molecular switch from viral translation to RNA replication [22]. Supposedly, after viral 3C-mediated cleavage, PTB and PCBP lose their enhancing function. Once IRES translation is stalled, replication of the viral RNA consequently is turned on. Taken together with our earlier observations [9], HAV translation is inhibited indirectly by its own product, $3C^{pro}$, through the proteolytic cleavage of PCBP and PTB.

The HAV 3B and 3C proteins are 23 and 219 amino acids in length, respectively [25]. The 3B moiety was found to be essential for the 3AB interaction with 3CD [26]. It seems that 3BC was more suppressive than 3C in cap-independent translation. Further studies will reveal the 3B function in the interaction with PTB and 3BC. In conclusion, HAV proteinase 3C cleaved PTB and suppressed HAV IRES-dependent translation.

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STATEMENT OF PERSONAL INTERESTS

None of the authors have personal interests relevant to this research to declare.

DECLARATION OF FUNDING INTERESTS

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