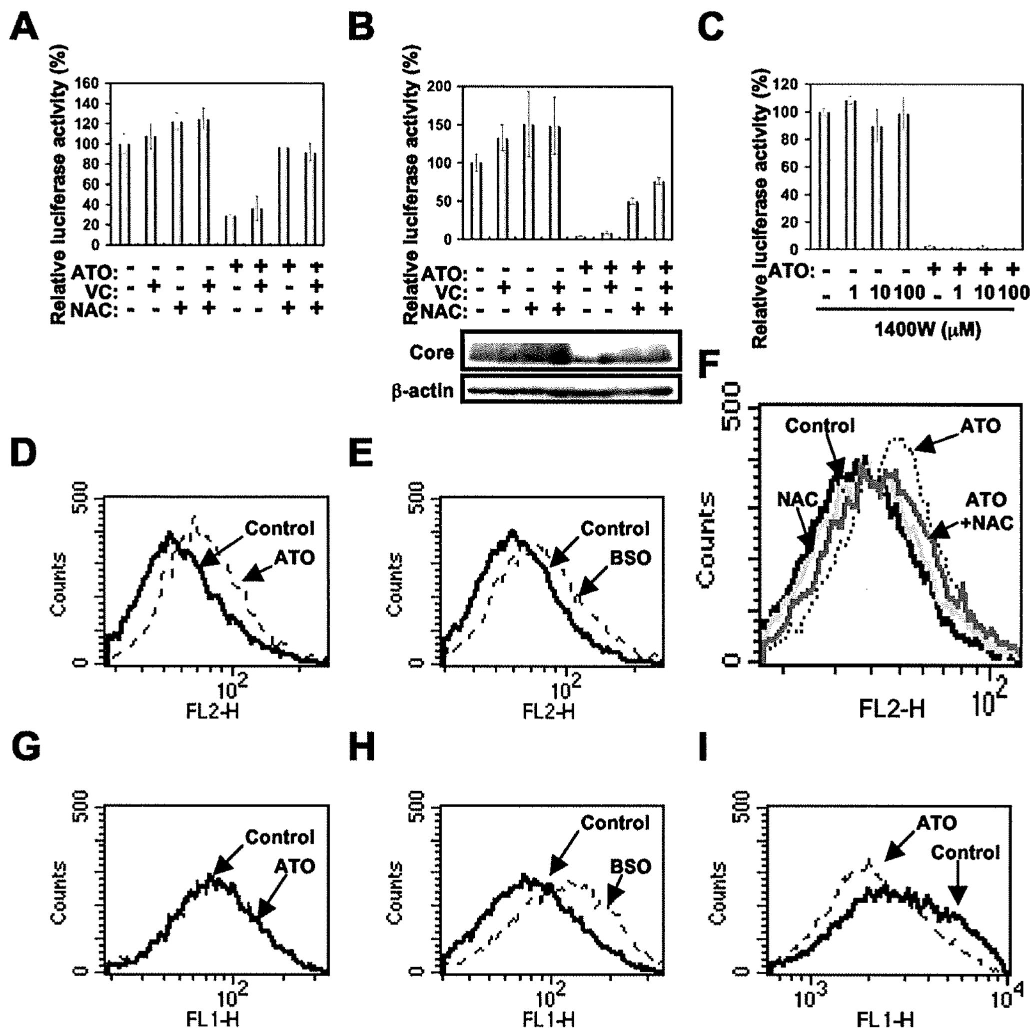

FIG. 6. The anti-HCV activity of ATO is associated with the glutathione redox system and oxidative stress. (A and B) The anti-HCV activity of ATO is eliminated by treatment with the antioxidant NAC. OR6 cells were treated with 1 μM ATO alone and in combination with 100 μM vitamin C (VC), with or without 10 mM NAC, for 24 h (A) or 72 h (B). The replication level of HCV RNA was monitored by the RL assay. The relative RL activity is shown. The results shown are means from three independent experiments; error bars indicate standard deviations. The results of Western blot analysis of cellular lysates with anti-HCV core or anti-β-actin antibody in OR6 cells at 72 h after the treatment with 1 μM ATO alone and in combination with 100 μM VC, with or without 10 mM NAC, are also shown. (C) Effect of combination treatment with ATO and the iNOS inhibitor 1400W on HCV RNA replication. OR6 cells were treated with 1 μM ATO alone and in combination with 1400W at the indicated concentrations for 72 h. The replication level of HCV RNA was monitored by the RL assay as described for panels A and B. (D and E) Effect of ATO on production of a ROS, O_2^-, in O cells. O cells were treated with 1 μM ATO (D) or 2 μM BSO (E) for 24 h. The intracellular O_2^- level was measured by flow cytometry using DHE as described in Materials and Methods. (F) Inhibition of ATO-dependent O_2^- induction by NAC. O cells were treated with either 1 μM ATO or 10 mM NAC alone and in combination with 10 mM NAC for 24 h. (G and H) Effect of ATO on production of a ROS, H_2O_2, in O cells. O cells were treated with 1 μM ATO (G) or 2 μM BSO (H) for 24 h. The intracellular H_2O_2 level was measured by flow cytometry using DCF as described in Materials and Methods. (I) Effect of ATO on the intracellular glutathione level in O cells. O cells were treated with 1 μM ATO for 72 h. The intracellular glutathione level was measured by flow cytometry using CellTracker Green CMFDA as described in Materials and Methods.

tively activate STAT3 and NF-κB, which are associated with HCV pathogenesis (19, 34, 36, 43, 49, 59, 60, 67). In fact, oxidative stress has been shown to trigger STAT3 tyrosine phosphorylation and nuclear translocation, which correlate with the activation of STAT3, leading to its DNA-binding activity (9). In contrast, ATO inhibited the STAT3 tyrosine phosphorylation through direct interaction with JAK kinase, thereby suppressing the transcriptional activity of STAT3 (12, 62). Importantly, STAT3 activation has been reported to be associated with HCV RNA replication (59, 69). The STAT3 Tyr705 dominant negative mutant has been shown to inhibit HCV RNA replication, suggesting that STAT3 positively regulates HCV replication (59). In contrast, others have reported that STAT3 induces anti-HCV activity (69). In this study, we analyzed the potential effect of ATO treatment on a set of stress-signaling events, including the NF-κB, AP-1, and STAT3 pathways, since ATO is known to modulate various signaling pathways. However, at 1 μM, which exerted an anti-HCV activity, the respective signaling pathways were not affected, arguing that the anti-HCV activity is independent of these

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pathways (Fig. 5). In this regard, these stress-signaling pathways have been reported to be constitutively activated in HCV core- or NS5A-expressing cells (19, 36, 49, 59, 60, 67). In addition, previous studies demonstrated that ATO modulates the NF-κB, AP-1, and STAT3 pathways at higher concentrations (NF-κB, >10 μM; AP-1, >30 μM; STAT3, >4 μM). Therefore, we may have only observed the marginal effect of ATO in this study (Fig. 5). On the other hand, the HCV core or NS3 protein as well as HCV infection induces NO, leading to induction of double-stranded DNA breaks and accumulation of mutations of cellular genes (35). However, the iNOS inhibitor 1400W could not suppress HCV RNA replication and the anti-HCV activity of ATO, indicating that NO is not associated with the anti-HCV activity or with HCV replication (Fig. 6C).

It has been indicated that oxidative damage plays an important role in the effect of ATO (38). ROS generated in response to ATO exposure lead to accumulation of intracellular H_2O_2. Glutathione peroxidase and catalase are key enzymes regulating the levels of ROS and protecting cells from ATO-induced damage (26). However, the gastrointestinal glutathione peroxidase was drastically downregulated in cells harboring HCV replicons, which are rendered more susceptible to oxidative stress (39). The glutathione redox system has been implicated in the cellular defense system (14, 20). Glutathione, a major antioxidant in cells, is a tripeptide synthesized from cysteine, glutamic acid, and glycine, and it can scavenge superoxide anion free radicals. ATO has been shown to bind to the sulfhydryl group of glutathione and deplete the intracellular glutathione, resulting in enhancement of the sensitivity to oxidative damage (20, 33). Conversely, the antioxidant NAC is readily taken up by cells and serves as a precursor to elevate intracellular glutathione (53). In fact, ATO-induced apoptosis has been shown to be inhibited by NAC (11, 14, 21, 28). In this study, we have demonstrated that the anti-HCV activity of ATO was completely eliminated by treatment with NAC for 24 h (Fig. 6A). In addition, we found that ATO increased intracellular O_2^- but not H_2O_2 and depleted the intracellular glutathione in HCV RNA-replicating cells (Fig. 6D to I). Importantly, NAC diminished the ATO-dependent O_2^- induction (Fig. 6F). This finding could strengthen the link between ATO-dependent oxidative stress and anti-HCV activity. Similarly, Wen et al. reported an increase in ROS and enhanced susceptibility to glutathione depletion in the HCV core-expressing HepG2 cells (61). Accordingly, ROS have been shown to significantly suppress RNA replication in HCV replicon-harboring cells treated with H_2O_2 (13). In addition, HCV replication has been shown to be inhibited by lipid peroxidation of arachidonate, and this peroxidation could be blocked by lipid-soluble antioxidants such as vitamin E (23). Conversely, several antioxidants, such as vitamin C, vitamin E, and NAC, enhanced HCV replication in the present study (Fig. 6A and B) (65). Thus, we suggest that ATO inhibited HCV RNA replication by modulating the glutathione redox system and oxidative stress. In contrast to the above findings with HCV, NAC has been shown to suppress HIV-1 replication by preventing the activation of HIV-1 long terminal repeat transcription by NF-κB, suggesting a correlation between a decrease in glutathione levels and activation of HIV-1 replication (46, 53, 54). In this context, ATO has shown opposite effects on HIV-1 and HCV replication, stimulating the former and inhibiting the latter. Considering all of these results together, ATO can be regarded as a useful, novel anti-HCV reagent. In addition, the host redox system may be critical for HCV replication and may represent a pivotal target for the clinical treatment of patients with chronic hepatitis C.

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Mini-review

Cancer stem cells in hepatocellular carcinoma: Recent progress and perspective

Tetsuhiro Chiba [a,d], Akihide Kamiya [b], Osamu Yokosuka [c], Atsushi Iwama [a,d,*]

[a] Department of Cellular and Molecular Medicine, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan
[b] Division of Stem Cell Therapy, Center for Stem Cell and Regenerative Medicine, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
[c] Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan
[d] JST, CREST, Sanbancho, Chiyoda-ku, Tokyo 102-0075, Japan

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ABSTRACT

Although the "cancer stem cell (CSC)" hypothesis was first proposed roughly 50 years ago, recent progress in stem cell biology and technologies has successfully achieved the identification of CSCs in a variety of cancers. CSCs are defined as a minor population which possesses a prominent ability to generate new tumors that faithfully reproduce the phenotype of original tumors in xenotransplant assays. Additionally, CSCs are able to self-renew and generate differentiated progenies to organize a hierarchical cell system in a similar fashion to normal stem cells. Although not all types of cancer follow the CSC theory, it provides an attractive cellular mechanism to account for the therapeutic resistance and recurrence of the disease. A minor population with CSC properties has been detected in a number of established hepatocellular carcinoma (HCC) cell lines and extensive analyses characterizing the CSC system in primary HCC samples are now ongoing. Considering that HCC has high rates of recurrence and mortality, novel therapeutic approaches are urgently required. Although the clinical relevance of CSCs remains elusive, deep understanding of the cellular organization of HCC may allow us to develop therapies targeting specific cell types such as CSCs.

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1. Introduction

Cancer is usually unicellular in origin [1,2], although cancer cells constitute functional heterogeneity in a wide variety of cancers [3]. Classically, two general theories have been debated in terms of carcinogenesis [4]. The stochastic model indicates that a few cells which acquired proliferative potential via stochastic events are responsible for tumor formation. The alternative hypothesis, namely, the hierarchical model, postulates that a small subset of cells generates a hierarchical organization containing varied downstream descendants, proliferates extensively, and initiates tumors at high frequency.

Stem cells, generally defined by an ability to differentiate into multiple cell lineages and self-renew, contribute to not only organogenesis but also regeneration in response to the injury of tissues and organs [5]. Recent advancements in stem cell biology have allowed for the identification and characterization of stem cells in a variety of tissues and organs. On the other hand, it has been documented that solid tumors such as breast cancer and colon cancer contain a small subset of tumorigenic cells which can generate new tumors in xenograft transplantation [6,7]. This minor population of cells, termed cancer stem cells (CSCs) or tumor initiating cells (TICs), possesses stem cell-like properties and contributes to a hierarchical structure containing varied progenies in a similar fashion to normal stem cells. Successful detection of CSCs in a wide variety of cancers supports the hierarchical carcinogenesis theory.

* Corresponding author. Address: Department of Cellular and Molecular Medicine, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. Tel.: +81 43 2262189; fax: +81 43 2262191.
E-mail address: aiwama@faculty.chiba-u.jp (A. Iwama).

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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 $RT1A1^-OX18^{low}ICAM\text{-}1^+$, $Liv2^+$, E-cadherin$^+$, $Dlk1^+$ or $c\text{-}Met^+CD49f^{+/low}c\text{-}Kit^-CD45^-TER119^-$ cells in the developing rodent liver utilizing cell sorting technology and clonal colony assays [18–23]. Interestingly, a portion of $c\text{-}Met^+CD49f^{+/low}c\text{-}Kit^-CD45^-TER119^-$ 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 $c\text{-}Met^+CD49f^{+/low}c\text{-}Kit^-CD45^-TER119^-$ 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			
$RTlA1^-OX18^{low}ICAM\text{-}l^+$	Rat (ED13)		[18]
$Liv2^+$	Mouse (ED10.5–12.5)	20–60	[19]
E-cadherin$^+$	Mouse (ED 12.5)		[20]
$Dlk\text{-}1^+$	Mouse (ED 14.5)	Nearly 10	[21]
$Dlk\text{-}1^+$	Rat (ED 14)	5.7 ± 0.9	[22]
$c\text{-}Met^+CD49f^{+/low}c\text{-}Kit^-CD45^-TER119^-$	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]
$Thy\text{-}1^+$	Rat (AAF/PH or AAF/CC14)		[28]
$Sca\text{-}1^+CD34^+CD45^+$	Mouse (DDC diet)		[29]
EpCAM$^+$	Rat (AAF/PH)		[32]

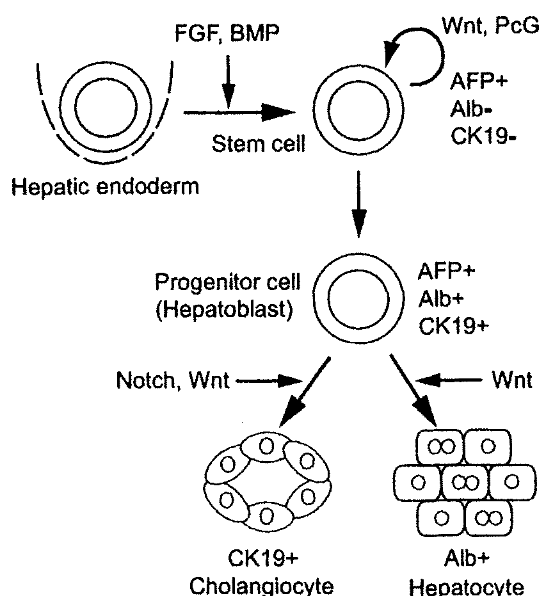

Fig. 1. Hepatogenesis and the regulatory machinery involved. In the developing liver, both fibroblast growth factor (FGF) signal from the cardiac mesoderm and bone morphogenetic protein (BMP) signal from the septum transversum mesenchyme cells promote the commitment of ventral endoderm to the liver bud. 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,

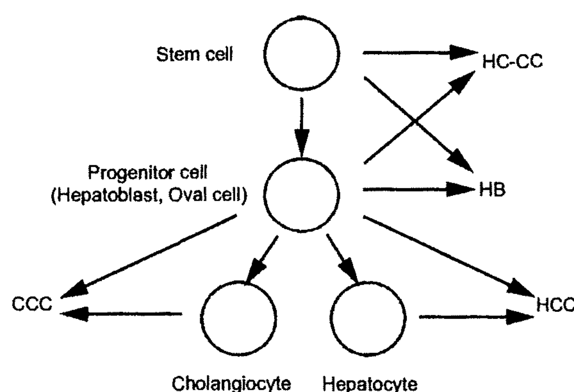

Fig. 2. Proposed model for the cellular origin of liver malignancies. Liver tumors develop from more heterogeneous cells as expected. Combined hepatocellular and cholangiocellular carcinoma (HC-CC) and hepatoblastoma (HB) are thought to be derived from primitive hepatoblasts and pluripotent stem cells. 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

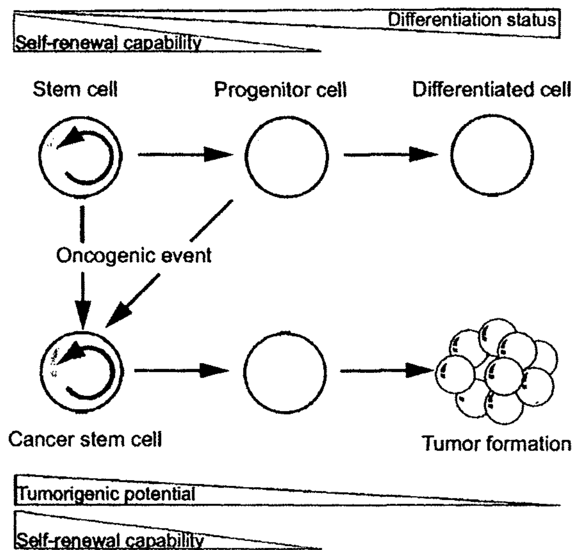

Fig. 3. Organization of cancer stem cell systems. Normal stem cells with self-renewal capability generate progenitor cells and subsequently various terminally differentiated cells. Oncogenic mutations in normal stem/progenitor cells or even in differentiated cells enhance or endow the self-renewal capability. 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

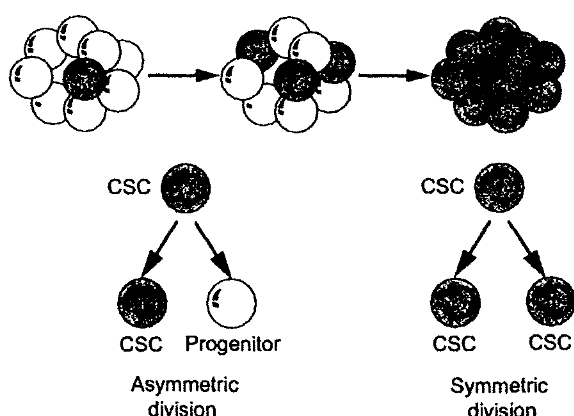

Fig. 4. Hierarchical diversity in cancer cells. Not all types of tumors have a cancer stem cell (CSC) system and CSCs do not necessarily represent a minor subpopulation of cancer cells. Asymmetrical self-renewal division of CSCs is crucial for the maintenance of a hierarchical organization in the tumor. 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.

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Association between mutations in the core region of hepatitis C virus genotype 1 and hepatocellular carcinoma development

Shingo Nakamoto, Fumio Imazeki*, Kenichi Fukai, Keiichi Fujiwara, Makoto Arai, Tatsuo Kanda, Yutaka Yonemitsu, Osamu Yokosuka

Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-Ward, Chiba City, Chiba 260-8670, Japan

Background & Aims: To determine whether amino acid mutations in the core region of hepatitis C virus (HCV) genotype 1 are associated with response to interferon (IFN) therapy and development of hepatocellular carcinoma (HCC).

Methods: We followed up 361 patients (median duration, 121 months), and IFN monotherapy was administered to 275 (76%) [sustained virological response (SVR) rate, 26.5%]. Using pretreatment sera, mutations at core residues 70 and 91 were analyzed [double wild (DW)-type amino acid pattern: arginine, residue 70; leucine, residue 91].

Results: A low aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio and low HCV load were independently associated with SVR, but core mutations were not. During follow-up, 12 of 81 (14.8%) patients with the DW-type pattern and 52 of 216 (24.1%) patients with non-DW-type pattern developed HCC ($p = 0.06$, Breslow–Gehan–Wilcoxon test). Multivariate analysis with the Cox proportional-hazards model revealed the following independent risk factors for HCC: male gender [$p < 0.0001$; risk ratio (RR), 3.97], older age ($p < 0.05$; RR, 2.08), advanced fibrosis ($p < 0.0001$; RR, 5.75), absence of SVR ($p < 0.01$; RR, 10.0), high AST level ($p < 0.01$; RR, 2.08), high AST/ALT ratio ($p < 0.01$; RR, 2.21), and non-DW-type pattern ($p < 0.05$; RR, 1.96). In patients with F0–F2 fibrosis at entry, non-DW-type was likely to lead to cirrhosis ($p = 0.051$).

Conclusions: In HCV genotype 1 patients, HCC risk could be predicted by studying core mutations, response to IFN, and host factors like age, gender, and liver fibrosis.

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Cancer

Introduction

Hepatitis C virus (HCV) infection is a global health problem and the number of chronic carriers worldwide is estimated at 170 million [1]. HCV causes chronic hepatitis, which may progress to liver cirrhosis and hepatocellular carcinoma (HCC); the speed of disease progression, though, varies among patients [2,3]. Age, gender, steatosis, liver fibrosis, and response to interferon (IFN) therapy are reported to be associated with disease progression and HCC development [4–7]. HCV has six major genotypes, of which genotype 1 is most common in Japan and reported to be associated with increased severity and progression of chronic liver disease [8,9]. HCV contributes to HCC by directly modulating the pathways promoting the malignant transformation of hepatocytes [10–13]. Studies on transgenic mice revealed that the HCV core protein has oncogenic potential [14], but other studies yielded conflicting results [15,16]. Recently, mutations at amino acids 70 and 91 in the core region were shown to predict virological response to therapy with IFN plus ribavirin and also HCC development [17–19]. However, few studies support these results, and hence, the clinical impact of core mutations on HCC development is still unclear. In order to determine the viral factors associated with HCC development, we performed a retrospective cohort study on 361 patients with chronic liver disease caused by HCV genotype 1 infection and analyzed the amino acids present at core residues 70 and 91. Additionally, we evaluated whether these mutations were associated with IFN treatment, cirrhosis development, or host factors like age and gender.

Patients and methods

Study population

We enrolled 361 consecutive HCV genotype 1-infected patients who had undergone liver biopsy between August 1986 and June 1998 at Chiba University Hospital. At the enrollment time, the absence of HCC was proven by abdominal ultrasonography (US), computed tomography (CT), or magnetic resonance imaging (MRI). All the patients tested positive for anti-HCV antibody, determined by second-generation enzyme-linked immunosorbent assay. Patients with chronic hepatitis B, autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, Wilson disease, or alcoholic liver disease were excluded, as were patients with a history of alcoholism, drug abuse, or IFN therapy. Written informed consent was obtained from all patients before performing liver biopsy.

Keywords: Hepatitis C virus; Core region; Hepatocellular carcinoma; Interferon; Sustained virological response.

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* Corresponding author. Tel.: +81 43 226 2083; fax: +81 43 226 2088.
E-mail address: imazekif@faculty.chiba-u.jp (F. Imazeki).

Abbreviations: HCV, hepatitis C virus; IFN, interferon; HCC, hepatocellular carcinoma; SVR, sustained virological response; DW-type, double wild-type; RR, risk ratio; AST, aspartate aminotransferase; ALT, alanine aminotransferase; US, ultrasonography; CT, computed tomography; MRI, magnetic resonance imaging; PCR, polymerase chain reaction; OR, odds ratio.

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Table 1. Baseline characteristics of 361 hepatitis C (HCV) genotype 1-infected patients according to hepatocellular carcinoma (HCC) development.

Patients	n = 361	HCC development		
		(+), n = 82	(−), n = 279	p value
Gender (male/female)	219/142	56/26	163/116	0.1
Age (years)	50.5 ± 12.2	56.8 ± 7.1	48.6 ± 12.7	<0.0001
BMI (kg/m²)	23.1 ± 2.9	23.1 ± 2.8	23.1 ± 3.3	0.82
Staging of fibrosis (F0-1/F2/F3/F4)	197/59/52/53	13/18/23/28	184/41/29/25	<0.0001
IFN treatment and response				
SVR/non-SVR/non-IFN	73/202/86	4/55/23	69/147/63	0.0004
Laboratory data				
AST (IU/L)	87 ± 62	109 ± 59	80 ± 61	0.0001
ALT (IU/L)	125 ± 93	139 ± 80	121 ± 96	0.13
AST/ALT	0.75 ± 0.26	0.84 ± 0.28	0.73 ± 0.25	0.0003
Platelets (10⁴/mm³)	17.7 ± 6.7	13.0 ± 3.3	18.2 ± 6.9	<0.0001
Albumin (g/dL)	4.2 ± 0.36	4.1 ± 0.39	4.3 ± 0.35	<0.0001
Total bilirubin (mg/dL)	0.8 ± 0.6	0.9 ± 0.3	0.8 ± 0.6	0.39
Core protein (pg/mL)	201 ± 245	283 ± 273	177 ± 231	0.001
Amino acid pattern				
70 Wild/non-wild/ND	168/129/64	32/32/18	136/97/46	0.23*
91 Wild/non-wild/ND	139/158/64	28/36/18	111/122/46	0.58*
DW/non-DW/ND	81/216/64	12/52/18	69/164/46	0.08*

BMI, body mass index; DW, double wild (arginine at residue 70 and leucine at residue 91 in the core region); ND, not detected; ND cases were excluded.

The clinical backgrounds of the patients are shown in Table 1. The study population was predominantly male (59% men), and the mean age of the patients was 50.5 ± 12.2 years, with 15% patients having liver cirrhosis.

Laboratory examination

Serum samples were obtained and stored at −30 °C until analysis. We assumed that genotype 1 corresponds to group 1 when determining the HCV RNA genotypes by serologic grouping of serum antibodies [20]. The serum HCV load of the patients was determined at the time of liver biopsy, using the HCV core protein detection kit (Eiken Chemical, Tokyo, Japan; detection limit, 8 pg/mL) [21].

Histopathological examination

Percutaneous liver biopsy was performed, and specimens were histopathologically assessed as described previously [22]. According to the criteria of Desmet et al. [23], the staging of fibrosis was defined as F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis), and F4 (cirrhosis).

Core nucleotide sequences

HCV RNA was extracted from the serum samples obtained at the time of liver biopsy, and it was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Nucleic acids were amplified by PCR with the

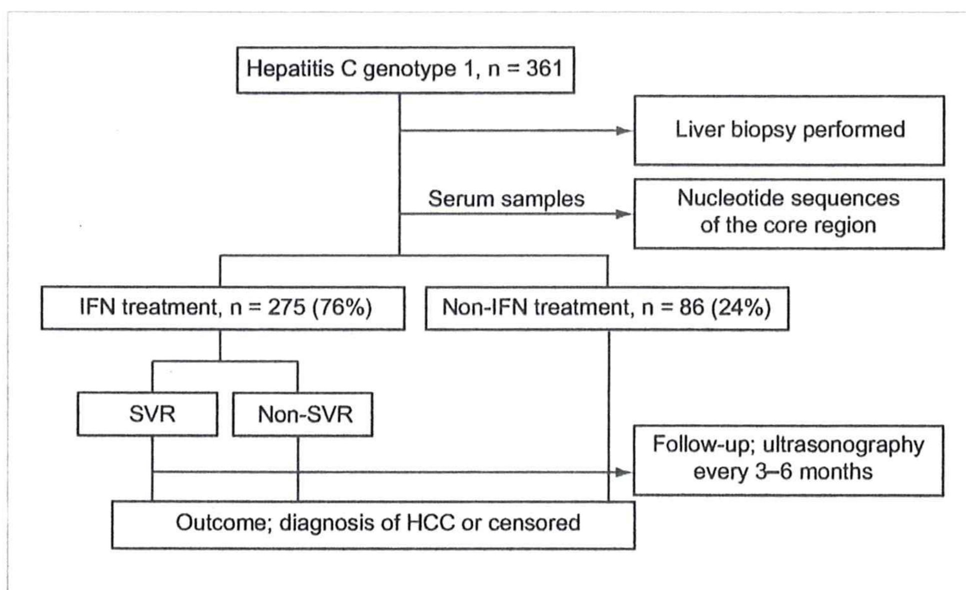

Fig. 1. Clinical courses after enrollment and the evaluation methods. IFN, interferon; SVR, sustained virological response; HCC, hepatocellular carcinoma. [This figure appears in colour on the web.]

Cancer

HotStart Taq Master Mix kit (Qiagen, Hilden, Germany) and primers that have been previously described [24]. Polymerase chain reaction (PCR) was initiated with a denaturation step at 95 °C for 15 min, followed by 45 cycles at 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 3 min, and subsequent extension for 7 min. PCR products were resolved by agarose gel electrophoresis, purified using the QIA quick PCR purification kit (Qiagen), and directly sequenced using a Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Tokyo, Japan). The sequences were determined using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

As described previously, the double wild-type (DW-type) amino acid pattern was defined as the presence of arginine at residue 70 (wild-type) and leucine at residue 91 (wild-type) [19].

IFN treatment

Depending on whether IFN was administered, the patients were divided into the IFN (76%) and non-IFN groups (24%) (Fig. 1). Patients who received IFN monotherapy during follow-up were divided into two subgroups: the sustained virological response (SVR) group, including patients who tested negative for HCV RNA at 24 weeks after completion of therapy, and non-SVR group (Fig. 1). Of the 275 patients in the IFN group, 73 (26.5%) achieved SVR.

Follow-up and diagnosis of cirrhosis and HCC

Clinical assessments were performed at least once every month during IFN treatment and every 3–6 months after the treatment. During follow-up, abdominal US was performed every 3–6 months to determine whether HCC had developed (Fig. 1). If necessary, additional procedures like CT, MRI, abdominal angiography, and US-guided tumor biopsy were performed to confirm HCC development. We also evaluated whether cirrhosis had developed in non-cirrhotic patients (F0–F2 stage). Cirrhosis was diagnosed according to the criteria of cirrhosis as described previously [25,26]. The follow-up period was the duration from the initial liver biopsy to HCC diagnosis or the last follow-up visit. For non-cirrhotic patients, this was the duration from the start point to cirrhosis diagnosis.

Statistical analysis

The χ^2 test was used to compare categorical variables, and Student's t test to compare continuous variables related to background characteristics among groups. Continuous variables were expressed as mean ± standard deviation. The cumulative incidence of HCC and cirrhosis was calculated using the Kaplan-Meier method and evaluated using the Breslow–Gehan–Wilcoxon test. Multivariate analysis was performed using the Cox proportional-hazards model or multiple logistic regression analysis. The Cochran–Armitage trend test was used for analyzing the association between the prevalence of mutation and subject age. Statistical significance was defined as $p < 0.05$.

Results

Cumulative HCC incidence

During follow-up (median duration, 121 months; range, 8–257 months), 82 (22.7%) patients developed HCC [HCC group; 13 of 197 (6.6%) from F0–F1, 18 of 59 (30.5%) from F2, 23 of 52 (44.2%) from F3, and 28 of 53 (52.8%) from F4 stage at entry] and 279 (77.3%) did not (non-HCC group). The cumulative HCC incidence at 5, 10, and 15 years of follow-up was 9.5%, 22.9%, and 30.9%, respectively.

Core nucleotide sequences

The core nucleotide sequence was determined for 297 of 361 (82.3%) patients. In the entire patient group, the proportions of DW-type and non-DW-type patterns were 22% and 60%, respectively (Table 1).

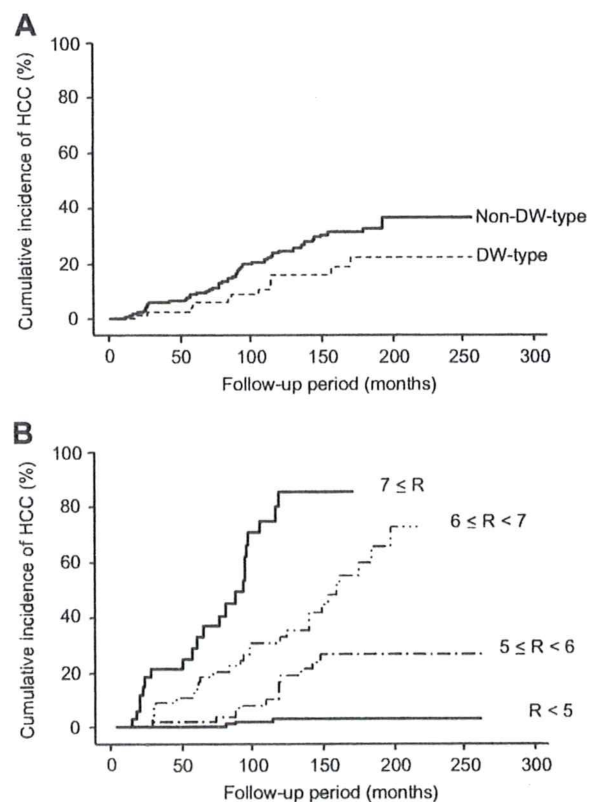

Fig. 2. Cumulative incidence of hepatocellular carcinoma (HCC) in hepatitis C genotype 1-infected patients. (A) Comparison between patients with double wild-type (DW-type: arginine, residue 70; leucine, residue 91) ($n = 81$) and non-DW-type ($n = 216$) amino acids in the core region ($p = 0.06$). (B) Comparison based on risk score (R) calculated using independent variables for HCC risk ($p < 0.0001$).

The core nucleotide sequence could not be determined for 64 patients because their samples showed significantly lower levels of the HCV core protein than those obtained from the 297 patients in whom the core sequence could be detected (119 vs. 217 pg/mL; $p = 0.0083$). There was no significant difference between the other variables shown in Table 1.

Cumulative HCC incidence according to core amino acid mutations

During follow-up, 12 of 81 (14.8%) patients with the DW-type pattern and 52 of 216 (24.1%) patients with the non-DW-type pattern developed HCC. Cumulative HCC incidence was 6.8% and 11% at 5 years, 19.1% and 27.7% at 10 years, and 26.6% and 38% at 15 years in the DW-type and non-DW-type groups, respectively. Cumulative HCC incidence in the DW-type group tended to be lower than that in the non-DW-type group ($p = 0.06$; Fig. 2A).

Predictive factors associated with HCC development

Potential predictive factors associated with HCC development are shown in Table 1. Univariate analysis revealed 10 parameters correlating with HCC development (Table 1). Multivariate analy-

Table 2. Factors associated with hepatocellular carcinoma development in hepatitis C genotype 1-infected patients, identified by multivariate analysis using the Cox proportional-hazards model.

Factor*	Category	Risk ratio (95% CI)	p value
Gender	Male	3.97 (2.05–7.63)	<0.0001
	Female	1.0	
Age (years)	⩾50	2.08 (1.01–4.33)	0.049
	<50	1.0	
Staging of fibrosis	⩾2	5.75 (2.68–12.35)	<0.0001
	<2	1.0	
IFN treatment and response	Absence of SVR	10.0 (2.29–43.48)	0.002
	SVR	1.0	
AST (IU/L)	>90	2.08 (1.20–3.62)	0.009
	⩽90	1.0	
AST/ALT	⩾0.8	2.21 (1.24–3.97)	0.007
	<0.8	1.0	
Amino acid pattern	Non-DW	1.96 (1.02–3.76)	0.04
	DW	1.0	

CI, confidence intervals; DW, double wild (arginine at residue 70 and leucine at residue 91 in the core region).
*Significant factors are shown.

sis with the Cox proportional-hazards model showed that the following seven independent parameters were significantly associated with HCC development: male gender ($p < 0.0001$), age ⩾ 50 years ($p = 0.049$), fibrosis ⩾ F2 ($p < 0.0001$), absence of SVR ($p = 0.002$), aspartate aminotransferase (AST) level > 90 IU/L ($p = 0.009$), AST/alanine aminotransferase (ALT) ratio ⩾ 0.8 ($p < 0.007$), and non-DW-type pattern in the core region ($p = 0.04$) (Table 2).

Prediction of HCC development based on risk score

Using the predictive variables from the previous step (Table 2), the risk score (R) for HCC development was calculated from the beta coefficients derived from the Cox proportional-hazards model as follows: $R = 0.671 \times$ (non-DW-type) + $2.307 \times$ (absence of SVR) + $0.733 \times$ (AST > 90 IU/L) + $0.733 \times$ (age ⩾ 50 years) + $1.752 \times$ (staging of fibrosis ⩾ 2) + $1.378 \times$ (male) + $0.795 \times$ (AST/ALT ⩾ 0.8) (each variable: yes = 1, no = 0). Fig. 2B shows the cumulative HCC incidence of four subgroups categorized by risk score, and the RR of each group is shown in Table 3. The cumulative HCC incidence increased with the risk score: from highest to lowest it was 84.7%, 35.1%, 18.5%, and 3.0% at 10 years.

Cumulative HCC incidence according to IFN treatment and response

During follow-up, 4 (5.5%) patients in the SVR, 55 (27.2%) in the non-SVR, and 23 (26.7%) in the non-IFN groups developed HCC; cumulative HCC incidence was 0%, 11.3%, and 13.2%, respectively, at 5 years; 7.8%, 25.6%, and 27.3%, respectively, at 10 years; and 7.8%, 36.5%, and 35.5%, respectively, at 15 years. Moreover, cumulative HCC incidence was significantly lower in the SVR group than other groups ($p < 0.001$; Fig. 3).

Table 3. Relative risk of HCC development based on risk score, using the Cox proportional-hazards model.

Score (R)	Risk ratio (95% CI)	p value
$R < 5$	1	
$5 \leqslant R < 6$	9.22 (2.60–32.7)	0.0006
$6 \leqslant R < 7$	26.9 (8.15–89.0)	<0.0001
$7 \leqslant R$	88.3 (25.8–302)	<0.0001

CI, confidence intervals.

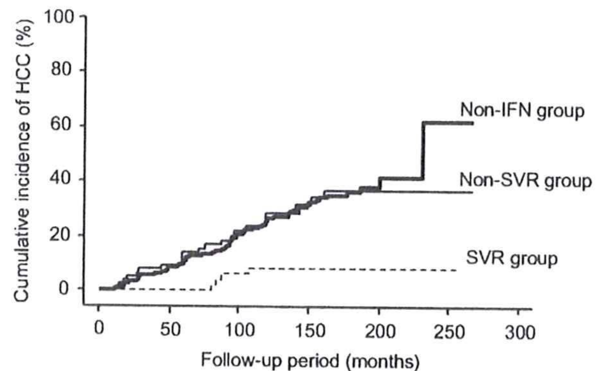

Fig. 3. Cumulative incidence of hepatocellular carcinoma (HCC). Comparison between the sustained virological response (SVR) ($n = 73$), non-SVR ($n = 202$), and non-interferon (IFN) ($n = 86$) groups ($p = 0.002$).

Analysis of SVR-associated factors

Compared to those in the non-IFN group, patients in the IFN group were younger (49 years vs. 54 years, $p = 0.003$), had higher aminotransferase levels (AST, 93 vs. 68 IU/L, $p = 0.001$; ALT, 137 vs. 87 IU/L, $p < 0.0001$) and lower core protein levels (183 vs. 263 pg/mL, $p = 0.01$). Table 4 shows baseline characteristics of patients according to interferon response. Univariate analysis revealed six SVR-associated parameters, whereas multiple logistic regression analysis revealed two independent significant predictors of SVR: AST/ALT ratio of <0.8 [$p = 0.005$; odds ratio (OR), 3.09; 95% confidence interval (CI), 1.40–6.82] and core protein level of <200 pg/mL [$p < 0.0001$; OR, 70.94; 95% CI, 9.56–526.2]. However, both univariate ($p = 0.64$) and multivariate analyses (data not shown) showed that the DW-type pattern in the core region was not associated with SVR.

Table 4. Baseline characteristics of patients according to interferon response.

Nature of the Regime	SVR n = 73	Non-SVR n = 202	p value
Gender (Male/Female)	47/26	126/76	0.76
Age (years)	46.6 ± 13.3	50.5 ± 11.5	0.02
BMI (kg/m^2)	22.7 ± 2.8	23.2 ± 3.0	0.24
Staging of fibrosis: (F0-1/F2/F3/F4)	45/12/9/7	104/34/34/30	0.42
Laboratory data			
AST (IU/L)	79 ± 56	97 ± 69	0.048
ALT (IU/L)	132 ± 92	139±100	0.60
AST/ALT	0.65 ± 0.22	0.75 ± 0.27	0.003
Platelets (10^4/mm^3)	18.6 ± 6.7	16.7 ± 6.1	0.03
Albumin (g/dL)	4.3 ± 0.3	4.2 ± 0.4	0.06
Total bilirubin (mg/dL)	0.7 ± 0.4	0.8 ± 0.4	0.02
Core protein (pg/mL)	31 ± 50	234 ± 226	<0.0001
Amino acid pattern			
70 Wild/Non-wild/ND	35/21/17	89/74/39	0.30
91 Wild/Non-wild/ND	24/32/17	76/87/39	0.62
DW/Non-DW/ND	14/42/17	46/117/39	0.64

BMI, body mass index; DW, double wild (arginine at residue 70 and leucine at residue 91 in the core region); ND, not detected.

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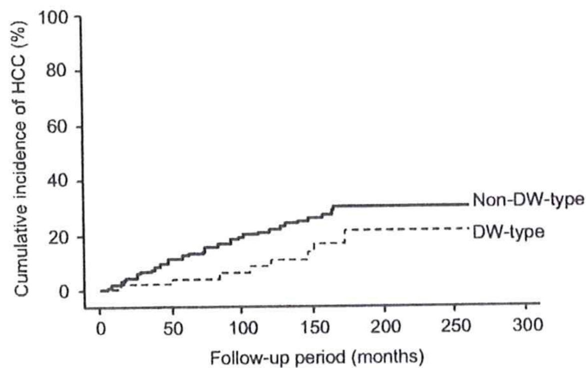

Fig. 4. Cumulative incidence of cirrhosis in non-cirrhotic patients (F0–F2). Comparison between patients with double wild-type (DW-type: arginine, residue 70; leucine, residue 91) ($n = 81$) and non-DW-type ($n = 216$) amino acids in the core region ($p = 0.051$).

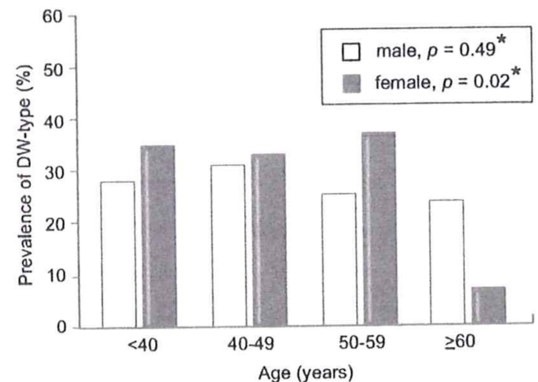

Fig. 5. Prevalence of double wild-type (DW-type: arginine, residue 70; leucine, residue 91) amino acids in the hepatitis C core region according to age and gender. *By the Cochran-Armitage trend test.

Cumulative cirrhosis incidence for non-cirrhotic patients (F0–F2)

Of the 256 non-cirrhotic patients (197 from F0–F1, 59 from F2), 50 (19.5%) developed cirrhosis (cirrhosis group) and 206 (80.5%) did not (non-cirrhosis group). The cumulative cirrhosis incidence at 5, 10, and 15 years of follow-up was 9.7%, 18.2%, and 26.4%, respectively. The HCC incidence was higher in the cirrhosis group [23/50 (46%)] than the non-cirrhosis group [8/206 (3.9%); $p < 0.0001$]. In the entire population, 71 of 82 (86.6%) patients who developed HCC had underlying cirrhosis and 11 (13.4%) did not, when HCC was detected ($p < 0.0001$).

Cumulative cirrhosis incidence according to the amino acid pattern in the core region for F0–F2 patients

The cumulative cirrhosis incidence tended to be higher in the non-DW-type group than the DW-type group (11.9% and 3.6% at 5 years, 21.5% and 10.4% at 10 years, and 29.7% and 20.7% at 15 years of follow-up, respectively; $p = 0.051$; Fig. 4).

Analysis of factors associated with cirrhosis development in F0–F2 patients

We analyzed the factors associated with cirrhosis development in patients with F0–F2 fibrosis at enrollment. Univariate analysis revealed nine parameters correlating with cirrhosis development: male gender ($p = 0.04$), older age ($p < 0.0001$), advanced fibrosis ($p < 0.0001$), absence of SVR ($p < 0.0001$), high AST level ($p < 0.0001$), high ALT level ($p = 0.01$), high AST/ALT ratio ($p = 0.001$), low platelet count ($p = 0.0009$), and high core protein level ($p = 0.02$). Multivariate analysis, including analysis of the amino acid pattern in the core region with the Cox proportional-hazards model, showed that the following three independent parameters were significantly associated with cirrhosis development: male gender ($p = 0.004$), fibrosis = F2 ($p = 0.004$), and absence of SVR ($p = 0.02$). Meanwhile, the presence of the non-DW-type pattern in the core region tended to lead to cirrhosis development (RR, 2.13; 95% CI, 0.93–4.91; $p = 0.07$).

Analysis of factors associated with mutations at core residues 70 and 91

Eighty-one patients with the DW-type pattern at core residues 70 and 91, who were at low risk for HCC, tended to be younger than the 216 patients with the non-DW-type pattern, who were at high risk for HCC (48.4 ± 11.8 years vs. 51.1 ± 11.8 years, respectively; $p = 0.08$). Separate analysis of men and women (Fig. 5) showed that the DW-type pattern was rare in women aged 60 years or above ($p = 0.02$).

Consistent with these results, HCC incidence was the same in men and women aged 60 or above (19% vs. 10% at 5 years and 32% vs. 38% at 10 years of follow-up, respectively; $p = 0.89$); however, in patients aged less than 60 years, HCC incidence was lower in women than in men (4% vs. 11% at 5 years and 15% vs. 22% at 10 years of follow-up, respectively; $p = 0.03$).

Discussion

Male gender, older age, advanced-stage fibrosis, and no IFN treatment are reported as important predictors of HCC development in chronic hepatitis C patients [4–7]. Viral factors associated with HCC development were also reported [27–29]. Several studies showed that mutations in the core protein are associated with HCC among HCV genotype 1b-infected patients, but the results varied between studies [18,30,31]. Consistent with a report by Akuta et al. [18], we showed that the presence of the non-DW-type pattern at core residues 70 and 91 is an independent risk factor for HCC development. Akuta et al. [18] studied 313 chronic hepatitis C patients who received IFN therapy (101 were excluded), and found that non-DW-type was an independent risk factor for HCC development (RR, 5.92; 95% CI, 1.58–22.2; $p = 0.008$) by using the Cox proportional-hazards model, and its correlation with HCC risk was stronger than that found in our study (RR, 1.96; 95% CI, 1.02–3.76; $p = 0.04$). We analyzed cirrhotic patients (14.7% of total population), most of whom developed HCC, and also non-cirrhotic patients, and found that the non-DW-type was still an independent risk factor for HCC development (RR, 2.90; 95% CI, 1.11–7.61; $p = 0.03$). Furthermore, we

found that the non-DW-type in patients with F0–F2 fibrosis was likely to lead to cirrhosis, diagnosed by US ($p = 0.051$). Moreover, the non-DW-type in patients with F0–F3 fibrosis was significantly associated with cirrhosis development ($p = 0.007$, data not shown). These results suggest that the non-DW-type may affect HCC development by accelerating cirrhosis development; however, prospective studies of histological findings are needed to confirm this.

It is unclear why the amino acids at residues 70 and 91 affect HCC development. The core protein cooperates with the Ras oncogene and transforms primary rat embryo fibroblasts into the tumorigenic phenotype [10]. The HCV core protein (residues 25–91) also interacts with the heterogeneous nuclear ribonucleoprotein K, which stimulates the c-myc promoter, downstream of the Wnt/beta-catenin signal [11]. Pavio et al. reported that the HCV core (residues 59–126, residues at 70 and 91 were non-wild-type) interacts with Smad3 and inhibits the TGF-beta pathway, important in apoptosis [12]. Mutations in the clustering variable regions (residues 39–76) are often seen in HCC patients [30], and mutations in the *N*-myristoylation sites (e.g., residue 91) in the core region, are associated with growth control and virus replication [31]. Delhem et al. have shown that the core protein with non-wild-type amino acids at residues 70 and 91 obtained from a HCC patient binds and activates PKR, which might cause carcinogenesis [13]. It was reported that the presence of a non-wild-type amino acid at residue 91 enhances internal initiation of HCV protein synthesis, leading to the expression of a core isoform, which may interact with viral and cellular components [32]. These results suggest that residues 70 and 91 themselves or via interactions with adjacent amino acids may be involved in HCC development; however, further studies are needed to evaluate the effect of core mutations on HCC development.

The presence of the DW-type pattern in the core region is also reportedly a predictor of the virological response to therapy with peginterferon and ribavirin [19]. With this therapy, an SVR of approximately 50% could be achieved by HCV genotype 1-infected patients having high viral load. We found the absence of an SVR and the non-DW-type pattern to be predictors of HCC development; however, the non-DW-type pattern was not a predictor of the absence of an SVR. This may be partly because we used IFN monotherapy without ribavirin, with which the SVR rate (26.5% in our study) was lower than that with peginterferon plus ribavirin [33,34]. Therefore, we believe that combination therapy, rather than IFN monotherapy, would more efficiently eradicate HCV with the DW-type pattern in the core region; however, further studies are required to test this hypothesis. Our current focus is on a prospective study to examine the association between core mutations and the outcome of combination treatment with peginterferon plus ribavirin.

Our study revealed that the DW-type pattern, associated with a low HCC risk, was rare in women aged 60 years or above. This may explain why HCC incidence in women was as high as that in men. The underlying mechanisms by which age or gender influence core-region mutations are unknown. In previous studies, a mutation at residue 70 was correlated with virological response to therapy with IFN plus ribavirin [17] and with AFP levels [35] in HCV genotype 1b-infected patients without HCC. Further follow-up studies must examine whether a mutation occurs in the wild-type amino acid.

We investigated two specific amino acid mutations in the HCV core region by direct sequencing. The HCV core sequence can be easily amplified using PCR because of its conservative nature and analysis of only two amino acid positions is timesaving; therefore, this method might be feasible for identifying predictive markers for HCC. A specific PCR method for detecting these mutations was reported [36]. Furthermore, we developed a rapid and sensitive real-time PCR method for quantitatively detecting these mutations [37]. We hope this method can be used to detect HCV sequences in case of a low viral load, and believe that it will be more useful for predicting HCC.

In conclusion, HCC risk could be predicted by studying mutations in the HCV core region, response to IFN, and host factors like age, gender, and liver fibrosis in HCV genotype 1-infected patients. These mutations might be involved in an oncogenic mechanism leading to HCC development in chronic HCV patients.

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