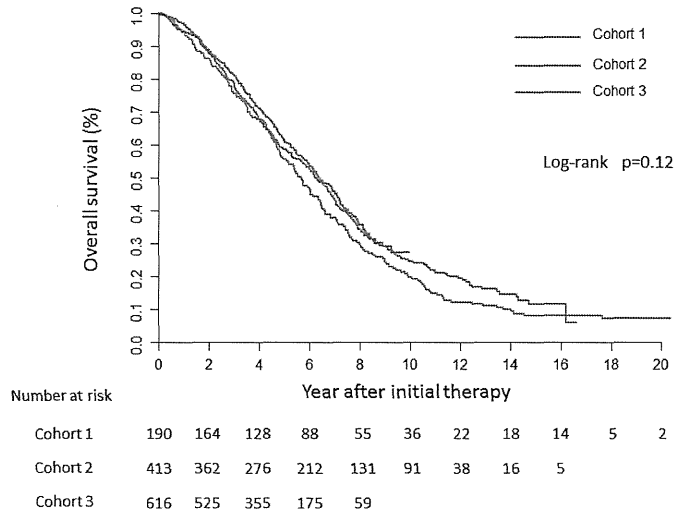

Figure 2. Overall survival in C-HCC patients according to the cohort. The survival rates at 1, 3, 5, and 7 years were 94.2, 75.8, 55.6 and 37.9% in cohort 1, 94.7, 78.6, 58.8 and 43.2% in cohort 2, and 97.1, 81.2, 61.1, and 45.2% in cohort 3, respectively.

Specific delivery of microRNA93 into HBV-replicating hepatocytes downregulates protein expression of liver cancer susceptible gene MICA

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ABSTRACT

Chronic hepatitis B virus (HBV) infection is a major cause of hepatocellular carcinoma (HCC). To date, the lack of efficient in vitro systems supporting HBV infection and replication has been a major limitation of HBV research. Although primary human hepatocytes support the complete HBV life cycle, their limited availability and difficulties with gene transduction remain problematic. Here, we used human primary hepatocytes isolated from humanized chimeric uPA/SCID mice as efficient sources. These hepatocytes supported HBV replication in vitro. Based on analyses of mRNA and microRNA (miRNA) expression levels in HBV-infected hepatocytes, miRNA93 was significantly downregulated during HBV infection. MiRNA93 is critical for regulating the expression levels of MICA protein, which is a determinant for HBV-induced HCC susceptibility. Exogenous addition of miRNA93 in HBV-infected hepatocytes using bionanocapsules consisted of HBV envelope L proteins restored MICA protein expression levels in the supernatant. These results suggest that the rescued suppression of soluble MICA protein levels by miRNA93 targeted to HBV-infected hepatocytes using bionanocapsules may be useful for the prevention of HBV-induced HCC by altering deregulated miRNA93 expression.

INTRODUCTION

Hepatitis B virus (HBV) infection is a major global health problem, and more than 350 million people globally are chronic carriers of the virus [1]. A significant number of these carriers suffer from either liver failure or hepatocellular carcinoma (HCC) during the late stages of the disease [2]. In fact, chronic infection with HBV is responsible for 60% of HCC cases in Asia and Africa and at least 20% those in Europe, Japan, and the United States [3].

While nucleoside and nucleotide analogs have been applied in the attempts to suppress HBV replication [4, 5], complete elimination of HBV (including cccDNA) remains difficult [6, 7], and an increased understanding of HBV replication and pathogenesis at the molecular level is essential for clinical management of chronic HBV infection. However, the lack of appropriate cell culture systems supporting stable and efficient HBV infection has been a major limitation. Although transient transfection or viral transfer of HBV genes or genomes are used in the study of specific steps of the HBV cell cycle [8-12], they do not accurately reflect the biology of HBV infection and replication. Thus, humanized mice are used for hepatitis virus research [13-18]. Although these mice are useful, immune deficient, chimeric mice are difficult to handle

and maintain. Therefore, a more convenient *in vitro* system is required for HBV research.

Primary human hepatocytes can support the complete HBV life cycle *in vitro* [7, 19], but a major drawback is their limited availability. To overcome difficulties regarding availability, we used chimeric mice as sources of primary human hepatocytes, which grow robustly during the establishment of chimeric mice, due to continual liver damage induced by urokinase-type plasminogen activator (uPA) [14, 15].

Another shortcoming of utilizing primary human hepatocytes is their difficulty with gene transduction due to the low transfection efficiency of their primary cell-like nature. Efficient gene delivery methods will significantly improve studies on primary hepatocytes for HBV replication. In addition, cell-specific targeting is required for efficient drug delivery *in vivo*. As a specific gene delivery method to liver-derived cells, bionanocapsules (BNCs) consisted of HBV envelope L particles have been tested for the selective delivery of genes, drugs, or siRNAs into liver-derived cells [20, 21]. Because these BNCs are consisted of HBV L protein, they may be applicable for drug delivery to HBV-infected primary human hepatocytes.

MicroRNAs (miRNAs) are endogenous ~22-nucleotide RNAs that mediate important gene-regulatory events by base-pairing with mRNAs and activating their repression [22]. We previously reported that modifying the expression of miRNAs in liver cells can efficiently regulate the expression levels of the MHC class I polypeptide-related sequence A (MICA) protein [23], which we previously identified as a crucial factor for the susceptibility of hepatitis virus-induced HCC and possibly hepatitis virus clearance [24, 25]. While emerging evidence suggests that miRNAs play crucial roles in chronic HBV infection [26], the comprehensive changes in miRNA expression levels induced by HBV infection in human hepatocytes or in alternative systems reflecting HBV-infected hepatocytes have not been explored.

In this study, we infected primary human hepatocytes isolated from chimeric mice with HBV and identified the transcripts and miRNAs whose expression levels changed. We explored whether BNCs carrying synthesized miRNAs could successfully deliver miRNAs into primary hepatocytes and rescue the modulated miRNA expression due to HBV replication. We found that BNCs carrying synthesized miRNA93 could efficiently restore deregulated soluble MICA protein levels in the supernatant of HBV-replicating primary hepatocytes. These results suggest that miRNA93 delivery into HBV-replicating hepatocytes using BNC methods may enhance HBV immune clearance or suppress HCC by altering miRNA93 levels in HBV-infected cells.

RESULTS

Changes in expression levels of transcripts and miRNAs during HBV replication in human primary hepatocytes

We examined changes in transcript and miRNA expression levels during HBV infection and replication in hepatocytes. Primary human hepatocytes were used for maintainng HBV replication *in vitro*. We first isolated primary hepatocytes from humanized chimeric mice. To examine the infectivity of HBV into the primary hepatocytes *in vitro*, HBsAg and HBV-DNA levels in the cell culture supernatant were measured after the cells were infected with approximately 1.5×10^7 copies of HBV/well in a 24-well plate at day 0. Although both HBsAg and HBV-DNA levels transiently decreased at approximately day 3, levels of both started to increase and were maintained until after day 23 post-infection (Figure 1a and b). These results suggested that human primary hepatocytes isolated from chimeric mice can efficiently support HBV replication *in vitro*, which can be used as an efficient *in vitro* HBV replication system.

To examine comprehensive changes in mRNA and miRNA expression levels in HBV-infected hepatocytes, cells at day 7 post-infection were collected and subjected to cDNA as well as miRNA microarrays. Among 24,460 genes examined, 65 were significantly upregulated by more than 4-fold, and 29 were downregulated to less than 25% (Supplementary Table 1 and 2); however, more than 800 total genes were upregulated or downregulated if the thresholds of the changes were set at 2-fold and 1.5-fold, respectively (Figure 1c; complete datasets have been deposited as GEO accession number: GSE55928). Among the upregulated genes, those associated with the cytochrome family, such as CYP2A7, CYP2C8, CYP2A6, CYP3A4, changed significantly, which was consistent with previous reports [27, 28]. However, few inflammatory cytokines or genes associated with cell growth changed significantly. Based on these results, host factors related to innate immunity may not sense HBV (at least under these replicating conditions), suggesting that this system may mimic the status of hepatitis B patients before seroconversion, in whom inflammation seldom occurs regardless of the high viral load.

Regarding changes in miRNA expression levels during HBV replication, among 2,019 mature miRNAs, 35 were upregulated and 14 downregulated by an increase or decrease of more than two-fold (Figure 1d and Supplementary Tables 3 and 4; complete datasets have been deposited as GEO accession number: GSE55929). Among these miRNAs, miR93-5p was significantly downregulated during HBV replication by more than 50%. Since miRNA93 regulates the expression levels

of the MICA protein [23, 29], which is involved in the susceptibility to hepatocellular carcinoma in chronic hepatitis patients [24, 25], we focused on this miRNA in further analyses.

Efficient delivery of miRNAs into liver cell lines using bionanocapsules

Efficient delivery methods of genes or compounds into targeted tissues or cells are essential to translate the *in vitro* results into clinical settings. Here, we utilized BNCs [21, 30, 31], which were originally developed to deliver genes and drugs with high efficiency and specificity to human liver-derived cells, as an efficient delivery method for miRNAs into human liver cells, including primary hepatocytes. Since BNCs are composed of HBV L proteins, the distribution of these BNCs and infected HBV should be similar. To confirm the efficiency of delivery of miRNAs into liver-derived cells by BNCs, we delivered BNCs carrying let-7g or miRNA93 to the human hepatocellular carcinoma cell lines, Huh7 and HepG2 cells, and to human normal hepatocytes immortalized with SV40 large T antigen, Fa2N4 cells [28]. The day after delivery of the BNCs, cells were collected and subjected to Northern blotting against let-7g, miRNA93, and U6, the loading control, and the results showed successful delivery of miRNAs into all cell lines tested (Figure 2a). The biological function of the delivered miRNAs was confirmed using luciferase-based reporters, which measured let-7g and miRNA93 functions [23]. Huh7 and Hep3B cells transfected with reporter constructs were delivered with let-7g or miRNA93 using BNCs, followed by a luciferase assay at the next day. Delivered miRNAs significantly decreased the corresponding luciferase activity, suggesting that the delivered miRNAs were functioning within the cells (Figure 2b).

We next examined the delivery of miRNAs into 293T cells (human embryonic kidney cell lines) to explore cell-specificity. Only a small increase in miRNA93 expression levels was observed 24 hours after transfer into 293T cells, based on Northern blots (Figure 2c), indicating that the BNCs had high specificity for hepatocyte-derived cells. The expression of transferred miRNA into Huh7 cells could be observed even 3 days after delivery (Figure 2d), suggesting that the delivered miRNAs are expressed

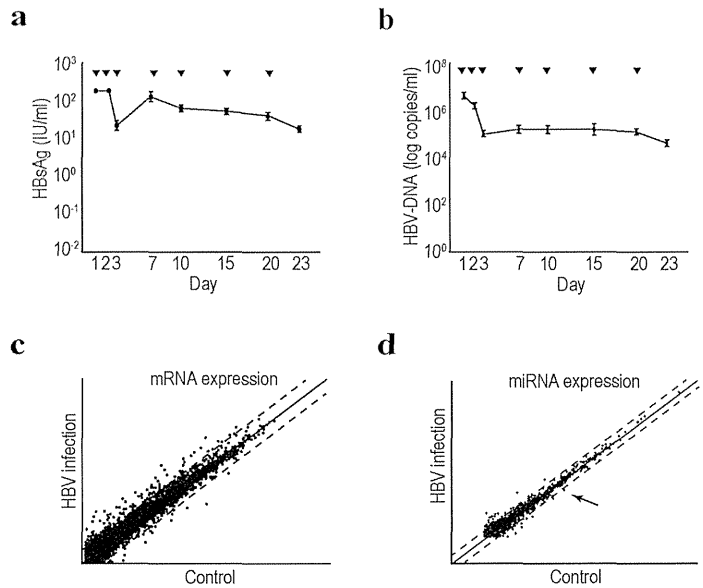

Figure 1: Comprehensive transcriptome and miRNA analyses in HBV-replicating human primary hepatocytes. a, b, Efficient HBV replication in human primary hepatocytes isolated from chimeric mice. Primary human hepatocytes isolated from chimeric mice were seeded into the wells of a 24-well plate. Serum from HBV-infected patients was added to infect the cells with HBV. Media was changed at the indicated days (▼). The supernatant was collected when the media was changed for the analyses of HBsAg levels (a) and HBV-DNA levels (b). Data represent the means ± s.d. of three independent experiments. c, Scatter plot reflecting the transcriptomic results comparing the control and HBV-replicating primary human hepatocytes. Cells at day 7 after HBV infection were used for the analyses. Intensity normalization was performed using global normalization based on the expression levels of all genes analyzed. Dashed lines indicate the thresholds: two-fold increase or 50% decrease in expression levels. The full data are deposited in NCBI GEO database accession: GSE55928. d, A scatter plot of the miRNA microarray results was used to determine the expression levels of comprehensive mature miRNAs. Total RNA from control and HBV-replicating primary hepatocytes at day 7 after infection was used. Dashed lines indicate the thresholds: two-fold increase or 50% decrease in expression levels. Intensity normalization was performed using global normalization based on the expression levels of all miRNAs. The arrow indicates the result for miRNA93. The full data are deposited in NCBI GEO database accession: GSE55929.

for several days.

miRNA delivery into human primary hepatocytes using bionanocapsules

Based on the efficient delivery of miRNA via BNCs into human liver-derived cell lines, we examined the BNC-mediated delivery of miRNAs into non-dividing human primary hepatocytes isolated from chimeric mice, as described above. BNCs could deliver miRNAs efficiently, even into non-dividing human primary hepatocytes, based on Northern blots (Figure 3a), irrespectively of the use of Polybren (Figure 3a).

Since the expression levels of miRNA93 were downregulated by HBV replication (Figure 1d and Supplementary Table 4), we delivered miRNA93 via BNCs into HBV replicating human hepatocytes to rescue the downregulation of miRNA93 levels and examine the effects of decreased miRNA93 on transcript levels (Figure 3b). The rescue of miRNA93 expression, recovered the baseline-level expression of some genes, such as 17-beta-hydroxysteroid dehydrogenase 14 (HSD17B14) and tripartite motif-containing protein 31 (TRIM 31), which

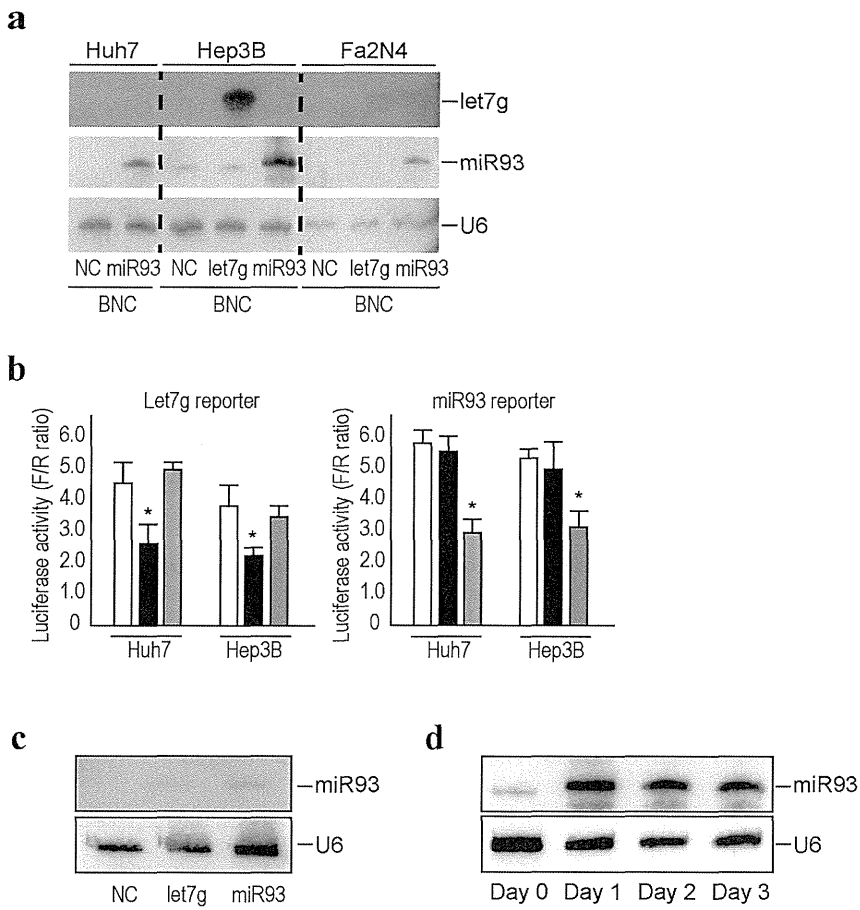

Figure 2: Efficient delivery of miRNAs into liver cell lines using BNCs. a, Northern blotting of miRNAs delivered into liver cells by BNCs. Liver cancer cell lines, Huh7 and Hep3B, and primary hepatocytes immortalized by SV40, Fa2N4, were incubated with BNCs harboring the indicated miRNAs (miRNA93 or let7g) or BNCs without miRNAs (NC). After 24 hours, cells were harvested and subjected to analysis. Membranes were re-probed for let7g, miRNA93, and U6 as a loading control. The results shown are representative of three independent experiments. b, miRNAs delivered using BNCs were biologically functional. Huh7 and Hep3B cells were transfected with the indicated reporter constructs, which indicate the activity of each miRNA function. Twenty-four hours after transfection, cells were mixed with BNCs containing let7g (black bar), miRNA93 (gray bar), or negative control (white bar). Forty-eight hours after transfection, cells were subjected to a dual luciferase assay. Data shown represent the means ± s.d. of the raw ratios (FL/RL), obtained by dividing the firefly luciferase values by the renilla luciferase values, of three independent experiments. $*p < 0.05$. c, Delivery of miRNAs via BNCs were liver cell-specific. The 293T cells (human embryonic kidney cells) were incubated with BNCs containing let7g, miRNA93, or negative control (NC). After 24 hours, cells were subjected to Northern blotting for miRNA93. U6 was used as a loading control. The results shown are representative of two independent experiments. d, miRNA93 expression in Huh7 cells after the delivery of miRNA93 via BNCs. Cells were sequentially collected after incubation with BNCs containing miRNA93 and subjected to Northern blotting. U6 was used as a loading control. The results shown are representative of three independent experiments.

were increased by HBV replication (Supplementary Table 1), suggesting that the mRNA levels of these genes may be directly or indirectly regulated by miRNA93. Although the enhanced decay of target transcripts by miRNAs has been reported [22, 32], miRNAs generally function as translational repressors [33]. However, these miRNA93 delivery results may not be accurate due to direct or indirect effects of miRNA93. In addition, changes in protein levels may differ from our transcript expression results.

Modulation of MICA protein expression levels by delivery of miRNA93 using BNCs

We previously identified miRNA93 as a critical regulator of MICA protein expression [23], which plays a role in the susceptibility to HBV-induced HCC [25]. MiRNA93 regulates MICA protein levels, but not transcript levels [23, 29]. Although it was found that miRNA93 expression levels decreased during HBV replication in primary hepatocytes (Figure 1d and Supplementary Table 4), MICA transcript levels were not affected (GEO accession number: GSE55928), suggesting that the effects of miRNA93 on MICA may be mediated by translational repression and not by mRNA decay, as we reported previously [23]. To confirm changes in the expression level of the MICA protein on the cell surface of primary hepatocytes induced by HBV infection, cells were subjected to FACS analyses. However, the protein expression levels on the cell surface did not change significantly (Figure 4a). MICA is a soluble protein released into the supernatant after shedding by ADAM10 and ADAM17[34]. Our results suggested that the

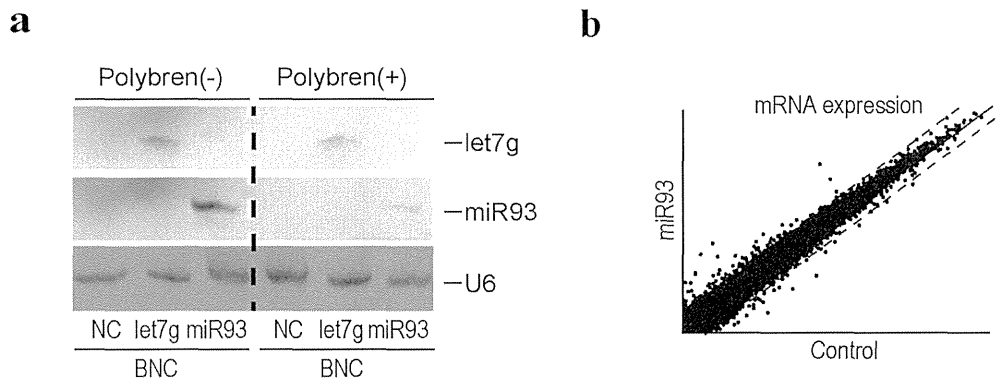

Figure 3: Efficient delivery of functional miRNAs into human primary hepatocytes using BNCs. a, Northern blotting for miRNAs delivered into cells using BNCs. Human primary hepatocytes isolated from chimeric mice were incubated with BNCs containing the indicated miRNAs (miRNA93 or let7g) or BNCs without miRNAs (NC), with or without Polybren. After 24 hours, cells were harvested and subjected to analysis. Membranes were re-probed for let7g, miRNA93, and U6 as a loading control. The results shown are representative of three independent experiments. b, A scatter plot reflecting the transcriptome results between the control and primary human hepatocytes treated with BNCs containing miRNA93. Cells were harvested 24 hours after BNC treatment. Intensity normalization was performed using global normalization based on the expression levels of all genes analyzed. Dashed lines indicate the thresholds: a two-fold increase or 50% decrease in expression levels. The full data are deposited in GEO database accession: GSE55928.

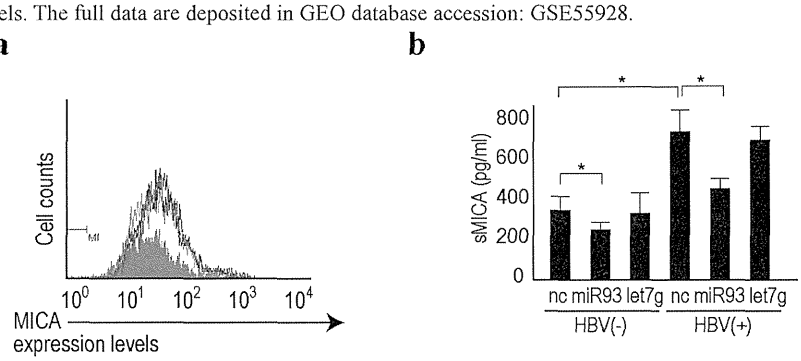

Figure 4: Soluble MICA protein levels were regulated by miRNA93 in human primary hepatocytes. a, Membrane-bound MICA protein expression was not affected by miRNA delivery into human primary hepatocytes. Flow cytometric analysis of membrane-bound MICA protein expression in cells delivered BNC-mediated control (green line), let7g (blue line), or miRNA93 (red line). Gray-shaded histograms represent background staining, assessed using isotype IgG. Representative results from three independent experiments are shown. b, Soluble MICA protein levels in the supernatants of primary hepatocytes after delivery of the indicated miRNAs (let7g or miRNA93) or negative control (NC) with or without HBV replication. Data represent the means ± s.d. of three independent experiments. *$p<0.05$.

modulated expression of MICA in primary hepatocytes during HBV replication affects this shedding process. To explore this possibility, we examined MICA protein levels in the supernatant using ELISA. As predicted, HBV infection significantly increased the protein concentration of MICA in the supernatant (Figure 4b).

Because an increase in soluble MICA levels in the serum of chronic hepatitis B patients is significantly associated with increased susceptibility to HCC [25], this increase during HBV replication needs to be prevented. Thus, we examined the effects of delivery of BNCs carrying miRNA93 into HBV-infected hepatocytes. Even though the MICA mRNA levels were not significantly affected by miRNA93 delivery based on microarray results (GEO accession: GSE55928), soluble MICA protein in the supernatant significantly decreased according to ELISA (Figure 4b). These results suggested that miRNA93 delivery into the liver decreases soluble MICA levels in the serum, which may be used to prevent HCC in chronic hepatitis B patients.

DISCUSSION

We report that HBV replication in human hepatocytes decreases miRNA93 expression and increases soluble MICA levels. Increased soluble MICA levels in the serum are strongly associated with HBV-related HCC [25], and the increased soluble MICA levels could be antagonized by the delivery of miRNA93 into hepatocytes using BNCs. Thus, BNCs carrying miRNA93 may be used to prevent HCC in patients with chronic HBV infection.

Methods of efficient long-term HBV replication *in vitro* are not commonly available. Although transient transfection assays using fragments or tandem-units of the HBV genome or the full-length HBV genome without vector backbone have been applied [8-12], these models can be analyzed only for short-term replication after transfection. Although stable cell lines carrying HBV genomes are also used, HBV particles are derived from the HBV genome and integrate into the host genome, which differs from natural infection, in which HBV replication mainly relies on HBV cccDNA [6, 7]. Although the most ideal system for HBV infection and replication studies *in vitro* are primary human hepatocytes, they are difficult to obtain. Freshly isolated human hepatocytes from chimeric mice used in this report are relatively easily to obtain, since they proliferate under immune-deficient and liver-damaging conditions. These cells could support HBV replication for a substantial period and are valuable resources for studies on HBV infection and replication.

Another essential tool used in this study is that of BNCs. Primary hepatocytes are generally difficult to transduce with exogenous genes via transfection. Although viral-mediated gene transfer is useful even for primary cells, we chose BNCs as the miRNA delivery method for several reasons. First, since BNCs are composed of HBV L particles, these BNCs preferentially target primary hepatocytes and theoretically target similar cells as does HBV. Second, since we want to develop future therapeutics based on our experimental results, we avoided using viral materials such as lentiviruses or retroviruses to improve biosafety. Third, although BNCs have been established to transfer genes or drugs [21, 31, 35], transfer of miRNAs has not yet been examined, which prompted us to investigate delivery of miRNAs. We found that BNCs could efficiently deliver miRNAs into primary hepatocytes. Although further studies are required, delivery of miRNAs into hepatocytes via BNCs may be a promising approach to target hepatocytes *in vivo*, as BNCs are efficient delivery vehicles in xenograft models using human liver-derived cells [21].

The present results regarding comprehensive transcriptome analyses using HBV replicating hepatocytes may be applicable for future HBV research. While similar experiments are typically performed using transfection in HBV protein-expressing cells, or other relatively artificial experimental settings, the results here may better reflect the *in vivo* situation for HBV-infected hepatocytes. The expression of approximately 0.3% of genes changed during HBV replication when the threshold was set to a greater than 4-fold increase or to less than a 25% decrease. Although some of these genes were consistent with previous transcriptomic studies [36-38], we observed several novel characteristics. First, few inflammation-related genes were included among genes whose expression levels were significantly changed. The reason for this discrepancy remains unclear, but the results were considered accurate, since inflammation is rare when HBV replicates prior to seroconversion in chronic HBV-infected patients. Thus, HBV may be able to evade the sensing system related to innate immunity [39-41]. It should be explored whether changes in HBV sequences or the presence of host cells other than hepatocytes affect gene expression in hepatocytes *in vivo*. Second, based on comprehensive analysis of transcript changes, many CYP-related genes were upregulated during HBV replication, which is consistent with previous reports [27, 28]. Since the biological significance of these changes remain unclear, further studies are required to explore the biological significance during HBV replication.

Microarray analyses of changes in miRNA expression levels in HBV-replicating cells revealed that miRNA expression levels were not affected by HBV replication (2.4% among 2,000 miRNAs when the threshold was set to more than a two-fold increase or less than a 50% decrease). However, the miRNAs whose expression levels changed may play crucial roles in the regulation of target gene expression without affecting transcript expression levels, for example, targeting of the MICA protein by miRNA93, whose expression levels were downregulated by HBV replication. The results of comprehensive miRNA expression level analysis in

HBV-replicating cells may increase our understanding of deregulated gene expression induced by HBV replication in hepatocytes.

MiRNA93 is a critical regulator of MICA protein expression [23, 29]. Thus, the decreased expression of miRNA93 by HBV suggested that the regulation of MICA expression by miRNA93 has biological significance. Polymorphisms in the MICA gene are associated with HBV and HCV-induced HCC [25, 42], and the increase in soluble MICA in the serum can be used as a susceptibility marker for HBV-induced HCC [25]. The increased levels of MICA protein expression agreed with the decreased miRNA93 expression. However, this increase was observed for soluble MICA protein levels and not membrane-bound MICA. While MICA is post-translationally dependent on the cell context or the status of viral infection [34], MICA may be readily processed from the cell surface in HBV-replicating primary hepatocytes and mainly released as soluble protein. Soluble MICA protein may function as a decoy for the NKG2D receptor in immune cells and as an evasion or immune surveillance system during chronic HBV infection. It may also be associated with HBV-induced HCC since HBV-infected hepatocytes may evade from the immune surveillance. Based on these results, BNCs carrying miRNA93 can be used to eliminate HBV-infected hepatocytes, which may be a novel approach for the prevention of subsequent virus-induced HCC.

MATERIALS AND METHODS

Cells

Primary human hepatocytes isolated fresh using the collagenase perfusion method from chimeric uPA/SCID mice with humanized livers [14, 17] were obtained from Phoenix Bio (Hiroshima, Japan). The purity of human hepatocytes was greater than 95%. A total of 3.0×10^5 cells/well were seeded on a type I collagen coated-24-well plate and maintained in DMEM with 10% FBS, 5 ng/ml EGF, 0.25 μg/ml insulin, 0.1 mM ascorbic acid, and 2% DMSO [43]. These cells were able be maintained at a high density for more than 3 weeks, supporting the long-term replication of HBV infection *in vitro*.

HBV infection *in vitro*

Serum from chronically HBV-infected patients with no HBe antibody before seroconversion was used for *in vitro* infection. Serum containing 9.0 log IU/ml of HBV genotype C in a volume of 3 μl, which is approximately 1.5×10^7 copies of HBV, was added to the 3.0×10^5 cells/well, followed by the addition of 4% PEG 8000 at day 0. Cells were washed, and the media was changed at days 1 and 2 and every 5 days thereafter. The media was collected to measure HBsAg and HBV-DNA at days 1, 2, 3, 7, 10, 15, 20 and 23 to confirm HBV replication. Measurements were performed at the clinical laboratory testing company SRL. Inc. (Tokyo, Japan).

cDNA array and miRNA microarray

Human 25K cDNA microarray and human 2K miRNA microarray analyses were performed using miRNA oligo chips according to the standard protocols (Toray Industries, Tokyo, Japan). The data and the experimental conditions were deposited in a public database (GEO: accession numbers: GSE55928 and GSE55929).

Bionanocapsules for miRNA delivery

Hollow particles consisting of HBV L proteins (pre-S1, pre-S2, and S regions) were used as the BNCs, as described previously [20, 21, 30]. The incorporation of miRNAs (miRNA93 or let-7g) into the hollow space and the delivery of miRNAs into human liver cells were performed as described previously [31]. Briefly, 32 μl BNC was added to 1 ml culture media at a final concentration of 50 nM miRNA 24 h before the indicated assays (unless otherwise specified).

Northern blotting of miRNAs

Northern blotting of miRNAs was performed as described previously. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA (10 μg) was resolved on denaturing 15% polyacrylamide gels containing 7 M urea in 1× TBE and then transferred to a Hybond N+ membrane (GE Healthcare) in 0.25× TBE. Membranes were UV-crosslinked and prehybridized in hybridization buffer. Hybridization was performed overnight at 42°C in ULTRAhyb-Oligo Buffer (Ambion) containing a biotinylated probe specific for miRNA93 (CTA CCT GCA CGA ACA GCA CTT TG) and let-7g (AAC TGT ACA AAC TACT ACC TCA), which was heated to 95°C for 2 min prior to hybridization. Membranes were washed at 42°C in 2× SSC containing 0.1% SDS, and the bound probe was visualized using the BrightStar BioDetect Kit (Ambion). Blots were stripped by boiling in a 0.1% SDS, 5 mM EDTA solution for 10 min prior to rehybridization using a U6 probe (CAC GAA TTT GCG TGT CAT CCT T).

Reporter plasmids, transient transfection, and dual luciferase assays

The firefly luciferase reporter plasmid was used to examine let7g and miRNA93 function. pGL4-TK, a renilla luciferase reporter, was used as an internal control [44]. Transfection and dual luciferase assays were performed as described previously [45].

Flow cytometry

The expression levels of MICA on the cell surface were determined using flow cytometry, as described previously [23]. Briefly, cells were hybridized with anti-MICA (1:500; R&D Systems, Minneapolis, MN, USA) and isotype control IgG (1:500; R&D Systems) in 5% BSA/1% sodium azide/PBS for 1 h at 4°C. After washing, cells were incubated with goat anti-mouse Alexa 488 (1:1,000; Molecular Probes, Eugene, OR, USA) for 30 min. Flow cytometry was performed and the data analyzed using Guava Easy Cyte Plus (GE Healthcare, Little Chalfont, UK).

ELISA for MICA

The concentration of MICA in the cell culture supernatant was measured using a sandwich ELISA, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

Significant differences between groups were determined using the Student's *t*-test when variances were equal and using Welch's *t*-test when variances were unequal. *P*-values less than 0.05 were considered statistically significant.

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Editorial note

This paper has been accepted based in part on peer-review conducted by another journal and the authors' response and revisions as well as expedited peer-review in Oncotarget

AUTHOR CONTRIBUTIONS

M.Ohno and M.Otsuka planned the research and wrote the manuscript. M.Ohno, T.K., C.S., T.Y., and A.T. performed the majority of the experiments. R.M., N.K., M.S. and N.K. measured performed ELISA. S.K. provided materials and wrote the manuscript. K.K. supervised the entire project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Original Article

Multiplication of alpha-fetoprotein and protein induced by vitamin K absence-II is a powerful predictor of prognosis and recurrence in hepatocellular carcinoma patients after a hepatectomy

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Aim: To evaluate the oncological implications of multiplication of α-fetoprotein (AFP) and protein induced by vitamin K absence or antagonists-II (PIVKA-II) in patients with hepatocellular carcinoma (HCC).

Methods: Data were prospectively collected from 516 consecutive patients who underwent a curative primary hepatectomy for HCC between 1998 and 2010. The AP-factor (AFP × PIVKA-II) was evaluated in relation to 2-year survival outcomes by receiver–operator curve analysis to determine the cut-off values. Patient survival, recurrence-free survival and risk factors were analyzed in accordance with the preoperative AP-factor.

Results: The AP-factor was categorized into three groups depending on the serum concentrations of AFP and PIVKA-II as follows: AP1 ($n = 206$; AFP < 200 ng/mL and PIVKA-II < 100 mAU/mL), AP2 ($n = 152$; AFP × PIVKA-II < 10^5) and AP3 ($n = 158$; AFP × PIVKA-II ≥ 10^5). The AP-factor was found to be significantly related to pathological factors such as differentiation, portal vein invasion, hepatic vein invasion and intrahepatic metastasis. Multivariate analysis was performed to identify the risk factors for survival and recurrence. Albumin, AP-factor and pathological factors including portal vein invasion, hepatic vein invasion and intrahepatic metastasis are independent risk factors for survival. Tumor number, AP-factor, and a non-cancerous liver were determinants of recurrence.

Conclusion: The AP-factor is closely related to differentiation and microscopic vascular invasion, and was selected by multivariate analysis as an independent factor for survival and recurrence, in HCC. Patients hopeful of obtaining good outcomes after a hepatectomy could be selected by the AP-factor evaluation.

Key words: alpha-fetoprotein, hepatocellular carcinoma, hepatectomy, protein induced by vitamin K absence or antagonists-II, prognosis, recurrence

INTRODUCTION

LIVER RESECTION HAS the highest capacity for local control of hepatocellular carcinoma (HCC) among all local treatment options and results in a good survival rate.[1] However, the recurrence rates of HCC continue to remain high even after curative hepatectomy.[2] Many factors related to the prognosis and recurrence of HCC have been reported with vascular invasion to the portal and/or hepatic vein identified as the most important factor that influences the outcome of hepatic resection.[3] Macroscopic vascular invasion is detectable by ultrasonography (US), computed tomography (CT) and magnetic resonance imaging (MRI). However, microscopic invasion can only be detected by performing a pathological examination just after hepatectomy and cannot be diagnosed preoperatively. Although tumor differentiation is reported to be an independent predictor of a poor outcome,[4] it also cannot be evaluated preoperatively. Hence, the serum levels of α-fetoprotein (AFP) and protein induced by vitamin K absence or antagonism factor-II (PIVKA-II), and the HCC tumor

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size and number are regarded as surrogate markers of microvascular invasion and tumor differentiation.[5,6]

α-Fetoprotein and PIVKA-II have shown utility as tumor markers of HCC and are associated with a poor prognosis after hepatectomy.[7] AFP is related to tumor differentiation,[8] whereas PIVKA-II is related to vascular invasion.[9] Individually, the presence of these tumor markers has less serious implications than microvascular invasion,[10] the latter being the most influential determinant of recurrence and survival in HCC patients undergoing a hepatectomy.[11] However, the oncological implications of determining a numerical value that would account for interaction of both AFP and PIVKA-II, namely, multiplication of the serum levels of AFP and PIVKA-II, have not yet been evaluated.

To further identify factors related to the prognosis and recurrence of HCC, we herein analyze the survival and recurrence outcomes in 516 consecutive patients who underwent a primary curative hepatectomy for HCC at our center. Specifically, we seek to evaluate the oncological implications of numerically determining the serum levels of AFP and PIVKA-II.

METHODS

Patients

BETWEEN JANUARY 1998 and December 2010, 516 consecutive adult patients underwent a hepatectomy for HCC at our center. The remaining patients were classified according to their preoperative serum levels of AFP and PIVKA-II. The mean age of these patients was 61.8 years and the age range was 18–88 years. Of the 516 HCC patients, 425 (82.4%) were male, 222 (43.0%) were hepatitis B virus surface antigen positive, 189 (36.6%) were hepatitis C virus antibody positive and 178 (34.5%) had cirrhosis. The preoperative serum AFP and PIVKA-II levels were simultaneously measured using standard methods at least 2 weeks before hepatectomy, when imaging studies were also performed. AFP was measured using an immune enzymometric assay with a commercially available kit (ST AIA-PACK AFP; TOSOH, Tokyo, Japan). PIVKA-II was measured by chemiluminescent immunoassay using a sensitive des-γ-carboxyprothrombin (DCP) antibody (Picolumi PIVKA-II; Eisai, Tokyo, Japan).

The patient subjects were divided into three groups according to their AFP levels (AFP low, ≤200 ng/mL; AFP mid, 200–1000 ng/mL; and AFP high: >1000 ng/mL). The patients were also divided into three groups according to their PIVKA-II levels (PII low, ≤100 mAU/mL; PII mid, 100–1000 mAU/mL; and PII high, >1000 mAU/mL). We evaluated multiplication of AFP and PIVKA-II to build a model that incorporates interaction effects of covariates of these two tumor markers in multivariate analysis of the Cox proportional hazards model. We evaluated the AP-factor, which was a tumor-related factor, the same as microvascular invasion, based on 2-year survival outcome, because the importance of microvascular invasion in regard to tumor recurrence and early death within 2 years after liver resection was reported in patients with small HCC.[12,13] Receiver–operator curve (ROC) analysis of the AP-factor (a product of the serum levels of AFP and PIVKA-II), AFP and PIVKA-II to evaluate the cut-off values for 2-year survival confirmed that the area under the curve (AUC) of the AP-factor (AUC = 0.74607) is significantly higher than that of AFP (AUC = 0.69804, $P = 0.0271$) and PIVKA-II (AUC = 0.69130, $P = 0.0065$) (Fig. 1). The patients were then classified into three groups in accordance with an AP-factor cut-off value of 10^5 (AUC = 0.74607, sensitivity = 63.27% specificity = 77.41%) as follows: AP1 (AFP < 200 ng/mL and PIVKA-II < 100 mAU/mL), AP2 (AFP × PIVKA-II < 10^5) and AP3 (AFP × PIVKA-II ≥ 10^5). AP1 (AFP < 200 ng/mL and PIVKA-II < 100 mAU/mL) was set accordingly because the 5-year patient survival (PS) rates for AFP low (AFP < 200 ng/mL) and PII low (PIVKA-II < 100 mAU/mL) were 76.4% and 81.3% and significantly higher than the other groups. The clinicopathological characteristics of these groups are summarized in Table 1. Among the 516 HCC patients in our cohort, 499 (96.7%) were categorized as Child–Pugh class A. These patients were followed up for a median of 107.7 months (range, 24.7–185.0). All the analyses in this study were performed in accordance with the ethical guidelines of Hokkaido University Hospital. This study was approved by the institutional review board of Hokkaido University.

Hepatectomy

Anatomical resection is defined as a resection in which lesion(s) are completely removed anatomically on the basis of Couinaud's classification (segmentectomy, sectionectomy and hemihepatectomy or extended hemihepatectomy) in patients with sufficient functional reserve. Non-anatomical partial but complete resection was achieved in our HCC patients. In all patients, R0 resections were performed, and the resection surface was found to be histologically free of HCC. An indocyanine green retention rate at 15 min (ICG-R15) was measured

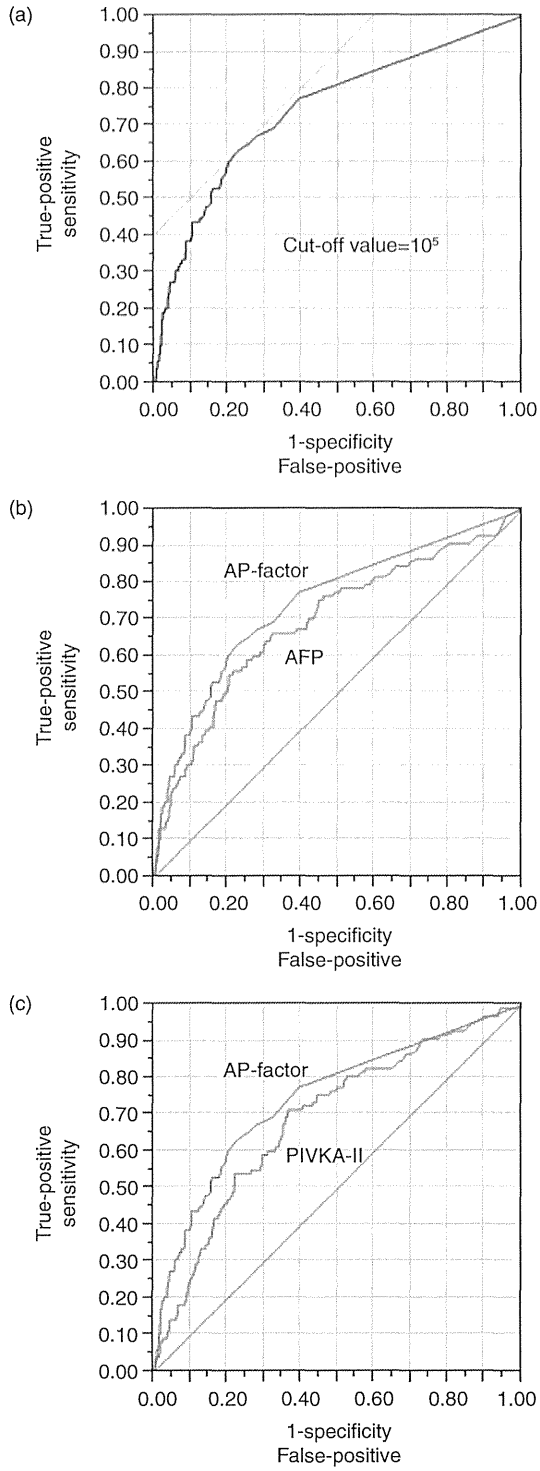

Figure 1 (a) The AP-factor – a product of the serum levels of AFP and PIVKA-II – was evaluated with respect to 2-year survival outcomes by ROC analysis which determined a cut-off value of 10^5 (AUC = 0.74607, sensitivity = 63.27% specificity = 77.41%). (b) ROC analysis of the AP-factor and AFP to evaluate the cut-off values for 2-year survival confirmed that the AUC of the AP-factor (AUC = 0.74607) is significantly higher than that of AFP (AUC = 0.69804, $P = 0.0271$). (c) ROC analysis of the AP-factor and PIVKA-II to evaluate the cut-off values for 2-year survival confirmed that the AUC of the AP-factor (AUC = 0.74607) is significantly higher than that of PIVKA-II (AUC = 0.69130, $P = 0.0065$). AFP, α-fetoprotein; AUC, area under the curve; PIVKA-II, protein induced by vitamin K absence or antagonist-II; ROC, receiver–operator curve.

for the evaluation of the liver function reserve, regardless of the presence or absence of cirrhosis.

HCC recurrence

For the first 2 years after hepatectomy, the patients underwent follow-up evaluations every 3 months comprising liver function tests, measurements of the tumor marker AFP and PIVKA-II, US and dynamic CT. After 2 years, routine CT was performed once in every 4 months. If recurrence was suspected, CT and MRI were performed, with CT during angiography and bone scintigraphy also performed if necessary. This enabled the precise diagnoses of the site, number, size and extent of invasiveness of the recurrent HCC lesions.

Statistical analysis

Patient survival and recurrence-free survival (RFS) rates were determined using the Kaplan–Meier method and compared between groups by the log–rank test. Univariate analysis of variables was also performed, but only significant variables were analyzed using the Cox proportional hazard model for multivariate analysis. Statistical analyses were performed by using standard tests (χ^2-test, Student's *t*-test) where appropriate. Significance was defined by *P*-values of less than 0.05. Statistical ROC analyses were performed using JMP version 10 for Windows (SAS Institute, Cary, NC, USA).

RESULTS

Clinicopathological characteristics and operative variables for the HCC patients

PATIENT CHARACTERISTICS, TUMOR-RELATED factors and perioperative outcomes are listed in Table 1. In the AP1, AP2 and AP3 groups, there were

Table 1 Clinicopathological characteristics of HCC patients classified according to AP-factor level

		AP-factor			*P*
		AP1 $n = 206$	AP2 $n = 152$	AP3 $n = 158$	
Sex	Male	177	121	127	0.2209
	Female	29	31	31	
Age (years)	<60	75	63	72	0.2060
	≥60	131	89	86	
HBsAg	Positive	72	82	68	0.0047
	Negative	134	76	84	
HCV	Positive	94	47	48	0.0024
	Negative	112	111	104	
Albumin (g/dL)	<4	75	57	73	0.1334
	≥4	131	95	85	
Total bilirubin (mg/dL)	<0.8	93	66	85	0.0740
	≥0.8	113	86	73	
ICG-R15 (%)	<15	93	92	98	0.0014
	≥15	113	60	60	
Tumor number	1	152	105	83	<0.0001
	2/3	50	33	45	
	≥4	4	14	30	
Tumor size (cm)	≤2	44	17	4	<0.0001
	3–4	128	71	43	
	≥5	34	64	111	
Macroscopic vascular invasion (portal vein, hepatic vein)	Absent	196	136	110	<0.0001
	Present	10	16	48	
Anatomical resection	Yes	124	120	136	<0.0001
	No	82	32	22	
Differentiation	Well	27	11	4	<0.0001
	Moderate	132	89	62	
	Poor	40	49	92	
	Unknown	7	3	0	
Microscopic portal vein invasion	Absent	188	81	114	<0.0001
	Present	18	71	44	
Microscopic hepatic vein invasion	Absent	201	139	127	<0.0001
	Present	5	13	31	
Microscopic intrahepatic metastasis	Absent	162	103	78	<0.0001
	Present	44	49	80	
Non-cancerous liver	Cirrhosis	83	46	49	0.0775
	Non-cirrhosis	123	106	109	

AP-factor – a product of the serum levels of AFP and PIVKA-II – was evaluated for 2-year survival by ROC analysis which determined a cut-off value for the AP-factor of 10^5. The HCC patients were classified into three groups accordingly: AP1 (AFP < 200 ng/mL and PIVKA-II < 100 mAU/mL), AP2 (AFP × PIVKA-II < 10^5) and AP3 (AFP × PIVKA-II ≥ 10^5).
AFP, α-fetoprotein; HBsAg, hepatitis B virus s antigen; HCV; anti-hepatitis C virus antibody; ICG-R15, indocyanine green retention rate at 15 min; PIVKA-II, protein induced by vitamin K absence or antagonism factor-II.

significant differences found in a number of variables including ICG-R15, tumor number, tumor size, macroscopic vascular invasion, anatomical resection, differentiation, microscopic portal vein invasion, microscopic hepatic vein invasion and microscopic intrahepatic metastasis. The *P*-values of the AP-factor for differentiation, microvascular portal invasion, microvascular hepatic vein invasion and intrahepatic metastasis were lower than or equal to those for AFP and PIVKA-II individually.

PS and RFS outcomes

The PS rates for the AP1, AP2 and AP3 groups at 5 years were 82.7%, 78.8% and 41.3%, respectively. The PS of the AP1 and AP2 patients was significantly higher than that of the AP3 cases ($P < 0.0001$ and <0.0001, respectively; Fig. 2a). The RFS outcomes for the AP1, AP2 and AP3 groups at 5 years were 34.0%, 40.7% and 17.1%, respectively. The RFS of AP3 was significantly lower than that of either AP1 or AP2 ($P < 0.0001$ and <0.0001, respectively; Fig. 2b).

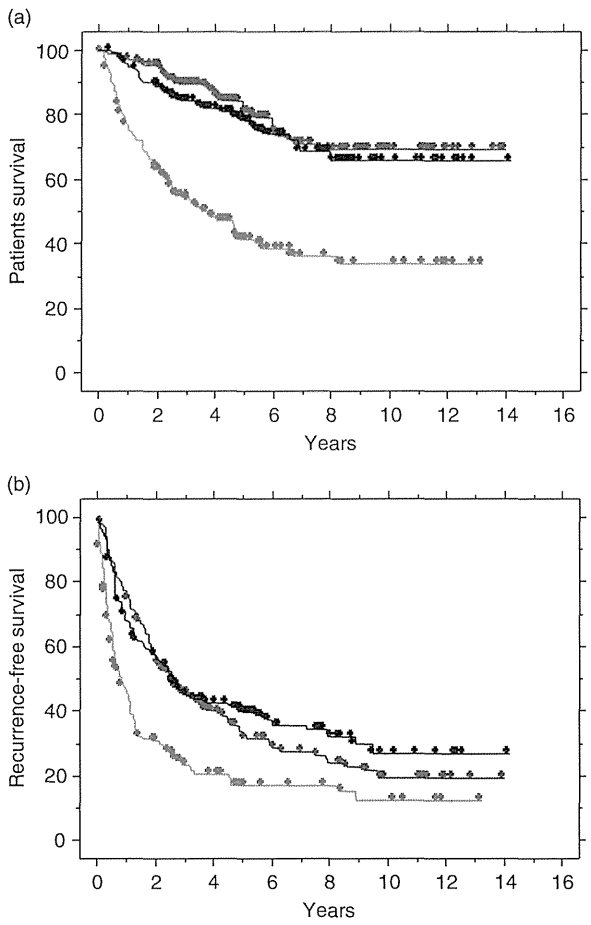

Figure 2 (a) Patient survival in accordance with the AP-factor classification. The PS rate for the AP1 group was significantly higher than those of the AP2 and AP3 groups (both $P < 0.0001$). (b) Patient recurrence-free in accordance with the AP-factor classification. The RFS rate of the AP3 group was significantly lower than that of the AP1 and AP2 patients (both $P < 0.0001$). —, AP1 ($n = 206$); —, AP2 ($n = 152$); —, AP3 ($n = 158$). PS, patient survival; RFS, recurrence-free survival.

Causes of death

There were 209 deaths among our 516 HCC patients (40.5%) due to HCC recurrence ($n = 164$; 78.5%), liver failure ($n = 17$; 8.1%) and other causes ($n = 28$; 13.4%).

Recurrent sites of HCC during follow up

Of the 139 cases of HCC recurrence in group AP1, 112 patients (80.6%) had a recurrence only in the liver and 27 (19.4%) in extrahepatic sites, including or excluding the liver. Of the 94 cases of recurrence in group AP2, 68 (72.3%) had recurrence only in the liver and 26 (27.7%) in extrahepatic sites, including or excluding the liver. Of the 122 cases of recurrence in group AP3, 63 (51.6%) had recurrence only in the liver and 59 (48.4%) in extrahepatic sites, including or excluding the liver. Importantly, recurrence in AP1 patients tended to occur only in the liver, whereas in AP3 patients it tended to occur in extrahepatic sites, including or excluding the liver ($P < 0.0001$).

Univariate and multivariate analyses of overall survival and RFS

When univariate analysis was performed to identify survival factors, serum albumin levels, tumor number, tumor size, macroscopic vascular invasion, AFP, PIVKA-II, AP-factor, differentiation, microscopic portal vein invasion, microscopic hepatic vein invasion and microscopic intrahepatic metastasis were found to be significant risk factors for survival outcomes (Table 2). When univariate analysis was also performed to identify the risk factors for recurrence, the serum albumin level, ICG-R15, tumor number, tumor size, macroscopic vascular invasion, AFP, PIVKA-II, AP-factor, differentiation, microscopic portal vein invasion, microscopic hepatic vein invasion, microscopic intrahepatic metastasis and a non-cancerous liver were identified as significant risk factors (Table 2).

The significant factors identified by univariate analysis for survival and HCC recurrence were included in multivariate analysis which showed that serum albumin levels ($P = 0.0056$), AP-factor ($P = 0.0062$), microscopic portal vein invasion ($P = 0.0027$), microscopic hepatic vein invasion ($P = 0.0056$) and microscopic intrahepatic metastasis ($P = 0.005$) are independent risk factors for survival (Table 3), and that tumor number ($P < 0.0001$), AP-factor ($P = 0.0161$) and non-cancerous liver ($P = 0.012$) are independent risk factors for recurrence (Table 3). ROC analysis of the AP-factor, AFP and PIVKA-II to evaluate the cut-off values for 2-year survival confirmed that the AUC of the AP-factor

Table 2 Univariate analysis of predictive values (clinical and tumor-associated factors) for patient survival and recurrence free survival

		n	*P* Survival	*P* Recurrence
Sex	Male	425	0.5111	0.3435
	Female	91		
Age (years)	<60	210	0.7956	0.5780
	≥60	306		
HBsAg	Positive	222	0.2211	0.2528
	Negative	294		
HCV	Positive	189	0.7128	0.7939
	Negative	327		
Albumin (g/dL)	<4	205	0.0001	<0.0001
	≥4	311		
Total bilirubin (mg/dL)	<0.8	264	0.6859	0.5098
	≥0.8	252		
ICG-R15 (%)	<15	283	0.7407	0.0084
	≥15	233		
Tumor number	1	340	<0.0001	<0.0001
	2/3	128		
	≥4	48		
Tumor size (cm)	≤2	65	<0.0001	<0.0001
	3–4	242		
	≥5 cm	209		
Macroscopic vascular invasion (portal vein, hepatic vein)	Absent	442	<0.0001	<0.0001
	Present	74		
Anatomical resection	Yes	380	0.7212	0.0756
	No	136		
AFP (ng/mL)	≤200	376	<0.0001	0.0021
	201–1000	42		
	>1000	98		
PIVKA-II (mAU/mL)	≤100	254	<0.0001	<0.0001
	101–1000	111		
	>1000	151		
AP-factor	AP1	206	<0.0001	<0.0001
	AP2	152		
	AP3	158		
Differentiation	Well	42	0.0021	<0.0001
	Moderate	283		
	Poor	181		
	Unknown	10		
Microscopic portal vein invasion	Absent	379	<0.0001	<0.0001
	Present	130		
Microscopic hepatic vein invasion	Absent	463	<0.0001	<0.0001
	Present	46		
Microscopic intrahepatic metastasis	Absent	340	<0.0001	<0.0001
	Present	170		
Non-cancerous liver	Cirrhosis	178	0.0656	0.0003
	Non-cirrhosis	338		

AFP, α-fetoprotein; AP-factor, a product of the serum levels of AFP and PIVKA-II; HBsAg, hepatitis B virus s antigen; HCV, anti-hepatitis C virus antibody; ICG-R15, indocyanine green retention rate at 15 min; PIVKA-II, protein induced by vitamin K absence or antagonism factor II.

Table 3 Multivariate analysis of values that are predictive for HCC patient survival and recurrence

		P	Risk ratio	95% CI
Survival				
Albumin (g/dL)	<4	0.0056	1.601	1.147–2.233
	≥4		1	
Tumor number		0.1855		
	1	0.0681	0.613	0.363–1.037
	2/3	0.2325	0.738	0.449–1.215
	≥4		1	
Tumor size (cm)		0.0776		
	≥5	0.0583	2.109	0.974–1.002
	3–4	0.3458	1.422	0.974–4.567
	≤2		1	
Macroscopic vascular invasion	Absent	0.7447	0.915	0.534–1.565
	Present		1	
AFP (ng/mL)		0.2125		
	>1000	0.2414	0.700	0.385–1.272
	≤200	0.0812	0.582	0.316–1.069
	201–1000		1	
PIVKA-II (mAU/mL)		0.3284		
	>1000	0.4571	0.840	0.531–1.329
	≤100	0.1471	0.595	0.295–1.201
	99–1000		1	
AP-factor		0.0062		
	AP1	0.0548	2.125	0.984–4.589
	AP3	0.0079	2.066	1.210–3.528
	AP2		1	
Differentiation		0.9550		
	Well	0.7066	1.488	0.188–11.789
	Moderate	0.6230	1.646	0.226–12.015
	Poor	0.6467	1.597	0.216–11.833
	Unknown		1	
Microscopic portal vein invasion	Absent	0.0027	0.517	0.336–0.796
	Present		1	
Microscopic hepatic vein invasion	Absent	0.0056	0.473	0.278–0.804
	Present		1	
Microscopic intrahepatic metastasis	Absent	0.0050	0.533	0.344–0.828
	Present		1	
Recurrence				
Albumin (g/dL)	<4	0.0667	1.239	0.985–1.557
	≥4		1	
ICG-R15 (%)	<15	0.0610	0.801	0.635–1.010
	≥15		1	
Tumor number		<0.0001		
	1	<0.0001	0.364	0.229–0.579
	2/3	0.0529	0.665	0.439–1.005
	4		1	
Tumor size (cm)		0.1443		
	≥5	0.0616	1.498	0.981–2.287
	3–4	0.3229	1.202	0.834–1.733
	≤2		1	
Macroscopic vascular invasion	Absent	0.7274	0.920	0.574–1.474
	Present		1	

Table 3 *Continued*

		P	Risk ratio	95% CI
AFP (ng/mL)		0.9638		
	>1000	0.7967	1.069	0.645–1.772
	≤200	0.9361	1.021	0.621–1.676
	201–1000		1	
PIVKA-II (mAU/mL)		0.4997		
	>1000	0.7397	1.060	0.750–1.499
	≤100	0.3008	0.766	0.463–1.269
	101–1000		1	
AP-factor		0.0161		
	AP1	0.0431	1.731	1.017–2.947
	AP3	0.0553	1.474	0.991–2.191
	AP2		1	
Differentiation		0.0760		
	Well	0.2922	2.184	0.510–9.348
	Moderate	0.1192	3.050	0.750–12.404
	Poor	0.0768	3.577	0.872–14.675
	Unknown		1	
Microscopic portal vein invasion	Absent	0.0561	0.716	0.508–1.009
	Present		1	
Microscopic hepatic vein invasion	Absent	0.2297	0.749	0.468–1.200
	Present		1	
Microscopic intrahepatic metastasis	Absent	0.2509	0.832	0.608–1.139
	Present		1	
Non-cancerous liver	Cirrhosis	0.0120	1.356	1.069–1.720
	Non-cirrhosis		1	

AFP, α-fetoprotein; AP-factor, a product of the serum levels of AFP and PIVKA-II; CI, confidence interval; HBsAg, hepatitis B virus s antigen; HCV, anti-hepatitis C virus antibody; ICG-R15, indocyanine green retention rate at 15 min; PIVKA-II, protein induced by vitamin K absence or antagonism factor-II.

(AUC = 0.74607) is significantly higher than that of AFP (AUC = 0.69804, $P = 0.0271$) and PIVKA-II (AUC = 0.69130, $P = 0.0065$).

DISCUSSION

IN OUR PRESENT study, the AP-factor was found to be closely related to both tumor differentiation and vascular invasion and was also identified as an independent factor related to PS and RFS outcomes with a *P*-value lower or equal to that of microscopic portal invasion, although AFP and PIVKA-II were not found to be independent survival factors. ROC analysis to evaluate 2-year survival in our HCC patient subjects who had received a hepatectomy confirmed that the AP-factor is a significantly superior indicator compared with AFP and PIVKA-II. Hence, the AP-factor is suggested to be a more reliable marker than other well-known indicators including AFP, PIVKA-II and microscopic portal invasion for the accurate prediction of survival and recurrence in HCC patients after a hepatectomy.

Previous reports have shown that AFP is an independent predictor of prognosis,[14] even in patients who have undergone a hepatectomy.[15] However, in our present analyses when the AP-factor was simultaneously inputted, AFP was not found by multivariate analysis to be an independent factor related to survival and recurrence in HCC. Although high levels of AFP in fully developed HCC or in the serum of the host are associated with more aggressive behavior and increased anaplasis,[16] it has been suggested that AFP regulates immune responses and induces either stimulatory or inhibitory growth activity.[17] On the other hand, it is well established that the AFP levels may increase in some patients with acute and chronic hepatitis without HCC,[18] and that the elevation of AFP levels correlates with the inflammation of background disease and hepatocyte regeneration.[19] Hence, because AFP does not always directly reflect tumor malignancy, its levels did not influence survival and recurrence in HCC cases according to multivariate analysis in our current study.

Protein induced by vitamin K absence or antagonists-II is also known as DCP. The specificity of PIVKA-II is approximately 95%, which is higher than that of AFP.[20] Recently, a highly sensitive assay for PIVKA-II was developed.[21] While sensitivity is still at approximately 50% for most small HCC,[22] the frequency of HCC patients in our present study with a lower than 40-mAU/mL PIVKA-II level was 36.6%. It is reported that the elevation of PIVKA-II correlates with the presence of vascular invasion.[9,23] DCP is reportedly an indicator of portal vein invasion of HCC,[24] as well as an independent prognostic indictor of recurrence and survival after hepatectomy.[7,10] However, in our present study, when we simultaneously inputted the AP-factor into our multivariate analysis, the results suggested that PIVKA-II is not an independent factor related to survival and recurrence. In previous studies that have assessed the value of DCP in predicting recurrence and survival after hepatectomy, the assays used were not highly sensitive. Hence, most of the cases that tested positive in these earlier studies had widespread or advanced HCC, and the biological nature of PIVKA-II positivity might have been overstated. Moreover, PIVKA-II may not reflect all of the factors related to the malignancy of HCC as it mainly indicates vascular invasion and not differentiation. PIVKA-II was therefore not selected an independent factor for HCC patient outcomes after hepatectomy in our current study.

The AP-factor – a product of the serum levels of AFP and PIVKA-II – was found in our current analyses to be significantly associated with all of the pathological factors tested including differentiation, microvascular portal invasion, microvascular hepatic vein invasion and intrahepatic metastasis (all $P < 0.0001$). From these results, we revealed that the AP-factor may have a duality in its relationship with AFP and PIVKA-II. It was previously reported that AFP has prognostic limitations in the case of microvascular hepatic vein invasion, as does PIVKA-II in the case of differentiation, in HCC.[8–10,23,25] The AP-factor overcomes these limitations because its *P*-value in relation to microvascular hepatic vein invasion was found to be very low ($P < 0.0001$). Because the AP-factor may represent the dual characteristics of both AFP and PIVKA-II, it may be a surrogate marker of both tumor differentiation and vascular invasion and more directly reflect tumor malignancy than either AFP or PIVKA-II individually. These findings may involve the fact that recurrence in AP1 patients tended to occur only in the liver, whereas in AP3 patients it tended to occur in extrahepatic sites, including or excluding the liver. Therefore, we identified the AP-factor as an independent factor very closely related to survival following microscopic vascular invasion, and closely related to recurrence in cases of increased tumor number.

Shimada *et al.* have reported that the positivity of both DCP and AFP is an independent indicator of a poor prognosis in HCC in terms of disease-free survival and PS.[7] For this reason, these authors suggested that both DCP and AFP produced by the HCC itself promote either tumor growth or tumor metastasis in an autocrine and/or paracrine fashion. Kaibori *et al.* have also reported that a positive status for both AFP and DCP at recurrence is an important prognostic indicator for HCC recurrence after hepatic resection.[26] However, our current patients were classified mainly by their AP-factor (AFP × PIVKA-II) levels because we hypothesized that this factor may be a surrogate marker of both tumor differentiation and vascular invasion and will more directly reflect tumor malignancy than either AFP or PIVKA-II individually. Moreover, ROC analysis of 2-year survival outcomes in our patients showed a significant superiority of the AP-factor over AFP and PIVKA-II as a prognostic indicator. For these reasons, the AP-factor may be a more reliable prognostic marker of PS and RFS of patients with HCC. Moreover, the classification of AP1, AP2 and AP3 is meaningful because it was possible to determine that AP2 was also equal to AP1, which was hoped to have the best outcome, and AP3 had the worst outcome in these three groups. Kiriyama *et al.* reported that triple positive tumor markers for HCC showed poor prognosis and invasive characteristics in pathological findings.[27] However, in this paper it was described that most of the patients in this study had less than the minimum detectable limit for *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3). Therefore, we evaluated the malignancy from AFP and PIVKA-II without AFP-L3.

Transplantation is considered to be the treatment of choice even for resectable small HCC in Child–Pugh class A patients.[28] Because the overall survival rates after hepatectomy for small HCC are shown to be equal to those after liver transplantation, hepatectomy before transplantation should be first performed for respectable HCC in patients with preserved liver function.[29] Moreover, because Poon *et al.* reported no differences in the cumulative survival curves of patients without microscopic venous invasion in resection and transplantation groups,[30] it is proposed that patients without microscopic portal invasion according to the Milan criteria should first be treated by hepatectomy. On the other hand, if HCC patients show microscopic portal invasion, the outcomes of liver transplantations are also