Chapter 7
Hepatic Differentiation of Human Embryonic Stem Cells and Induced Pluripotent Stem Cells by Two- and Three-Dimensional Culture Systems In Vitro

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Abstract Hepatocytes differentiated from human embryonic stem cells (hESCs) or induced pluripotent stem cells (hiPSCs) have a wide range of potential applications in biomedical research, drug discovery, and the treatment of liver disease. In this review, we provide an up-to-date overview of the wide variety of hepatic differentiation protocols. Moreover, we discuss the application of these protocols to three-dimensional culture systems in an attempt to induce hepatocyte-like cells with high hepatic functions.

Keywords Differentiation • ES cells • Hepatocytes • iPS cells • Three-dimensional culture

7.1 Hepatocytes in Cell-Based Therapy and Drug Discovery

The incidence of liver disease such as viral hepatitis, autoimmune hepatic disorders, fatty liver disease, and hepatic carcinoma is increasing worldwide [1]. Although the optimal treatment for end-stage liver disease is orthotopic liver transplantation, the

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major limitation of such treatment is the shortage of donor livers. The liver is composed of several types of cells, including hepatocytes, endothelial cells, Kupffer cells, stellate cells, and hematopoietic cells. Of these cells, hepatocytes play the most important role in major liver functions. Hepatocytes have many functions, including carbohydrate metabolism, glycogen storage, lipid metabolism, urea synthesis, drug detoxification, production of plasma proteins, and destruction of erythrocytes [2]. Therefore, the transplantation of hepatocytes has been considered an effective treatment alternative to orthotopic liver transplantation [3]. However, such a treatment requires an unlimited source of hepatocytes. Hepatocytes are useful for not only regenerative medicine but biomedical research and drug discovery. They are particularly useful for drug screenings, such as for the determination of metabolic and toxicological properties of drug compounds in in vitro models. Primary human hepatocytes are the current standard in vitro model, but isolated hepatocytes lose their functions rapidly even under optimized culture conditions [4, 5]. The use of human hepatocytes is limited by the scarcity of primary tissue from healthy donors. Donor-to-donor and batch-to-batch variations are also significant problems. Moreover, human hepatocytes can no longer proliferate in in vitro culture [6]. These are crucial issues for various applications, and new and unlimited sources of human hepatocytes are urgently required to address them.

7.2 Hepatic Differentiation of hESCs/hiPSCs in Two-Dimensional Culture

hESCs and hiPSCs could be established as promising new resources for obtaining human hepatocytes. Abe et al. [7] and Levinson-Dushnik et al. [8] demonstrated that mouse ESCs (mESCs) were capable of differentiating into endodermal cells. Hamasaki et al. [9] reported that hepatocyte-like cells were induced from mESCs by using humoral factors. Rambhatla et al. demonstrated the differentiation of hESCs into hepatocyte-like cells for the first time [10]. Since then, many studies have been initiated to enhance the hepatic differentiation efficiency and the functional qualities of the hepatocyte-like cells [11–16].

Hepatic differentiation from hiPSCs has been achieved using similar protocols as for hESCs [17–20]. iPSCs were generated from somatic cells as a result of the overexpression of four reprogramming factors (Oct3/4, Sox2, Klf-4, and c-Myc) [21, 22]. Consequently, hiPSCs provide the opportunity to generate individual-specific hepatocyte-like cells. For example, drug metabolism capacity differs among individuals [23], and thus it is difficult to make a precise prediction of drug toxicity by using hepatocytes isolated from a single donor or hESC-derived hepatocytes. A hepatotoxicity screening utilizing hiPSC-derived hepatocyte-like cells would allow the investigation of individual drug metabolism capacity. Moreover, hiPSC-derived hepatocytes generated from patients suffering from a particular disease could provide a source for the disease study and disease modeling [24, 25]. These application would be expected to lead to the discovery of novel drugs.

7.2.1 *Stepwise Hepatic Differentiation from hESCs/hiPSCs*

The general strategy for hepatic differentiation from hESCs/hiPSCs is a stepwise culture with the addition of growth factors or cytokines [11, 20] (Fig. 7.1), which mimics the in vivo microenvironment during liver development [26, 27] (Fig. 7.2).

Gastrulation of the vertebrate embryo starts with the formation of three germ layers: the ectoderm, mesoderm, and endoderm. The endoderm differentiates into various organs, including the liver, pancreas, lungs, intestine, and stomach. To examine the molecular mechanisms of endoderm specification during early embryogenesis, endoderm differentiation from ESCs has been widely investigated as an in vitro model [28].

In definitive endoderm (DE) differentiation, it is well known that nodal signaling, which involves members of the transforming growth factor-β super family, plays a crucial role and induces the expression of endoderm-related genes [29]. Activin A, a member of the nodal family, is a ligand of the type II activin receptor and can transmit a downstream signal by using Smad adaptor proteins [30–32]. D'Amour et al. accomplished the differentiation of hESCs to DE by using activin A [32]. Recently, protocols using the combination of activin A with other factors such as fibroblast growth factor (FGF) 2 or Wint3a have been also applied to efficiently induce the DE [33, 34, 14].

Hepatic differentiation from the DE is divided into two steps: hepatic specification and hepatic maturation. In the hepatic specification step, the DE differentiates into hepatoblasts that express alpha-fetoprotein (AFP), transthyretin, and albumin (ALB) [35–37]. At this stage, the interaction of FGFs with bone morphogenetic

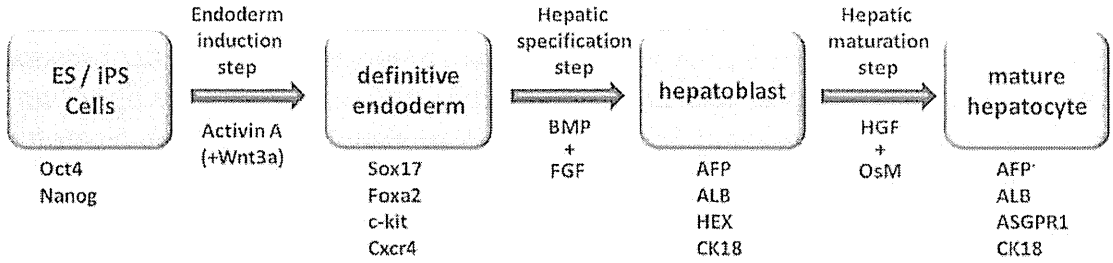

Fig. 7.1 In vitro hepatic differentiation from hESCs/hiPSCs

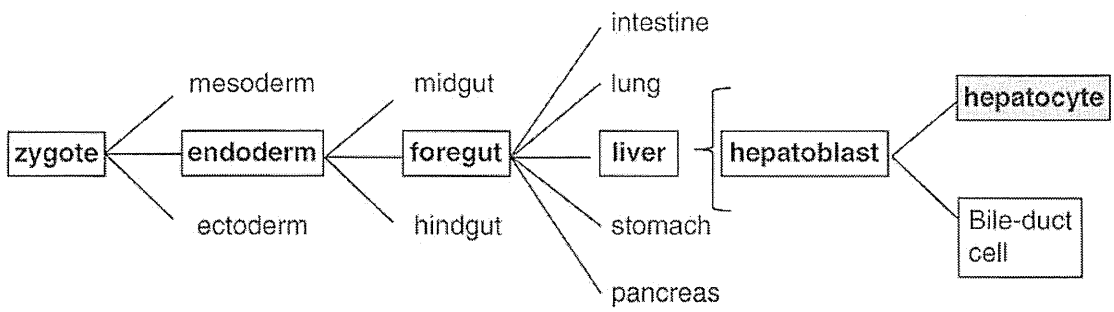

Fig. 7.2 The cell lineage steps during hepatic development

protein (BMP) 2 or BMP4 is important for the induction of hepatocyte-related genes [27, 38]. The combination of FGF4 and BMP2 promotes hepatic specification from human ESC-derived DE cells [13]. Similar results were obtained by using the combinations of aFGF and BMP4, bFGF and BMP4, or FGF4 and BMP4 [13, 33].

It is known that hepatoblasts differentiate into two distinct lineages, hepatocytes and cholangiocytes. During the fetal hepatic maturation, growth factors that are secreted by surrounding non-parenchymal liver cells, such as hepatocyte growth factor (HGF) and oncostatin M (OsM), are essential for hepatic maturation [39]. HGF enhances hepatocyte proliferation but inhibits biliary differentiation by blocking notch signaling [40]. Although HGF is widely used for inducing hepatic phenotypes (e.g., ALB and dipeptidyl peptidase IV expression) [16, 41, 42], this is not enough to induce functional mature hepatocytes [42, 43]. OsM, which is expressed in hematopoietic cells in the fetal liver [43, 44], promotes the hepatic differentiation from hepatoblast cells [39, 40, 45]. Furthermore, supplementation of the culture medium with dexamethasone, a glucocorticoid hormone, induces the production of mature hepatocyte-specific proteins and also supports the maturation process of the hepatocytes together with OsM [14, 15, 18].

7.2.2 *Hepatic Differentiation from hESCs/hiPSCs by Transduction of Hepatic Lineage-Specific Transcription Factors*

DE differentiation methods using growth factors are useful strategies for generating a DE with the ability to differentiate into hepatic or pancreatic lineages; however, these methods are not sufficient for generation of homogenous DE populations [46, 47]. To improve the efficiency of DE differentiation, several groups have attempted the modulation of expression levels in endoderm-related transcription factors. It has been demonstrated that overexpression of SOX17, which is an integral transcription factor for DE formation, promotes DE differentiation, resulting in an efficiency of DE differentiation of over 80 % based on the estimation of c-KIT/CXCR4 double-positive cells [47, 48]. The FOXA2 transcription factor also functions as a crucial regulator of the initial intracellular signaling pathways in DE differentiation [49, 50]. Overexpression of FOXA2 also enhances the efficiency of DE differentiation [51–53].

Several studies have demonstrated that, in the hepatic lineage specification stage, homogeneous hepatoblast populations can be generated by modulating the expression levels of hepatocyte-lineage-specific transcription factors as in the DE differentiation stage. Overexpression of HEX, which is an integral transcription factor for hepatic specification, has been shown to promote hepatic specification, resulting in enhanced expression levels of ALB and AFP in the HEX-transduced cells [54–56].

To generate functional hepatocyte-like cells which have characteristics similar to primary human hepatocytes, transduction of HNF4α genes, which are central regulators of liver development, in hESC-/hiPSC-derived hepatoblasts has been shown to successfully induce mature hepatocyte-like cells that have characteristics similar to

primary human hepatocytes [57]. Furthermore, the combination of overexpression of FOXA2 and HNF1α also could effectively induce mature hepatocyte-like cells [52]. The transduction of differentiation-related genes into hESCs/hiPSCs would be a powerful strategy to generate mature hepatocyte-like cells.

7.2.3 Hepatic Differentiation from hESCs/hiPSCs by a Co-culture System

In order to facilitate maturation of the hESC-/hiPSC-derived hepatocyte-like cells and to enhance the efficiency of hepatic differentiation, development of a differentiation system that more closely mimics progenitor development in vivo will be needed. The normal culture conditions of hepatocytes in vitro differ substantially from the environment in vivo. Cell–cell interactions are important in embryogenesis and organogenesis. In particular, heterotypic cell–cell interactions in the liver, such as interactions of parenchymal cells with non-parenchymal cells, play a fundamental role in liver function [58, 59]. Moreover, it is known that cell–cell interactions between the embryonic cardiac mesoderm and definitive endoderm are essential for liver development [60]. Transcription factors that are critical for hepatic development have been identified from these cell–cell interactions [60]. ES cells co-cultured with cardiac mesoderm showed spontaneous differentiation into hepatocyte-like cells [61]. It seems that the growth factors, including FGF and BMP, secreted from the cardiac mesoderm facilitate differentiation into hepatocyte-like cells. These results suggest that the combined differentiation methods, such as addition of soluble factors into the culture medium, transduction of differentiation-related genes, or co-cultivation with other lineage cells, may further enhance the differentiation and maturation efficiency of hepatocyte-like cells.

7.3 Hepatic Differentiation of hESCs/hiPSCs in Three-Dimensional Culture

Recently, numerous three-dimensional (3D) culture methods have been reported. Among these, the spheroid culture methods, which include the hanging-drop method and the float-culture method using culture dishes coated with non-adherent polymer, have been widely used to culture primary hepatocytes in vitro [62, 63] (Fig. 7.3). Spheroid culture methods allow better maintenance of the liver function of primary hepatocytes compared to two-dimensional (2D) culture [64, 65]. Moreover, various micro-patterned substrates, employing both surface engineering and synthetic polymer chemistry for utilizing spheroid culture, have been reported [66, 67] (Fig. 7.3). One of these technologies uses a nanopillar plate with an arrayed μm-scale hole structure at the bottom of each well and nanopillars that are aligned at the bottom of the respective holes. The seeded cells evenly drop into the holes,

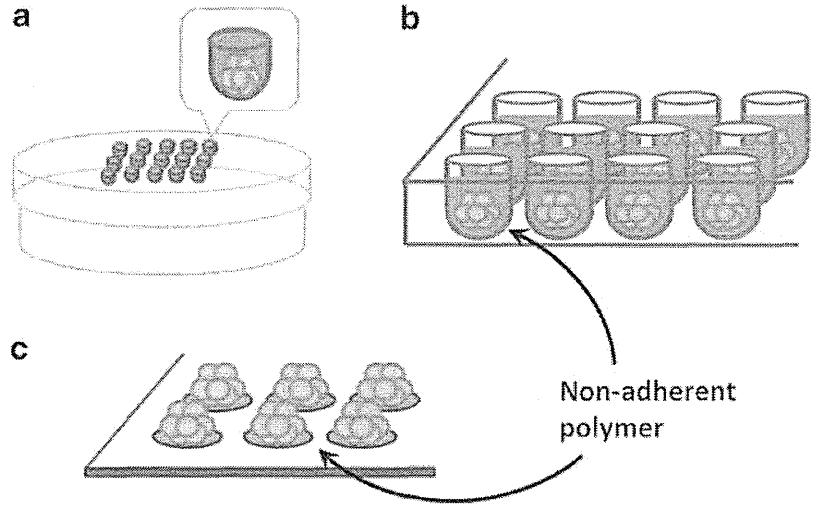

Fig. 7.3 The various spheroid culture methods: (**a**) the hanging-drop method, (**b**) the float-spheroid culture method using culture plate coated with non-adherent polymer, and (**c**) the spheroid culture method on micro-patterned plate

then migrate and aggregate on the top surface of the nanopillars, and thereby tend to form uniform spheroids in each hole. 3D spheroid culture systems using a nanopillar plate of hepatocyte-like cells have been used to promote hepatocyte maturation [68].

As a large-scale culture system of primary hepatocytes, the bioreactor methods have been used. By employing various optimized conditions, including flow conditions [69] and cell densities [70], the bioreactor method has been shown to have advantages for maintaining the functions of primary hepatocytes in vitro in comparison with 2D culture [71, 72] and also for achieving effects of spontaneous differentiation from hESCs into hepatocytes [73]. It has been reported that 3D culture using a bioreactor induces more functional hepatocyte-like cells differentiated from hESCs than in the case of 2D culture [73]. The 3D culture methods using polymer scaffold systems have also demonstrated effectiveness both in culturing primary hepatocytes [74, 75] and in differentiation from ESCs into hepatocyte-like cells in vitro [76–78]. These data showed that hepatocyte-like cells could be differentiated from hESCs on a polymer scaffold.

Furthermore, cell-sheet engineering has recently been reported [79, 80]. Cell-sheet culture was performed by using a culture dish coated with a temperature-responsive polymer, poly (*N*-isopropylacrylamide) [81–83]. Several groups have adopted culture methods with a combination of 3D culture and co-culture (3D co-culture) and showed that the liver function of primary hepatocytes could be maintained at a higher level and for longer than without the coculture conditions [84–86]. Furthermore, the hepatic maturation of hESC-/hiPSC-derived hepatocyte-like cells by stratification of a Swiss 3T3 cell sheet using cell-sheet engineering was demonstrated. The hESC-/hiPSC-derived hepatocyte-like cells in the 3D co-culture system showed significantly up-regulated ALB expression in comparison with the case of 2D culture [87]. A 3D co-culture system would be expected to enhance the degree of maturation compared with a 2D culture.

In the last decade, the hepatic differentiation from hESCs/hiPSCs has been subjected to numerous challenges. Many groups have been struggling to develop the best differentiation protocols from hESCs/hiPSCs to hepatocyte-like cells. The hepatic differentiation efficiency, which is the population of ALB-positive cells, of over 80 % has been achieved in vitro from hESCs/hiPSCs. However, several hepatic functions, including expression levels of cytochrome P450 enzyme, of hESCs-/hiPSCs-derived hepatocyte-like cells are still lower than freshly isolated hepatocytes. New approaches that generate more effective and more functional hepatocyte-like cells may be developed in the near future. The hESC-/hiPSC-derived hepatocyte-like cells are expected to be a useful source of cells not only for drug discovery but also for the treatment of liver disease in the future medicine.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Impact of alpha-fetoprotein on hepatocellular carcinoma development during entecavir treatment of chronic hepatitis B virus infection

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Abstract

Background Entecavir (ETV) is one of the first-line nucleoside analogs for treating patients with chronic hepatitis B virus (HBV) infection. However, the hepatocellular carcinoma (HCC) risk for ETV-treated patients remains unclear.

Methods A total of 496 Japanese patients with chronic HBV infection undergoing ETV treatment were enrolled in this study. The baseline characteristics were as follows: age 52.6 ± 12.0 years, males 58 %, positive for hepatitis B e antigen 45 %, cirrhosis 19 %, and median HBV DNA level 6.9 log copies (LC) per milliliter. The mean treatment duration was 49.9 ± 17.5 months.

Results The proportions of HBV DNA negativity (below 2.6 LC/mL) were 68 % at 24 weeks and 86 % at 1 year, and the rates of alanine aminotransferase (ALT) level normalization were 62 and 72 %, respectively. The mean serum alpha-fetoprotein (AFP) levels decreased significantly at 24 weeks after ETV treatment initiation (from 29.0 ± 137.1 to 5.7 ± 27.9 ng/mL, $p < 0.001$). The cumulative incidence of HCC at 3, 5, and 7 years was 6.0, 9.6, and 17.2 %, respectively, among all enrolled patients. In a multivariate analysis, advanced age [55 years or older, hazard ratio (HR) 2.84; $p = 0.018$], cirrhosis (HR 5.59, $p < 0.001$), and a higher AFP level (10 ng/mL or greater) at 24 weeks (HR 2.38, $p = 0.034$) were independent risk

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factors for HCC incidence. HCC incidence was not affected by HBV DNA negativity or by ALT level normalization at 24 weeks.
Conclusions The AFP level at 24 weeks after ETV treatment initiation can be the on-treatment predictive factor for HCC incidence among patients with chronic HBV infection.

Keywords Hepatitis B virus · Entecavir · Risk factors for hepatocellular carcinoma incidence · Alpha-fetoprotein

Abbreviations

AFP	Alpha-fetoprotein
ALT	Alanine aminotransferase
cccDNA	Covalently closed circular DNA
ETV	Entecavir
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
IFN	Interferon
NA	Nucleos(t)ide analog
ROC	Receiver operating characteristic

Introduction

More than 350 million people worldwide have hepatitis B virus (HBV) infection, and persistent hepatic damage following HBV infection is associated with liver disease progression [1–3]. Chronic HBV infection accounts for approximately 52.3 % of hepatocellular carcinoma (HCC) cases worldwide [4], and antiviral treatment such as interferon (IFN) or nucleos(t)ide analogs (NAs) that aims to improve the prognosis of patients with chronic HBV infection has been developed [5]. Entecavir (ETV), one of the first-choice NAs, is a more potent antiviral agent with a higher genetic barrier to resistance than lamivudine; ETV administration over the long term has been reported to enable most patients to maintain a state of viral suppression [6–9]. With regard to the suppressive effect of NAs on HCC, in a randomized controlled trial of patients who were treated with lamivudine or placebo, the lamivudine-treatment group showed a significantly lower HCC rate than the placebo group during the observation period of 32.4 months (3.9 % vs 7.4 %, $p = 0.047$) [10]. In other cohort studies of patients who were treated with lamivudine, HCC incidence has been reported to be significantly lower in those who maintained low HBV DNA levels [less than 4 or 5 log copies (LC) per milliliter], especially in those with cirrhosis [11–13]. In contrast, the suppressive effect of ETV on HCC incidence remains unclear because a randomized controlled study of patients treated with ETV or placebo has not been performed.

To date, many studies have assessed the relationship between clinical factors and HCC incidence, such as male gender, advanced age, presence of cirrhosis, and high HBV DNA levels, during the natural course of chronic HBV infection [14, 15]. Among patients who were treated with IFN, it has been reported that hepatitis B e antigen seroconversion achieved with IFN treatment was associated with lower HCC incidence rates compared with nonseroconversion [16]. However, neither the pretreatment factors nor the on-treatment factors that are associated with HCC incidence among patients receiving ETV have been fully examined. ETV treatment for patients with chronic HBV infection reduces serum HBV DNA levels and may also have anti-inflammatory and antineoplastic effects. That is, among patients receiving ETV, various factors, such as HBV DNA, alanine aminotransferase (ALT), total bilirubin, albumin, and alpha-fetoprotein (AFP) levels, have the possibility to change and be associated with HCC suppression.

In this study, we evaluated the risk factors for HCC, especially the on-treatment factors in patients with chronic HBV infection who were undergoing ETV treatment.

Patients and methods

Study population

This study was a retrospective, multicenter study conducted by Osaka University Hospital and other institutions

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that participate in the Osaka Liver Forum. A total of 840 NA-naïve patients chronically infected with HBV started treatment with 0.5 mg of ETV per day between July 2004 and July 2012. Of these patients, we excluded 51 patients with HBV DNA levels under 3 LC/mL at the baseline, 13 patients who were co-infected with hepatitis C virus (HCV) or with human immunodeficiency virus, one patient who had undergone liver transplantation, and 140 patients with a history of HCC at the baseline. In addition, we excluded 51 patients who had been treated with ETV for less than 1 year and 88 patients who developed HCC within 1 year after the initiation of ETV treatment. As a result, 496 patients were enrolled in this cohort study. This study was conducted according to the ethical guidelines of the Declaration of Helsinki, amended in 2002, and was approved by the Institutional Review Board of Osaka University Hospital (approval number 12380-2).

HCC surveillance and data collection

The patients were followed up once every 3–6 months, and clinical symptoms, HBV DNA and other virological markers, complete blood count, liver biochemistry, and AFP levels were assessed. AFP levels measured between 20 and 28 weeks from the initiation of ETV treatment were regarded as valid AFP levels at 24 weeks. Ultrasonography of the abdomen, computed tomography, and/or magnetic resonance imaging was performed every 3–6 months for HCC surveillance. HCC was diagnosed by the presence of typical hypervascular characteristics evident on the computed tomography and/or magnetic resonance imaging scans. If no typical signs of HCC were observed, either hepatic angiography or fine-needle aspiration biopsy was performed with the patient's consent, or the patient was carefully followed until a diagnosis was possible on the basis of a definite observation. Liver cirrhosis was defined by a shrunken, small liver with a nodular surface as noted on liver imaging and by clinical features of portal hypertension.

Definition of treatment response

The surveillance start date was defined as the time of ETV treatment initiation. HBV DNA was measured by the COBAS Amplicor HBV Monitor Test (Roche Diagnostics, Tokyo, Japan) with a linear range of detection from 2.6 to 7.6 LC/mL or by the COBAS Taqman HBV Test v2.0 (Roche Diagnostics) with a linear range of detection from 2.1 to 9.0 LC/mL. The achievement of a virological response by ETV treatment was defined by serum HBV DNA levels that were continuously under 2.6 LC/mL. ALT level normalization was defined by serum ALT levels that were 30 IU/L or less.

Statistical analyses

Statistical analyses were performed using SPSS version 19.0 (IBM, Armonk, NY, USA) and SAS for Windows version 9.3 (SAS Institute, Cary, NC, USA). The continuous variables were expressed as the mean ± standard deviation or standard error of the mean or as the median (range), as appropriate, whereas the categorical variables were expressed as frequencies. The Wilcoxon signed-rank sum test was used to analyze differences between continuous variables before and after treatment. The cutoff value of AFP levels at 24 weeks from the initiation of ETV treatment for prediction of HCC incidence was assessed by the time-dependent receiver operating characteristic (ROC) curve, and the 95 % confidence interval for the area under the ROC curve was constructed using the bootstrap method. The Kaplan–Meier method was used to assess the cumulative HCC incidence, and the groups were compared using the log-rank test. The Cox proportional-hazards model was used to identify the independent factors associated with HCC incidence. The factors that were selected as significant by simple Cox regression analysis were evaluated by multiple Cox regression analysis. The risks were expressed as hazard ratios and 95 % confidence intervals. We considered $p < 0.05$ as significant.

Results

The characteristics of the 496 patients at the baseline and at 24 weeks after ETV treatment initiation are summarized in Table 1. The average age of the patients was 52.6 ± 12.0 years at the baseline, and there were 288 males (58 %) and 92 patients with cirrhosis (19 %). The patients were followed up for an average of 49.9 ± 17.5 months.

The cumulative incidence of virological response (HBV DNA level less than 2.6 LC/mL) at 24 weeks, 1 year, and 3 years after the initiation of ETV treatment was 68, 86, and 95 %, respectively. The median levels of HBV DNA were significantly decreased among noncirrhotic (6.9 LC/mL to less than 2.6 LC/mL, $p < 0.001$) and cirrhotic (6.9 LC/mL to less than 2.6 LC/mL, $p < 0.001$) patients from the baseline to 24 weeks after ETV treatment initiation (Table 1). ALT level normalization (30 IU/L or lower) was achieved in 62 % of patients at 24 weeks and in 72 % of patients at 1 year. The median ALT levels were significantly decreased among noncirrhotic (72.0–25.0 IU/L, $p < 0.001$) and cirrhotic (51.0–29.0 IU/L, $p < 0.001$) patients from the baseline to 24 weeks after ETV treatment initiation. The following parameters were also significantly increased from the baseline to 24 weeks after ETV treatment initiation: platelet counts and serum albumin levels

Table 1 Characteristics of patients at the baseline and 24 weeks after initiation of entecavir (ETV) treatment

	All patients, $n = 496$		Noncirrhotic patients, $n = 404$		Cirrhotic patients, $n = 92$	
	Baseline	24 weeks	Baseline	24 weeks	Baseline	24 weeks
Age (years)	52.6 ± 12.0 (15–82)		51.3 ± 12.1 (15–82)		58.2 ± 9.8 (32–81)	
Gender: male/female	288/208 (58 %)		233/171 (58 %)		55/37 (60 %)	
HBeAg[a]: positive/negative	220/270 (45 %)		181/219 (45 %)		39/51 (43 %)	
Histology[b], activity: A0/1/2/3	3/82/74/14		3/75/63/12		0/7/11/2	
Histology[b], fibrosis: F0/1/2/3/4	8/63/51/32/20		8/63/52/32/0		0/0/0/0/20	
History of IFN therapy: presence	50 (11 %)		44 (11 %)		6 (7 %)	
Platelet count ($\times 10^4/\mu L$)	16.0 ± 5.8	16.5 ± 6.4*	17.3 ± 5.2	17.7 ± 5.3*	10.3 ± 5.8	11.5 ± 7.9
Total bilirubin (mg/dL)	1.01 ± 1.48	0.83 ± 0.45*	0.91 ± 0.95	0.78 ± 0.42*	1.45 ± 2.78	1.09 ± 0.48
Albumin (g/dL)	3.94 ± 0.52	4.11 ± 0.44*	4.03 ± 0.44	4.18 ± 0.39*	3.56 ± 0.64	3.79 ± 0.50*
PT (%)	83.8 ± 16.3		86.7 ± 15.7		72.4±16.3	
ALT (IU/L)	143.7 ± 199.3 (9–1,885)	29.6 ± 16.5* (6–166)	156.1 ± 210.8 (9–1,885)	29.2 ± 16.9* (6–166)	89.2 ± 124.7 (12–763)	31.5 ± 14.0* (10–84)
ALT ≤ 30 (IU/L)	11 %	62 %	10 %	64 %	13 %	53 %
30 < ALT ≤ 60 (IU/L)	31 %	33 %	28 %	31 %	48 %	43 %
60 < ALT (IU/L)	58 %	5 %	62 %	5 %	39 %	4 %
HBV DNA (LC/mL) (median)	6.9	<2.6*	6.9	<2.6*	6.9	<2.6*
HBV DNA < 2.6 (LC/mL)	–	68 %	–	68 %	–	70 %
2.6 ≤ HBV DNA < 4.0 (LC/mL)	4 %	24 %	4 %	21 %	3 %	30 %
4.0 ≤ HBV DNA (LC/mL)	96 %	8 %	96 %	11 %	97 %	0 %
AFP (ng/mL)[c]	29.0 ± 137.1 (1–2,225)	5.7 ± 7.9* (1–126)	29.5 ± 152.7 (1–2,225)	4.9 ± 4.6* (1–126)	27.4 ± 48.0 (1–318)	9.3 ± 14.6* (1–52)
Observation periods (months)	49.9 ± 17.5 (14–109)		49.2 ± 17.6 (14–109)		52.8 ± 16.6 (18–82)	

Data are expressed as the mean ± standard deviation except for hepatitis B virus (*HBV*) DNA (median)

AFP alpha-fetoprotein, *ALT* alanine aminotransferase, *HBeAg* hepatitis B e antigen, *IFN* interferon, *LC* log copies, *PT* prothrombin time

* $p < 0.05$ (Wilcoxon signed-rank sum test)

[a] HBeAg measurement at the baseline was missing in six patients

[b] Liver biopsy was performed in 174 patients

[c] AFP data were missing in 78 noncirrhotic patients and five cirrhotic patients with cirrhosis

among noncirrhotic patients ($p = 0.008$ and $p < 0.001$, respectively) and serum albumin levels in cirrhotic patients ($p < 0.001$).

Mean serum AFP levels decreased significantly from 29.0 ± 137.1 ng/mL at the baseline to 5.7 ± 7.9 ng/mL at 24 weeks after the initiation of ETV treatment ($p < 0.001$). Mean AFP levels were assessed according to the severity of liver disease and decreased significantly from the baseline to 24 weeks in both the noncirrhotic group and the cirrhotic group (noncirrhotic group 29.5 ± 152.7 to 4.9 ± 4.6 ng/mL, $p < 0.001$; cirrhotic group 27.4 ± 48.0 to 9.3 ± 14.6 ng/mL, $p < 0.001$; Table 1). The proportion of patients with AFP levels below 10 ng/mL increased from 73 % at the baseline to 95 % at 24 weeks among noncirrhotic patients and from 48 % at the baseline to 76 % at 24 weeks among cirrhotic patients (Fig. 1).

A total of 42 patients developed HCC during the observation period (16 noncirrhotic patients, 26 cirrhotic patients). The cumulative incidence of HCC at 3, 5, and 7 years was 6.0, 9.6, and 17.2 %, respectively. The mean time point of HCC development was 34.0 ± 18.4 months from the initiation of ETV treatment. AFP levels among patients who developed HCC decreased from 24 weeks (13.1 ± 3.9 ng/mL) (mean ± standard error of the mean) to 48 weeks (10.2 ± 3.0 ng/mL) after the initiation of ETV treatment and increased again from 24 weeks before HCC incidence (7.6 ± 1.6 ng/mL) to the time of HCC incidence (35.4 ± 12.8 ng/mL) (Fig. S1). The cutoff value of AFP levels at 24 weeks from the initiation of ETV treatment for prediction of HCC incidence was set as 10 ng/mL on the basis of the calculated cutoff value (12.1 ng/mL) assessed using the time-dependent ROC curve (Table S1).

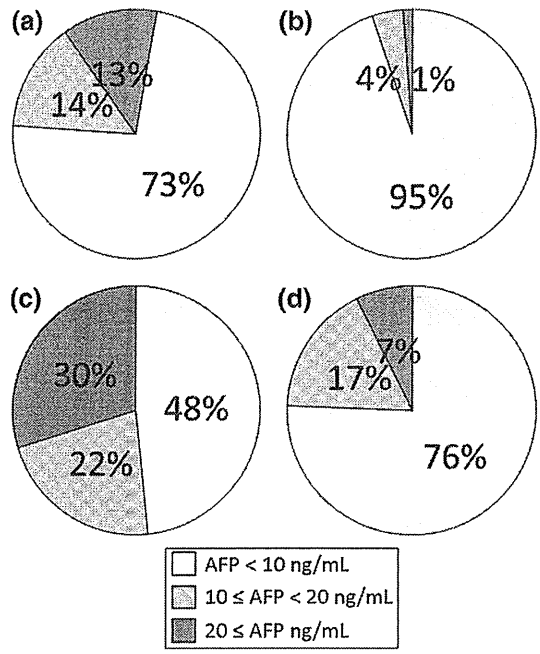

Fig. 1 Distribution of alpha-fetoprotein (*AFP*) levels at the baseline and at 24 weeks after the initiation of entecavir (ETV) treatment according to the severity of liver disease: **a** patients without cirrhosis at the baseline ($n = 326$); **b** patients without cirrhosis at 24 weeks after ETV treatment initiation ($n = 326$); **c** patients with cirrhosis at the baseline ($n = 87$); **d** patients with cirrhosis at 24 weeks after ETV treatment initiation ($n = 87$)

Factors associated with HCC incidence at the baseline

In a univariate analysis, factors at the baseline such as advanced age, cirrhosis, lower platelet counts, and higher total bilirubin, lower albumin, and higher AFP levels were significant, and a multivariate analysis demonstrated that advanced age (55 years or older) and cirrhosis were significant independent risk factors for HCC incidence (Table 2). After a stratified analysis of HCC incidence according to those risk factors at the baseline, the cumulative incidence of HCC at 5 years was 2.5 % in younger patients (younger than 55 years) and 18.6 % in older patients (55 years or older, $p < 0.001$; Fig. 2a). The cumulative incidence of HCC at 5 years was 5.3 % in noncirrhotic patients and was 30.0 % in cirrhotic patients ($p < 0.001$; Fig. 2b).

Factors associated with HCC incidence at 24 weeks after the initiation of ETV treatment

The association between HCC incidence and posttreatment factors at 24 weeks after the initiation of ETV treatment was estimated. In a univariate analysis, advanced age, cirrhosis, lower platelet counts,and lower albumin, higher total bilirubin, and higher AFP levels at 24 weeks were significant, and a multivariate analysis showed that a higher AFP level (10 ng/mL or greater) at 24 weeks was the only additional factor independently associated with HCC incidence other than advanced age and cirrhosis, which were found to be significant risk factors at the baseline (Table 3). The cumulative incidence of HCC at 5 years was 8.2 % among patients with an AFP level below 10 ng/mL at 24 weeks and was 34.2 % among patients with an AFP level of 10 ng/mL or higher at 24 weeks (Fig. 3a). Although the American Association for the Study of Liver Disease practical guidelines for chronic hepatitis B indicate that the aims of treatment for patients infected with HBV are to achieve a reduction in the serum HBV DNA levels and a normalization of serum ALT levels [17], in this study, neither virological response nor biochemical response (ALT level of 30 IU/L or lower) at 24 weeks by ETV treatment affected HCC incidence (Table 3). The cumulative incidence of HCC was almost equivalent between patients with and without virological response at 24 weeks in the analysis among all enrolled patients ($p = 0.685$; Fig. 3b). Additionally, there was no significant difference in the cumulative incidence of HCC between patients with or without normalization of ALT levels at 24 weeks ($p = 0.076$; Fig. 3c). The cumulative incidence of HCC significantly increased with higher AFP levels (10 ng/mL or greater) at 24 weeks even among patients who achieved virological response ($p = 0.023$) or normalization of ALT levels at 24 weeks ($p = 0.002$). The AFP levels at 24 weeks were closely related to HCC incidence irrespective of the virological response or biochemical response at 24 weeks in patients with HBV infection who were undergoing treatment with ETV.

The impact of AFP at 24 weeks on HCC incidence according to baseline factors

Because AFP levels at 24 weeks were found to be a significant factor related to HCC incidence among multiple factors that varied during treatment, the impact of AFP at 24 weeks on HCC incidence was assessed in the subgroups stratified by HCC-related factors at the baseline: age and the severity of liver disease. In the subgroup analysis stratified by age, AFP levels at 24 weeks were significantly related to HCC incidence, and the cumulative incidence of HCC at 5 years was significantly higher in patients with AFP levels of 10 ng/mL or higher at 24 weeks than those with AFP levels below 10 ng/mL, irrespective of age (younger than 55 years, 16.1 % vs 2.2 %, $p = 0.009$; 55 years or older, 45.4 % vs 14.9 %, $p < 0.001$; Fig. 4a, b). In the subgroup analysis that was stratified according to the severity of liver disease, the AFP level at 24 weeks was a significant factor in the cirrhotic group ($p = 0.029$) but not in the noncirrhotic group ($p = 0.377$); the cumulative incidence of HCC at 5 years in the cirrhotic group was

Table 2 Risk factors at the baseline for hepatocellular carcinoma (HCC) incidence in chronic hepatitis B patients receiving ETV treatment (Cox proportional-hazards model)

Factors	Category	Univariate analysis		*p*	Multivariate analysis		
		HR	95 % CI		HR	95 % CI	*p*
Age (years)	0:<55	1	2.601–13.243	<0.001	1	1.592–8.560	0.002
	1:≥55	5.869			3.691		
Gender	0:male	1	0.365–1.319	0.265			
	1:female	0.694					
Severity of liver disease	0:no cirrhosis	1	4.050–14.085	<0.001	1	2.415–9.404	<0.001
	1:cirrhosis	7.553			4.765		
HBeAg	0:negative	1	0.412–1.436	0.410			
	1:positive	0.770					
Histology: activity	0:A0-1	1	0.352–3.800	0.810			
	1:A2-3	1.157					
Histology: fibrosis	0:F0-2	1	0.865–5.910	0.096			
	1:F3-4	2.262					
History of IFN therapy	0:none	1	0.032–1.718	0.154			
	1:presence	0.236					
Platelet count ($\times 10^4/\mu L$)	0:<15	1	0.103–0.449	<0.001			
	1:≥15	0.215					
Total bilirubin (mg/dL)	0:<1.0	1	1.235–4.141	0.008			
	1:≥1.0	2.261					
Albumin (g/dL)	0:<4.0	1	0.201–0.725	0.003			
	1:≥4.0	0.381					
PT (%)	0:<80	1	0.301–1.056	0.074			
	1:≥80	0.564					
ALT (IU/L)	0:<80	1	0.345–1.246	0.197			
	1:≥80	0.656					
HBV DNA(LC/mL)	0:<6.5	1	0.748–2.701	0.283			
	1:≥6.5	1.422					
AFP (ng/mL)	0:<10	1	1.040–3.721	0.038			
	1:≥10	1.967					

CI confidence interval, *HR* hazard ratio

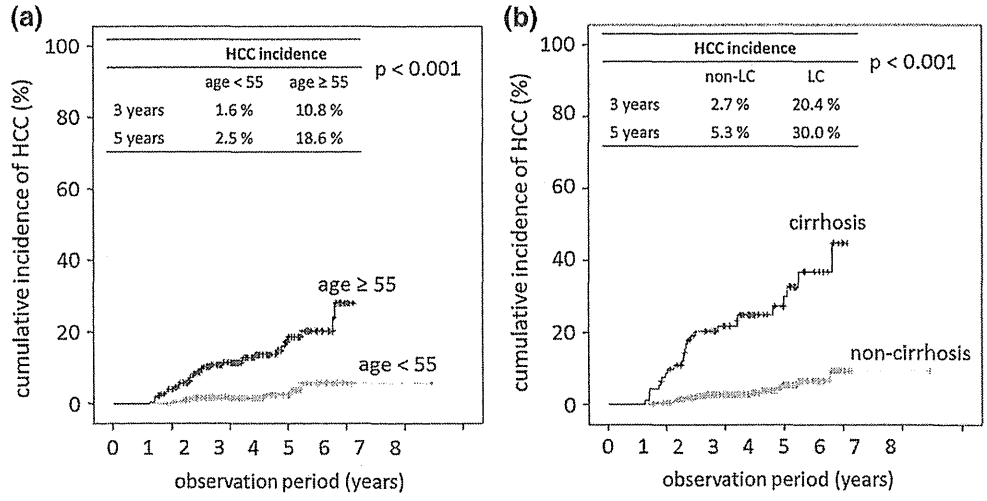

Fig. 2 Cumulative hepatocellular carcinoma (*HCC*) incidence among patients with hepatitis B virus (HBV) infection according to factors at the baseline (log-rank test). **a** Cumulative HCC incidence according to the age at the baseline (*black line* 55 years or older, *gray line* younger than 55 years). **b** Cumulative HCC incidence according to the severity of liver disease (*black line* cirrhosis, *gray line* no cirrhosis)

Table 3 Risk factors at 24 weeks after initiation of ETV treatment for HCC incidence in chronic hepatitis B patients receiving ETV treatment (Cox proportional-hazards model)

Factors	Category	Univariate analysis			Multivariate analysis		
		HR	95 % CI	*p*	HR	95 % CI	*p*
Age (years)	0:<55	1	2.601–13.243	<0.001	1	1.198–6.748	0.018
	1:≥55	5.869			2.843		
Gender	0:male	1	0.365–1.319	0.265			
	1:female	0.694					
Severity of liver disease	0:no cirrhosis	1	4.050–14.085	<0.001	1	2.518–12.411	<0.001
	1:cirrhosis	7.553			5.590		
Platelet count ($\times 10^4/\mu L$) at 24 weeks	0:<15	1	0.114–0.473	<0.001			
	1:≥15	0.233					
Total bilirubin (mg/dL) at 24 weeks	0:<1.0	1	1.360–4.569	0.003			
	1:≥1.0	2.493					
Albumin (g/dL) at 24 weeks	0:<4.0	1	0.201–0.725	0.003			
	1:≥4.0	0.381					
ALT (IU/L) at 24 weeks	0:≤30	1	0.938–3.157	0.080			
	1:>30	1.720					
VR[a] at 24 weeks	0:none	1	0.461–1.664	0.685			
	1:presence	0.875					
AFP (ng/mL) at 24 weeks	0:<10	1	2.589–11.496	<0.001	1	1.066–5.316	0.034
	1:≥10	5.456			2.381		

VR virological response

[a] VR is defined as HBV DNA of less than 2.6 LC/mL

higher in patients with AFP levels of 10 ng/mL or greater at 24 weeks than in those with AFP levels below 10 ng/mL (50.0 % vs 24.7 %; Fig. 4c, d).

Risk analysis for HCC incidence among patients who achieved virological response by ETV treatment

Among patients with HBV infection who achieved virological response by ETV treatment, the risk analysis for HCC incidence was performed in a Cox proportional-hazards model according to the number of the following three risk factors: AFP levels at 24 weeks, age, and the presence of cirrhosis (Fig. S2). When the AFP level remained high (10 ng/mL or higher) at 24 weeks, the cumulative incidence of HCC at 5 years was 6.7 % with no other risk factors (Fig. S2a), 14.8 % with the factor of age of 55 years or older, 27.9 % with the factor of cirrhosis, and 57.7 % with the factors of age of 55 years or older and cirrhosis (Fig. S2b).

Discussion

ETV treatment has been reported to reduce serum HBV DNA levels and ALT levels in patients with chronic HBV infection and to improve hepatitis [18]. On the basis of a study that showed that a higher HBV DNA level at the baseline is associated with a higher HCC incidence in the natural history cohort (the REVEAL study) [15], a reduction of HBV DNA levels by ETV treatment has been considered to have the possibility to suppress HCC incidence among patients with chronic HBV infection. However, it was still unknown whether a lower or an undetectable level of serum HBV DNA, which was achieved by ETV treatment, has a suppressive effect on HCC incidence as shown in the natural course. In the present study, factors associated with HCC incidence during ETV treatment among patients with chronic HBV infection were investigated.

In a previous study that used a historical control group, a significant suppressive effect of ETV on HCC incidence was shown in cirrhotic but not noncirrhotic patients [19]. Furthermore, Wong et al. [20] reported that HCC incidence was significantly lower among patients with cirrhosis who had undetectable levels of HBV DNA compared with those with detectable levels of HBV DNA. In the present study, reduced serum HBV DNA levels were associated with a decrease in the cumulative incidence of HCC only in patients with cirrhosis, and not in those without cirrhosis (Fig. S3). Originally, HBV covalently closed circular DNA (cccDNA) levels in the hepatocyte nuclei were nearly parallel to the serum HBV DNA levels in the natural

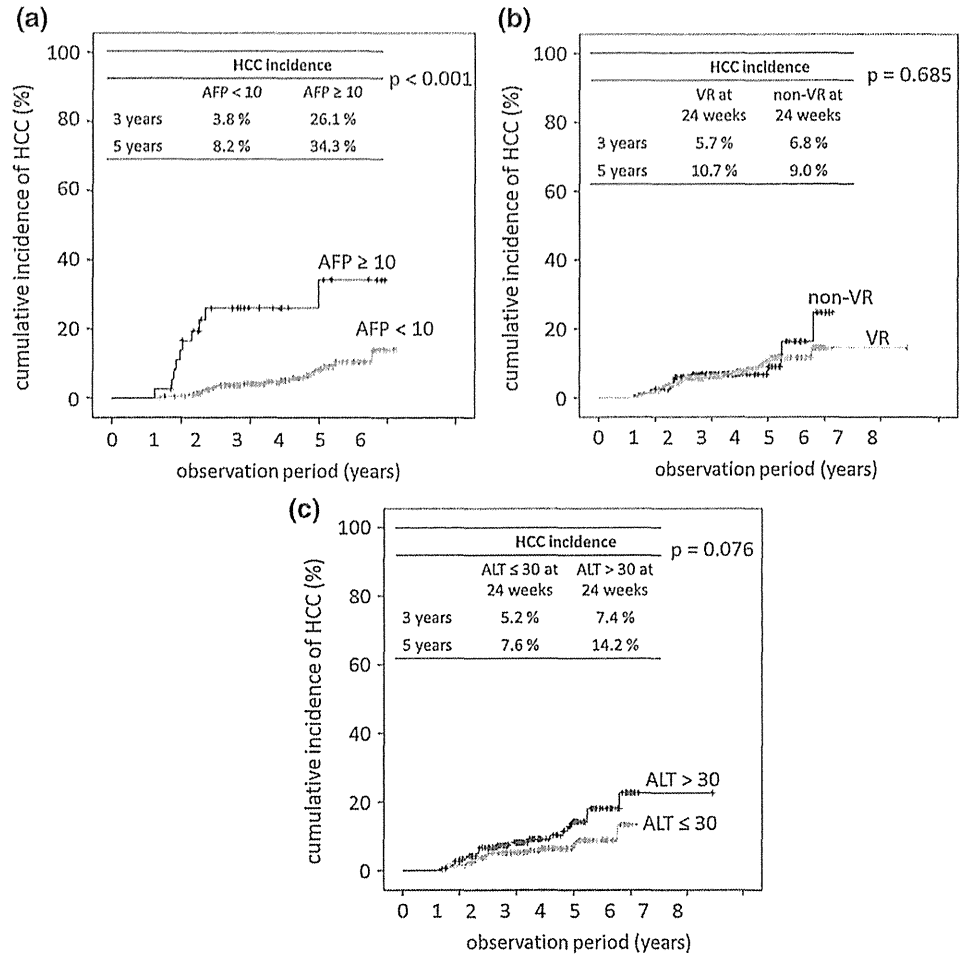

Fig. 3 Cumulative HCC incidence among patients with HBV infection according to factors at 24 weeks after ETV treatment initiation (log-rank test). Virological response (*VR*) is defined as HBV DNA of less than 2.6 log copies per milliliter. **a** Cumulative HCC incidence according to AFP levels at 24 weeks (*back line* AFP level of 10 ng/mL or greater at 24 weeks, *gray line* AFP level below 10 ng/mL at 24 weeks). **b** Cumulative HCC incidence according to virological response at 24 weeks (*black line* no VR at 24 weeks, *gray line* VR at 24 weeks). **c** Cumulative HCC incidence according to biochemical response at 24 weeks [*black line* alanine aminotransferase (*ALT*) level above 30 IU/L at 24 weeks, *gray line* ALT level of 30 IU/L or lower at 24 weeks]

course. However, low levels of serum HBV DNA achieved by ETV treatment do not always indicate low intracellular HBV cccDNA levels [21, 22]. Therefore, it is possible that an insufficient decrease of intracellular HBV DNA levels cannot bring the apparent HCC suppression in noncirrhotic liver with low malignant potential. A longer observation period is required to clarify the suppressive effect on HCC incidence among noncirrhotic patients. The relationship between HBV cccDNA levels in the liver and HCC incidence should also be examined.

In this study, in the analysis of the relationship between on-treatment factors and HCC incidence, only higher AFP levels (10 ng/mL or higher) at 24 weeks after the initiation of ETV treatment were found to be associated with HCC incidence. This is the first study to investigate the significance of AFP levels as a representative marker for the potential of HCC development among patients with chronic HBV infection undergoing ETV treatment. Originally, AFP was known as a tumor-associated antigen in HCC and as a target for immunotherapy. AFP has been used in the surveillance of HCC and in the evaluation of treatment response in HCC patients. The use of AFP as a marker to identify HCC among patients with HBV infection has previously been shown in patients with a natural course of the disease [23]. In recent reports that have focused on AFP levels for HCC diagnosis in patients undergoing ETV treatment, elevated AFP levels at 6 months before or at the time of HCC incidence were shown to be useful in detecting existing HCC [24, 25]; that is, elevated AFP levels implied the existence of cancer cells. However, the present study clarified that a high AFP level at 24 weeks did not suggest the existence of cancer cells, but indicates a potential for HCC incidence before the initiation of carcinogenesis. A possible reason is as follows. The AFP levels among patients who developed HCC decreased from 24 to 48 weeks after the initiation of ETV treatment and increased again from 24 weeks before HCC incidence to the time of HCC incidence. Furthermore, it took a considerably long time before HCC incidence, on average 32.6 months of the observation period (Fig. S1). With regard to the relationship between serum AFP levels and HCC incidence among HCV-infected patients, AFP levels at 24 weeks after the end of IFN treatment have been associated with HCC [26, 27]. AFP levels after the

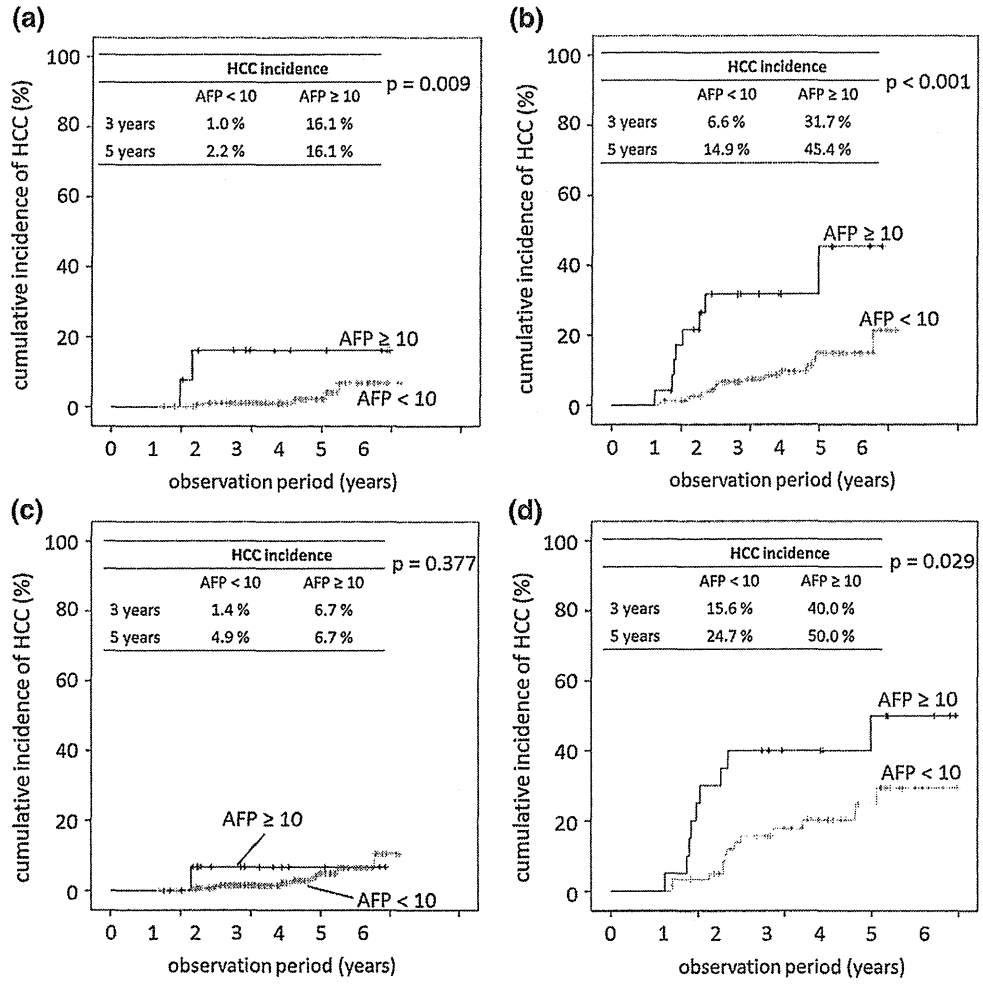

Fig. 4 Cumulative HCC incidence among patients with HBV infection according to AFP levels at 24 weeks after ETV treatment initiation, stratified with baseline factors (log-rank test). **a** Patients younger than 55 years. **b** Patients 55 years or older. **c** Patients without cirrhosis. **d** Patients with cirrhosis. *Black line* AFP level of 10 ng/mL or higher at 24 weeks, *gray line* AFP level below 10 ng/mL at 24 weeks

initiation of treatment of both HBV infection and HCV infection appear to have important implications for HCC incidence.

What the AFP levels at 24 weeks actually represent in patients undergoing ETV treatment is uncertain. The AFP level is a surrogate marker that appears to predict a disease condition from various pathological factors including inflammation, fibrosis, and liver regeneration, which involve carcinogenesis. Moreover, a previous study reported that the activation of natural killer cells by dendritic cells was inhibited when they were co-cultured with AFP; this result suggests an association between HCC development and the maintenance of high AFP levels [28]. Therefore, AFP is thought to be an important biomarker that can reflect various aspects of liver disease.

American Association for the Study of Liver Disease practice guidelines for the management of HBV have defined the goal of NA treatment as to decrease serum HBV DNA levels to undetectable levels to suppress HCC development. In this study, the HBV DNA levels and ALT levels were rapidly lowered in most patients. However, this study shows that the virological and biochemical treatment responses had no association with HCC development, whereas advanced age, liver cirrhosis, and a higher AFP level at 24 weeks after the initiation of ETV treatment were independent risk factors that were significantly associated with HCC development. It is considered that decreasing serum HBV DNA levels to undetectable levels is the necessary, but not sufficient condition to suppress HCC development. In fact, the HCC incidence rate even in patients undergoing ETV treatment who achieved virological response at 24 weeks with the three factors of age of 55 years or older, liver cirrhosis, and AFP level of 10 ng/mL or higher increased to as high as approximately 60 % at 5 years (Fig. S2). Accordingly, the undetectable HBV DNA level in patients with chronic HBV infection undergoing ETV treatment is in itself of little consequence and does not mean a riskless environment.

The limitation of this study is that analysis including other HCC-related factors, such as hepatitis B surface antigen levels, precore and core promotor mutations, and family history of HCC or alcohol consumption, was not