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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Predictive value of tumor markers for hepatocarcinogenesis in patients with hepatitis C virus

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Abstract
Background Increases in tumor markers are sometimes seen in patients with chronic liver disease without hepatocellular carcinoma (HCC). The aim of this study was to determine the relationship between the levels of three tumor markers [alpha-fetoprotein (AFP), *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3%), and des-γ-carboxy prothrombin (DCP)] and hepatic carcinogenesis to identify hepatitis C virus (HCV) carriers at high risk for cancer development.
Methods A total of 623 consecutive HCV carriers with follow-up periods of >3 years were included. The average integration values were calculated from biochemical tests, and tumor markers, including AFP, AFP-L3%, and DCP, and factors associated with the cumulative incidence of HCC were analyzed.
Results HCC developed in 120 (19.3%) of the 623 patients. Age >65 years [adjusted relative risk, 2.303 (95% confidence interval, 1.551–3.418), $P < 0.001$], low platelet count [3.086 (1.997–4.768), $P < 0.001$], high aspartate aminotransferase value [3.001 (1.373–6.562), $P < 0.001$], high AFP level [≥10, <20 ng/mL: 2.814 (1.686–4.697), $P < 0.001$; ≥20 ng/mL: 3.405 (2.087–5.557), $P < 0.001$] compared to <10 ng/mL, and high AFP-L3% level [≥5, <10%: 2.494 (1.291–4.816), $P = 0.007$; ≥10%: 3.555 (1.609–7.858), $P < 0.001$] compared to <5% were significantly associated with an increased incidence of HCC on multivariate analysis.
Conclusions Increased AFP or AFP-L3% levels were significantly associated with an increased incidence of HCC. Among HCV carriers, patients with ≥10 ng/mL AFP or patients with ≥5% AFP-L3% are at very high risk for the development of HCC even if AFP is less than 20 ng/mL or AFP-L3% is less than 10%, which are the most commonly reported cutoff values.

Keywords Alpha-fetoprotein (AFP) · *Lens culinaris* agglutinin-reactive fraction of AFP · Hepatic regeneration · Necroinflammatory activity · Hepatocarcinogenesis

Introduction

Serum alpha-fetoprotein (AFP) is a widely used marker for hepatocellular carcinoma (HCC) [1]. However, serum AFP levels are increased in patients with liver diseases other than HCC, including viral hepatitis [2–4], with a prevalence of 10–42% [2, 5–7]. Increases in AFP are a marker of hepatic regeneration following hepatocyte destruction in viral hepatitis [8]. However, the pathogenesis and clinical significance of this phenomenon remain unclear.

The *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3%) and des-γ-carboxy prothrombin (DCP) are also markers for HCC [9–12]. Available data suggest that these tumor markers are more highly specific for HCC than AFP alone [9]. However, there are no reports examining the prognostic value of these markers in hepatocarcinogenesis.

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Results of biochemical tests, including tumor markers, can fluctuate for a given patient and can vary between different patients, and repeated measurements over time may provide a more accurate picture of disease development or progression. The arithmetic mean value is often used to assess biochemical parameters over time, but this value can be greatly affected by the interval between measurements such that a short period of very high values can inappropriately skew the mean. We have previously argued that the average integration value is more meaningful than the arithmetic mean value for the purposes of monitoring disease progression [13, 14].

The aim of this study was to determine the relationship between three tumor markers (AFP, AFP-L3%, and DCP) to better identify hepatitis C virus (HCV) carriers at high risk for the development of HCC. Of note, we used the average integration values of these parameters in our analysis.

Patients, materials, and methods

Patient selection

A total of 1623 consecutive patients positive for anti-HCV antibody visiting the Department of Gastroenterology at Ogaki Municipal Hospital during the period January 1995 to December 1997 were considered for enrollment. The present study cohort included the following criteria for enrollment: (1) positive for anti-HCV antibody by second- or third-generation enzyme-linked immunosorbent assay and detectable HCV RNA for at least 6 months; (2) no evidence of positivity for hepatitis B surface antigen; (3) exclusion of other causes of chronic liver disease (i.e., alcohol consumption lower than 80 g/day, no history of hepatotoxic drug use, and negative tests for autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and Wilson's disease); (4) follow-up period greater than 3 years; (5) measurement of AFP, AFP-L3%, and DCP at least every 6 months; (6) no evidence of HCC for at least 3 years from the start of the observation periods; and (7) interferon (IFN) therapy completed greater than 3 years before the detection of HCC in patients who received IFN therapy. A total of 623 patients fulfilled these criteria (Fig. 1).

Fibrosis was histologically evaluated in 187 of the 623 patients and staged according to Desmet et al. [15] as follows: F0, no fibrosis; F1, mild fibrosis; F2, moderate fibrosis; F3, severe fibrosis; and F4, cirrhosis. The remaining 436 patients were evaluated by ultrasound (US) findings and biochemical tests. The diagnosis of cirrhosis was made according to typical US findings, e.g., superficial nodularity, a coarse parenchymal echo pattern, and signs of portal hypertension (splenomegaly >120 mm, dilated portal vein diameter >12 mm, patent collateral veins, or ascites) [16–18]. In this study patients who did not satisfy these criteria were classified as having chronic hepatitis. Four hundred and sixty-three patients were diagnosed with chronic hepatitis and 160 patients with cirrhosis.

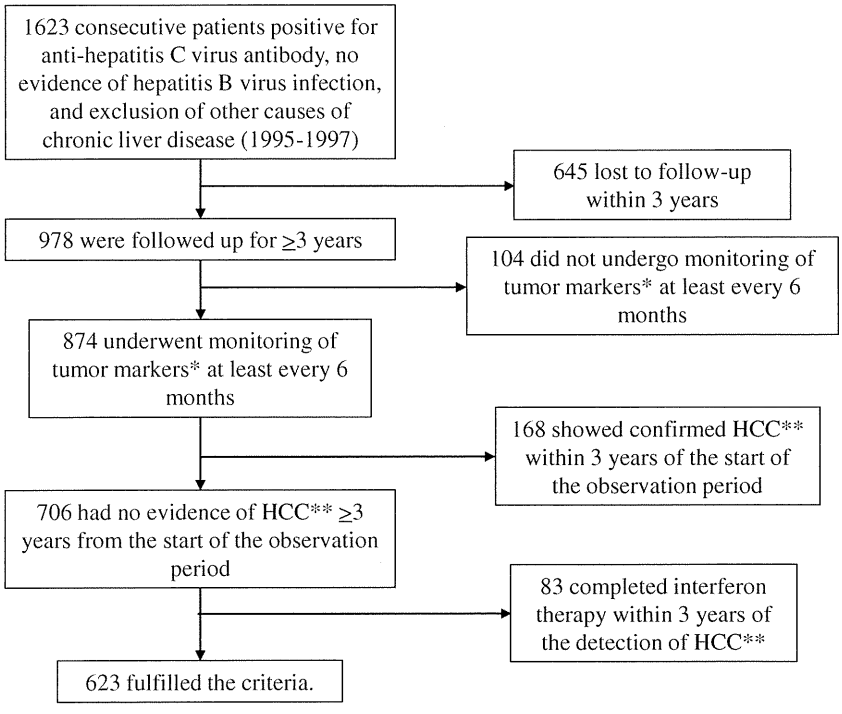

Fig. 1 Schematic flowchart of enrolled patients. *Serum alpha-fetoprotein (AFP), *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3%), and des-γ-carboxy prothrombin (DCP). **Hepatocellular carcinoma (*HCC*)

All patients were followed up at our hospital at least twice a year. During each follow-up examination, platelet count, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transpeptidase (γ-GTP), total bilirubin, cholinesterase, alkaline phosphatase (ALP), lactate dehydrogenase (LDH), albumin, total cholesterol, AFP, AFP-L3%, and DCP were measured. Platelet count and ALT, AST, γ-GTP, total bilirubin, cholinesterase, ALP, LDH, albumin, total cholesterol, AFP, AFP-L3%, and DCP values were expressed as average integration values [13, 14]. Briefly, using ALT as an example, the area of a trapezoid is calculated by multiplying the sum of two ALT values by one-half of the interval between the measurements. This value is then divided by the observation period to obtain the average integration value, and this technique provides a better representation of values over time when there are extremes of high and low values [14, 16]. In patients who developed HCC during the observation period, AFP, AFP-L3%, and DCP values obtained at least 1 year before the diagnosis of HCC were assessed. Serum AFP concentration was determined with a commercially available kit. AFP-L3% was measured by lectin-affinity electrophoresis and antibody-affinity blotting with the AFP Differentiation Kit L (Wako Pure Chemical Industries, Osaka, Japan) [10]. DCP was measured with a DCP reagent (Picolumi PIVKA-II; Eizai, Tokyo, Japan) [11]. Cutoff levels for AFP, AFP-L3%, and DCP were set at 20 ng/mL, 10%, and 40 mAU/mL, respectively, according to previous reports [10–12]. HCV genotype and quantification of HCV RNA (Amplicor 2; Roche Diagnostics, Tokyo, Japan) were determined in 513 cases. All patients underwent imaging modalities (US, computed tomography [CT], or magnetic resonance imaging [MRI]), every 3 months in patients with cirrhosis and every 6 months in patients with chronic hepatitis.

The diagnoses of HCC were confirmed by histologic examination of resected hepatic tumors or US-guided needle biopsy specimens. When biopsy of the tumor was contraindicated, the HCC diagnosis was made using clinical criteria and imaging findings obtained from B-mode US, CT angiography, or MRI [19, 20]. HCC was histologically diagnosed in 46 patients, and in the remaining 74 patients, the diagnosis was made based on clinical criteria [19, 20]. All tumors were 3 cm or less in maximum diameter, and there were 3 nodules or less on diagnosis.

One hundred eighty-nine patients received IFN therapy. Patients were classified into three groups according to the type of response to IFN therapy: sustained virologic response (SVR), defined as the absence of serum HCV RNA at 6 months after IFN therapy; the non-SVR group, defined as the presence of serum HCV RNA at 6 months after IFN therapy; and the no IFN therapy group.

Patients were classified into three groups for each of the tumor markers according to the average integration values of AFP, AFP-L3%, and DCP: A1, <10 ng/mL ($n = 452$); A2, ≥10, <20 ng/mL ($n = 80$); and A3, ≥20 ng/L ($n = 91$); L1, <5% ($n = 588$); L2, ≥5, <10% ($n = 18$); and L3, ≥10% ($n = 17$); and D1, <20 mAU/mL ($n = 379$); D2, ≥20, <40 mAU/mL ($n = 170$); and D3, ≥40 mAU/mL ($n = 51$), respectively.

The present study ended on 31 December 2008 or the date of identification of HCC occurrence. The median follow-up period was 9.0 years (range 3.0–13.0 years). The total number of blood examinations was 25.721, and the median number of blood examinations was 23 (range 6–105) per subject.

Statistical analysis

Statistical analysis was performed with the Statistical Program for Social Science (SPSS ver.17.0 for Windows; SPSS Japan, Tokyo, Japan). Continuous variables are shown as medians (ranges). The Mann–Whitney *U*-test was used for continuous variables, and Fisher's exact test was used for categorical variables. Actuarial analysis of the cumulative incidence of hepatocarcinogenesis was performed by the Kaplan–Meier method, and differences were tested by the log-rank test. The Bonferroni correction was performed for multiple comparisons. The Cox proportional hazards model and forward selection method were used to estimate the relative risk of HCC development associated with age (≤65 or >65 years), sex (female or male), body mass index (BMI ≤25.0 or >25.0 kg/m^2), HCV genotype (type 1 or type 2), viral concentration (≤100 or >100 KIU/mL), platelet count (<12.0 × 10^4/mm^3 or ≥12.0 × 10^4/mm^3), ALT (≤35 or >35 IU/mL), AST (≤40 or >40 IU/mL), total bilirubin (≤1.2 or >1.2 mg/dL), γ-GTP (≤56 or >56 IU/mL), ALP (≤338 or >338 IU/mL), cholinesterase (<431 or ≥431 IU/mL), LDH (≤250 or >250 IU/mL), albumin (<3.5 or ≥3.5 g/dL), total cholesterol (<130 or ≥130 mg/dL), cirrhosis (presence or absence), and IFN treatment (no therapy, non-SVR, or SVR) for univariate and multivariate analyses. We used the lower or upper limit of the reference values at our institute as cutoff values for platelet count, ALT, AST, total bilirubin, γ-GTP, ALP, cholinesterase, LDH, albumin, and total cholesterol levels. Statistical significance was set at $P < 0.05$.

The study protocol was approved by the Ethics Committee at Ogaki Municipal Hospital in January 2009 and the study was performed in compliance with the Helsinki Declaration. Informed consent was obtained from each patient for analyzing patient records and images.

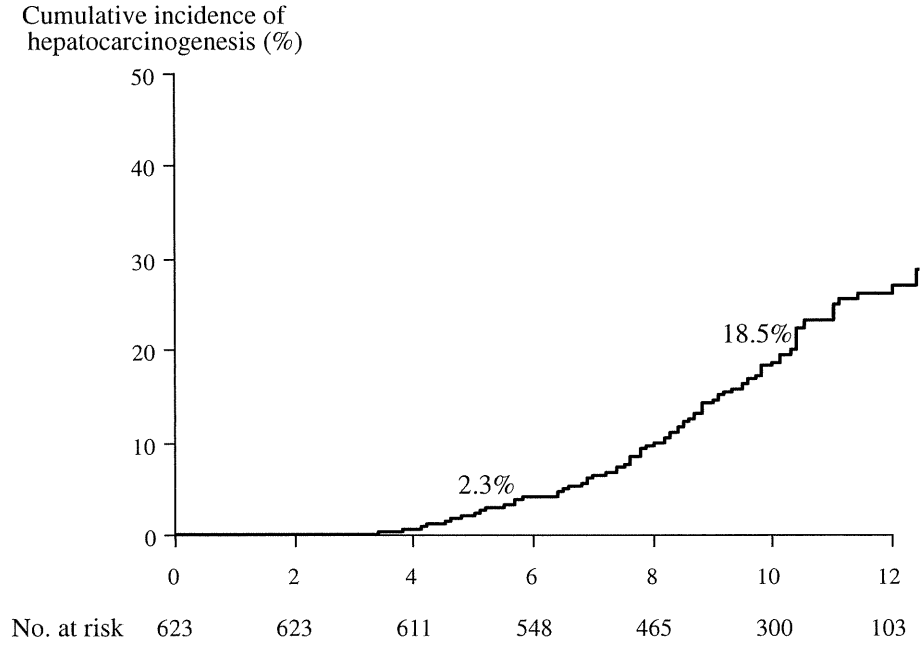

Fig. 2 Overall cumulative incidence rate of HCC

Table 1 Patient characteristics

Age (years)	61 (26–84)
Sex (F/M)	265/358
BMI (kg/m^2)	22.5 (12.0–34.9)
HCV genotype (type 1/type 2)	356/157
Viral concentration (KIU/mL)	270 (0.5–6300)
AFP (ng/mL)	4.8 (0.8–341.5)
AFP-L3 (%)	0.1 (0.0–32.5)
DCP (mAU/mL)	18.1 (8.5–99.6)
Platelets ($\times 10^4$/mm^3)	14.8 (3.0–33.9)
ALT (IU/L)	46.4 (10.1–340.4)
AST (IU/L)	48.5 (13.3–168.9)
γ-GTP (IU/L)	37.6 (9.9–2207)
Total bilirubin (mg/dL)	0.6 (0.2–2.7)
ALP (IU/L)	276.4 (86.8–845.5)
Cholinesterase (IU/L)	242.9 (38.8–545.30)
LDH (IU/L)	196.4 (118.4–650.1)
Albumin (g/dL)	4.0 (2.4–4.9)
Total cholesterol (mg/dL)	155.8 (77.9–264.1)
Fibrosis (F0/F1/F2/F3/F4)[a]	32/73/56/24/2
Cirrhosis (present/absent)	160/463
IFN therapy (none/non-SVR/SVR)	434/146/43

Continuous variables are quoted as medians (ranges)

BMI body mass index, *HCV* hepatitis C virus, *AFP* alpha-fetoprotein, *AFP-L3Lens culinaris* agglutinin-reactive fraction of AFP, *DCP* des-γ-carboxy prothrombin, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *GTP* gamma glutamyl transpeptidase, *ALP* alkaline phosphatase, *LDH* lactate dehydrogenase, *IFN* interferon, *SVR* sustained virologic response

[a] Staging of chronic hepatitis according to Desmet et al. [15]

Results

HCC developed in 120 (19.3%) of the 623 patients. The 5- and 10-year cumulative incidences of HCC were 2.3 and 18.5%, respectively (Fig. 2). Demographic and medical data for the 623 patients are summarized in Table 1.

Factors associated with the incidence of hepatic carcinogenesis on univariate analysis

Factors associated with the incidence of HCC are listed in Table 2. Age ≥65 years, high AFP level, high AFP-L3% level, high DCP level, low platelet count, high ALT level, high AST level, high LDH level, high ALP level, low cholinesterase level, low albumin level, presence of cirrhosis, and response to IFN therapy were significantly associated with the development of HCC on univariate analysis.

The 5-, 7-, and 10-year cumulative incidences of HCC were 1.1, 2.1, and 7.5% in group A1; 2.6, 9.6, and 42.1% in group A2; and 6.6, 18.3, and 50.0% in group A3, respectively, and the cumulative incidence of HCC differed significantly between groups A1 and A2 and groups A1 and A3 (Fig. 3). The 5-, 7-, and 10-year cumulative incidences of HCC were 1.4, 4.6, and 15.6% in group L1; 19.6, 39.7, and 73.6% in group L2; and 12.5, 25.0, and 56.7% in group L3, respectively, and the cumulative incidence of HCC differed significantly between groups L1 and L2 and groups L1 and L3 (Fig. 4). The 5-, 7-, and 10-year cumulative incidences of HCC were 0.5, 4.6, and

Table 2 Factors associated with hepatocarcinogenesis (univariate analysis)

	Crude hazard ratio (95% CI)	*P*
Age (years)		
≤65	1	
>65	2.318 (1.580–3.400)	<0.001
AFP (ng/mL)		
A1; <10	1	
A2; ≥10, <20	6.061 (3.768–9.750)	<0.001
A3; ≥20	8.985 (5.874–13.744)	<0.001
AFP-L3 (%)		
L1; <5	1	
L2; ≥5, <10	8.032 (4.388–14.700)	<0.001
L3; ≥10	3.781 (1.838–7.778)	<0.001
DCP (mAU/mL)		
D1; <20	1	
D2; ≥20, <40	1.209 (0.788–1.855)	0.385
D3; ≥40	4.535 (2.840–7.241)	<0.001
Platelets ($\times 10^4$/mm^3)		
≥12.0	1	
<12.0	5.887 (3.982–8.702)	<0.001
ALT (IU/L)		
≤35	1	
>35	2.632 (1.574–4.400)	<0.001
AST (IU/L)		
≤40	1	
>40	8.120 (4.115–16.024)	<0.001
LDH (IU/L)		
≤250	1	
>250	1.970 (1.249–3.106)	<0.001
ALP (IU/L)		
≤338	1	
>338	2.509 (1.724–3.650)	<0.001
Cholinesterase (IU/L)		
>431	1	
≤431	3.288 (2.209–4.893)	<0.001
Albumin (g/dL)		
≥3.5	1	
<3.5	3.948 (2.635–5.917)	<0.001
Cirrhosis		
Absent	1	
Present	3.474 (2.413–5.002)	<0.001
IFN therapy		
No therapy	1	
Non-SVR	0.312 (0.180–0.539)	<0.001
SVR	0.215 (0.075–0.620)	0.004

Continuous variables are quoted as medians (ranges)

CI confidence interval, *AFP* alpha-fetoprotein, *AFP-L3Lens culinaris* agglutinin-reactive fraction of AFP, *DCP* des-γ-carboxy prothrombin, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *LDH* lactate dehydrogenase, *ALP* alkaline phosphatase, *IFN* interferon, *SVR* sustained virologic response

14.8% in group D1; 1.8, 4.3, and 16.3% in group D2; and 10.0, 25.0, and 48.2% in group D3, respectively, and the cumulative incidence of HCC differed significantly between groups D1 and D3 and groups D2 and D3 (Fig. 5).

Factors associated with the incidence of hepatic carcinogenesis on multivariate analysis

Factors associated with the incidence of HCC as analyzed by the Cox proportional hazards model and the forward selection method are listed in Table 3. Age >65 years, low platelet count, high AST level, high AFP level, and high AFP-L3% level were significantly associated with the incidence of HCC. Factors associated with the incidence of HCC were analyzed in patients with chronic hepatitis and cirrhosis (Table 4). High age, low platelet count, high AST level, and high AFP level were significantly associated with the incidence of HCC in chronic hepatitis, and male sex, high age, low platelet count, high AFP level, and high AFP-L3% level were significantly associated with the incidence of HCC in cirrhosis. Factors associated with the incidence of HCC were analyzed in patients with and without IFN treatment (Table 5). Male sex, low platelet count, low cholinesterase level, and high AFP level were significantly associated with the incidence of HCC in patients with IFN therapy and male sex, high age, low platelet count, high AFP level, and high AFP-L3% level were significantly associated with the incidence of HCC in patients without IFN therapy.

Discussion

Advances in US, CT, and MRI have allowed for the more frequent and earlier detection of small HCC tumors less than 2 cm in diameter during the routine follow-up of patients with chronic liver disease [21–23]. However, the performance and resolution of the imaging device, the skills of individual operators, and the diagnostic acumen of the interpreting radiologist all affect the early detection of HCC. AFP, AFP-L3%, and DCP levels have been used as prognostic markers rather than diagnostic markers for HCC [9]. However, the detection rate of small HCC tumors with these markers is low; AFP-L3% and DCP have low sensitivity, and AFP has low specificity. Sassa et al. [12] reported detection rates of 22.6 and 48.4% for AFP-L3% and DCP, respectively, in patients with small HCC tumors. It is currently thought that serum markers are useful for follow-up after HCC therapy in patients with high tumor marker levels before treatment [24].

We have previously reported that the average integration value of ALT correlates with the cumulative incidence of hepatocarcinogenesis, even within the normal range [13, 14]. In the present study, the average integration value of AFP was not selected as a factor associated with the

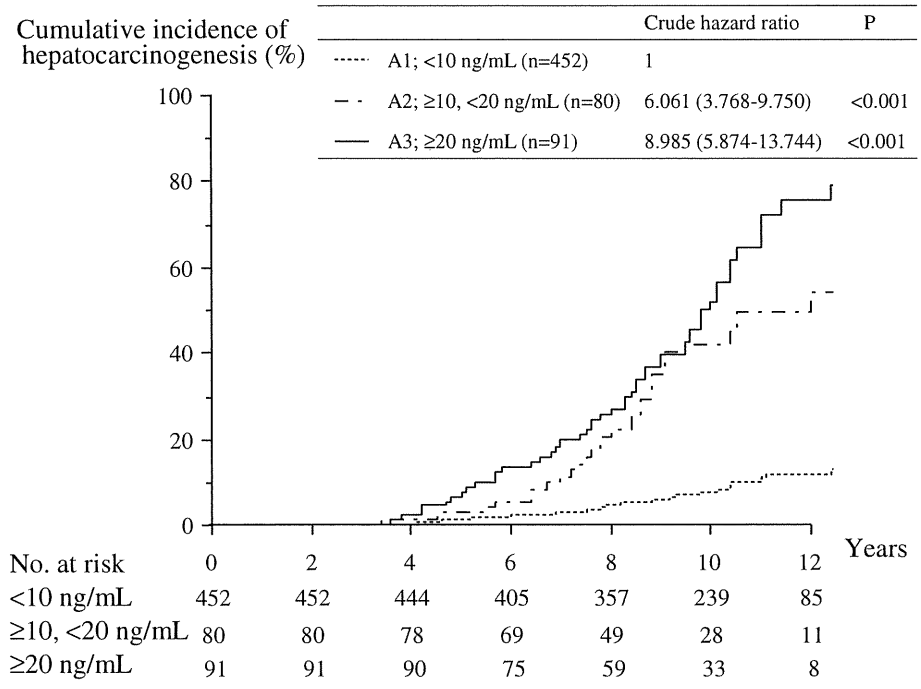

Fig. 3 Incidence of HCC according to the average integration value of AFP. The cumulative incidence of HCC differed significantly between groups A1 (<10 ng/mL) and A2 (≥10, <20 ng/mL) and groups A1 and A3 (≥20 ng/L)

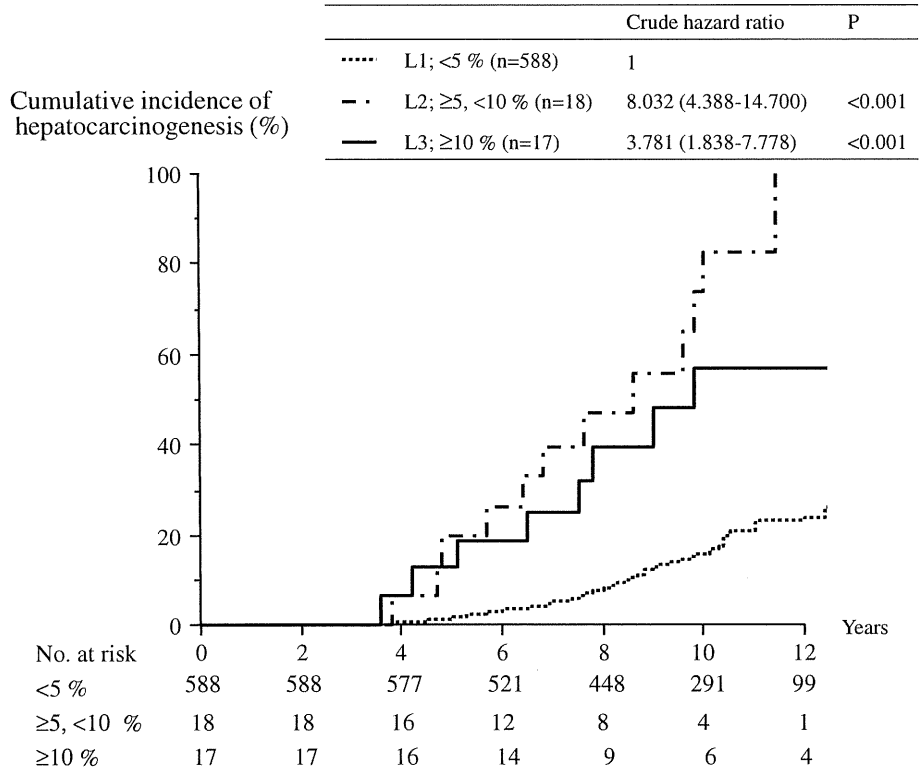

Fig. 4 Incidence of HCC according to the average integration value of AFP-L3%. The cumulative incidence of HCC differed significantly between groups L1 (<5%) and L2 (≥5, <10%) and groups L1 and L3 (≥10%)

incidence of HCC on multivariate analysis. AFP production is thought to be increased in response to injury, possibly due to increased hepatocyte turnover, in patients with HCV who do not have HCC [25]. In contrast, increased ALT levels are correlated with hepatocellular necrosis but not with hepatocyte proliferation. This difference may at least partially explain the absence of correlation between ALT and AFP levels.

The multivariate analysis in our series was carried out to minimize the influence of confounding factors, and 5 factors were selected by the forward selection method. Age >65 years, low platelet count, high AST value, high AFP

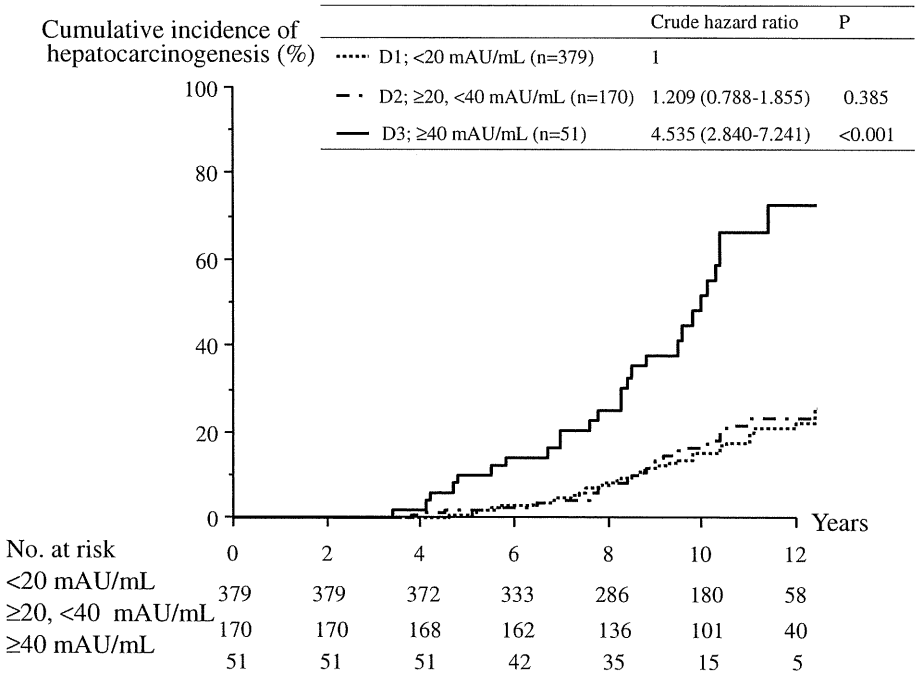

Fig. 5 Incidence of HCC according to the average integration value of DCP. The cumulative incidence of HCC differed significantly between groups D1 (<20 mAU/mL) and D3 (≥40 mAU/mL) and groups D2 (≥20, <40 mAU/mL) and D3

Table 3 Factors associated with hepatocarcinogenesis (multivariate analysis)

	Adjusted hazard ratio (95% CI)	*P*
Age (years)		
≤65	1	
>65	2.303 (1.551–3.418)	<0.001
Platelets ($\times 10^4$/mm^3)		
≥12.0	1	
<12.0	3.086 (1.997–4.768)	<0.001
AST (IU/L)		
≤40	1	
>40	3.001 (1.373–6.562)	0.006
AFP (ng/mL)		
A1; <10	1	
A2; ≥10, <20	2.814 (1.686–4.697)	<0.001
A3; ≥20	3.405 (2.087–5.557)	<0.001
AFP-L3 (%)		
L1; <5	1	
L2; ≥5, <10	2.494 (1.291–4.816)	0.007
L3; ≥10	3.555 (1.609–7.858)	0.002

AST aspartate aminotransferase, *AFP* alpha-fetoprotein, *AFP-L3 Lens culinaris* agglutinin-reactive fraction of AFP

level, and high AFP-L3% level were significantly associated with hepatic carcinogenesis in our multivariate analysis, but serum ALT level was not a risk factor for developing HCC. Ikeda et al. [26] reported that the cumulative incidence of HCC increased significantly in cirrhotic patients with an AFP level ≥10 ng/mL compared to those with an AFP level <10 ng/mL, and the adjusted risk ratio was 15.788 in HCV patients. They speculated that AFP is a marker of disease activity or severity and cellular regeneration, and it acts as a better predictor of HCC with viral etiology of cirrhosis. As an index of hepatic regeneration, the AFP level better represents the risk of hepatic carcinogenesis than an index of liver injury (e.g., ALT level). In addition to AFP, AFP-L3% was identified as a factor predicting the development of HCC, and this is a specific marker for the existence of HCC. Therefore, elevations in AFP-L3% may reflect an occult cancer that is undetectable with current imaging modalities. More intensive surveillance is needed for patients such as those who fulfill the criteria of groups L2 and L3 in our series, although these groups were very small in size. However, similar to other laboratory values, as high AFP-L3% values may be associated with severe liver damage, it is necessary to interpret these values carefully. DCP is well known to be also a specific marker of HCC. DCP is more closely related to tumor size than AFP and AFP-L3% [27]. Therefore, it is thought that these were the reasons that DCP was not selected as a predictive marker for HCC in our multivariate analysis.

Among the other risk factors we identified for the development of HCC, a low platelet count stands out. The platelet count is a useful marker for the diagnosis of cirrhosis [28], and cirrhosis is an established risk factor for HCC in HCV carriers [26, 28–30]. Taken together with our other findings, the low platelet count suggests that HCC develops in patients with progressive or advanced liver disease. We additionally used ultrasound (US) to distinguish cirrhotic patients from non-cirrhotic patients [16–18]. The presence of cirrhosis on US was strongly associated with an increased

Table 4 Factors associated with hepatocarcinogenesis on multivariate analysis in patients with chronic hepatitis and cirrhosis

	Chronic hepatitis ($n = 463$)	Cirrhosis ($n = 160$)
Age (years): ≤65 vs. >65	<0.001	0.008
Gender: female vs. male		<0.001
Platelets ($\times 10^4/mm^3$): ≥12.0 vs. <12	0.001	0.007
AST (IU/L): ≤40 vs. >40	0.043	
AFP (ng/mL): <10 vs. ≥10, <20 vs. ≥20	<0.001	0.003
AFP-L3 (%): <5 vs. ≥5, <10 vs. ≥10		0.017

AST aspartate aminotransferase, *AFP* alpha-fetoprotein, *AFP-L3 Lens culinaris* agglutinin-reactive fraction of AFP

Table 5 Factors associated with hepatocarcinogenesis on multivariate analysis in patients with and without IFN treatment

	With IFN ($n = 189$)	Without IFN ($n = 434$)
Age (years): ≤65 vs. >65		0.001
Gender: female vs. male	0.005	<0.001
Platelets ($\times 10^4/mm^3$): ≥12.0 vs. <12.0	0.047	<0.001
Cholinesterase (IU/L): ≥431 vs. <431	0.007	
AFP (ng/mL): <10 vs. ≥10, <20 vs. ≥20	<0.001	<0.001
AFP-L3 (%): <5 vs. ≥5, <10 vs. ≥10		<0.001

IFN interferon, *AFP* alpha-fetoprotein, *AFP-L3 Lens culinaris* agglutinin-reactive fraction of AFP

incidence of HCC on univariate analysis, but US-determined cirrhosis was not identified as a risk factor on multivariate analysis. Histologic assessment of fibrosis and cirrhosis was obtained in only 187 patients (30.0%), and patients with F4 fibrosis had a higher incidence of HCC in our univariate analysis. However, the population of patients with material available for histologic review was only one-third the size of the entire study population, and this small number may have negatively affected our ability to detect the predictive nature of fibrosis at all levels of severity. In contrast to serum ALT, serum AST levels were significantly associated with the incidence of HCC. AST levels are often abnormal in patients with cirrhosis when ALT values are in the normal range, and the AST/ALT ratio is frequently greater than 1 in cirrhotic patients [31]. Elevated AST activity is a surrogate marker for cirrhosis. Aging is associated with a number of events at the molecular, cellular, and physiological levels that influence carcinogenesis and subsequent cancer growth [32]. It has been hypothesized that an age-associated decrease in DNA repair [33] contributes to the development of HCC.

Recent reports have shown that AFP levels fall following the administration of IFN with or without ribavirin [34, 35]. IFN has been shown to have antiviral, anti-inflammatory, and anticancer activities [36]. One study demonstrated an anticancer effect of IFN when this agent was given following intrahepatic recurrence after HCC resection [37], and in our study, previous treatment with IFN was a factor associated with a reduced incidence of HCC on univariate analysis. The median ages of our patients with and without IFN treatment were 53 years (range 28–71) and 65 years (range 26–84), respectively; the age in those receiving IFN was significantly lower than the age in the group without IFN ($P < 0.0001$). It is thought that age and IFN therapy are confounding factors because IFN therapy has better results in younger patients. Although IFN was not identified as a predictive factor on multivariate analysis, the possibility cannot be denied that IFN may play an important role in modulating AFP levels prior to the onset of HCC.

In conclusion, increased AFP or AFP-L3% levels were significantly associated with an increased incidence of HCC. Among HCV carriers, patients with ≥10 ng/mL AFP or patients with ≥5% AFP-L3% are at very high risk for the development of HCC even if AFP is less than 20 ng/mL or AFP-L3% is less than 10%, which are the most commonly reported cutoff values. Intensive imaging modalities including US, CT, and MRI are recommended every 3–6 months for these patients.

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Conflict of interest There is no conflict of interest to disclose.

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Original Article

Phase I and pharmacokinetic clinical trial of oral administration of the acyclic retinoid NIK-333

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Aim: NIK-333 (an acyclic retinoid) has been reported to prevent recurrence of hepatocellular carcinoma (HCC) in patients after curative treatment. This study was conducted to determine the maximum tolerated dose, dose-limiting toxicities (DLT) and pharmacokinetics of NIK-333 administrated p.o. at doses ranging 300–900 mg/day.

Methods: Patients who were cancer-free after percutaneous local ablation or surgical resection of HCC were enrolled. The total daily dose was administrated as a single dose (single-dose stage) followed by a week of rest, and then in two equally divided doses administrated after breakfast and supper for 48 consecutive weeks (repeated-dose stage).

Results: No patients at the dose levels of 300 mg/day and 600 mg/day developed any DLT. At the final dose level of 900 mg/day, three of the nine patients developed grade 3 hypertension as a DLT. There were no significant difference values of maximum drug concentration (C_{max}) and log(C_{max}) between fasting and postprandial condition. In the repeated-dose stage, there was no significant difference between the start and week 24 of NIK-333 administration within any dose cohort in either the mean area under the blood concentration time curve (0–6 h) or the C_{max}. NIK-333 was well-tolerated when administrated p.o. at doses of up to 600 mg/day for 48 weeks.

Conclusion: Hypertension was noted as a DLT at the dose level of 900 mg/day, and this dose was considered to be inappropriate. The recommended dose for the phase II/III clinical trial is thought to be 300 mg/day and 600 mg/day.

Key words: acyclic retinoid, hepatocellular carcinoma, NIK-333, peretinoin, phase I

INTRODUCTION

A NUMBER OF treatment options are available for the treatment of hepatocellular carcinoma (HCC), including percutaneous local ablation, surgical resection and transcatheter arterial embolization. However, even after successful treatment, a considerable risk of recurrence exists and no standard therapy for preventing HCC recurrence has yet been established.

In this context, the concept of chemoprevention with retinoids has been proposed as an approach to delaying or preventing post-treatment HCC recurrence.[1] NIK-333 ([*2E,4E,6E,10E*]-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid, Table 1) is a synthetic polyprenoic acid discovered by Muto *et al.* in 1980, that exhibits retinoid-like activities by binding to cellular retinoic acid-binding protein.[2] Muto *et al.*[3,4] reported that p.o. administration of NIK-333 (600 mg/day, twice daily) for 1 year in patients with treated HCC inhibited the development of a second primary hepatoma and improved survival. The effect of NIK-333 is believed to be exerted by its differentiation-inducing[5,6] and apoptosis-inducing[7,8] actions on human hepatoma-derived cells via various actions such as an arrest of the cell cycle, an inactivation of the Ras-extracellular signal-regulated kinase signaling system and an inhibition of the activation of receptor tyrosine kinase.[9,10]

We conducted a phase I clinical trial with patients who were completely treated by local ablative therapy or surgical resection of HCC to determine the maximum tolerated dose (MTD), dose-limiting toxicities (DLT) and pharmacokinetics of NIK-333 administrated p.o. at doses ranging 300–900 mg/day.

METHODS

Patient eligibility

THE ELIGIBILITY CRITERIA for study enrollment were: (i) HCC completely treated by local ablative therapy or surgical resection; (ii) chronic liver disease of

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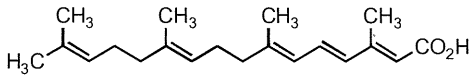
Table 1 Characteristics of NIK-333

Structural formula	
Generic name	Peretinoin
Chemical name	*(2E,4E,6E,10E)*-3,7,11,15-Tetramethylhexadeca-2,4,6,10,14-pentaenoic acid
Molecular weight	302.45
Dosage	Soft capsules containing 75 mg/NIK333 per capsule

Child–Pugh class A or B; (iii) absence of HCC confirmed by imaging; (iv) age of 20 years or more and less than 75 years; (v) adequate blood cell counts (white blood cell count ≥3000/mm^3, hemoglobin ≥10 g/dL, platelet count ≥50 000/μL), hepatic functions (serum aspartate and alanine transaminase levels ≤5.0 × the upper limit of normal [ULN], serum total bilirubin ≤2.0 × ULN) and renal functions (serum creatinine ≤1.0 × ULN); and (vi) availability of written informed consent from the patient.

The exclusion criteria were: (i) bone mineral density of 80% or less of the young adult mean (YAM) as measured by dual-energy X-ray absorptiometry (DXA); (ii) serious underlying disease of the liver, kidneys, heart or hematopoietic system; (iii) liver angioma; (iv) esophageal leukoplakia; (v) severe diabetes with insulin therapy; (vi) other confirmed malignancy; (vii) pregnancy, potential pregnancy or the desire to become pregnant; (viii) lactating women; (ix) history of allergy to retinoid-related compounds (e.g. vitamin A); (x) etretinate, known as a retinoid, therapy within 2 years prior to study entry; (xi) participation in another clinical trial within 6 months prior to study entry; and (xii) any patient considered by the principal or other investigator(s) to be ineligible for the trial.

This clinical trial was performed with the approval of the National Cancer Center's Institutional Review Board for clinical investigation and according to the provisions of the Declaration of Helsinki, International Conference on Harmonization of Good Clinical Practice guidelines, and local laws and regulations.

Study design

This study was conducted in two sequential stages in the same patients: a single-dose, placebo-controlled stage (including food-effect examination with 300 mg dose); and a subsequent 48-week repeated-dose stage (Fig. 1). Three dose levels were investigated, employing a dose escalation design: level 1 (initial dose), 300 mg; level 2, 600 mg; level 3, 900 mg.

The drug was administrated p.o. at each dose level to 12 patients. In the single-dose stage, two patients in each dose cohort were randomly assigned to receive the placebo in order to obtain control data on these patients (single-blind stage). In contrast, the active drug was administrated to all patients in the subsequent repeated-dose stage (open-label stage).

The recommended NIK-333 dose for the phase II/III clinical trial was to be determined based on the type and frequency of DLT observed during the single-dose stage, repeated-dose stage, follow-up period and also in accordance with the recommendations of the Independent Data Monitoring Committee.

Single-dose stage (including food-effect examination)

In the initial stage of the single-dose stage, a food-effect examination was conducted to determine whether NIK-333 should be taken in fasting or feeding condition. In a two-phase study with a cross-over design with a washout period of at least 1 week, the patients in the level 1 dose cohort received 300 mg NIK-333 in either the fasting state or within 30 min of a meal. The pharmacokinetic data obtained following fasting and postprandial administration of NIK-333 were comparatively analyzed to investigate the effects of meal intake on drug pharmacokinetics. Based on the pharmacokinetic analysis results, the timing of NIK-333 administration was determined and uniformly employed in the subsequent single-dose stage for the level 2 and level 3 dose cohorts, as well as in the repeated-dose stage. The daily dose defined for each dose level cohort was administrated as a single dose. Follow-up observation was continued for at least 1 week after study drug administration.

Repeated-dose stage

After completion of the single-dose stage, a 48-week repeated-dose stage was conducted in the three dose level cohorts, with NIK-333 administrated at a total daily dose identical to that in the single-dose stage, but

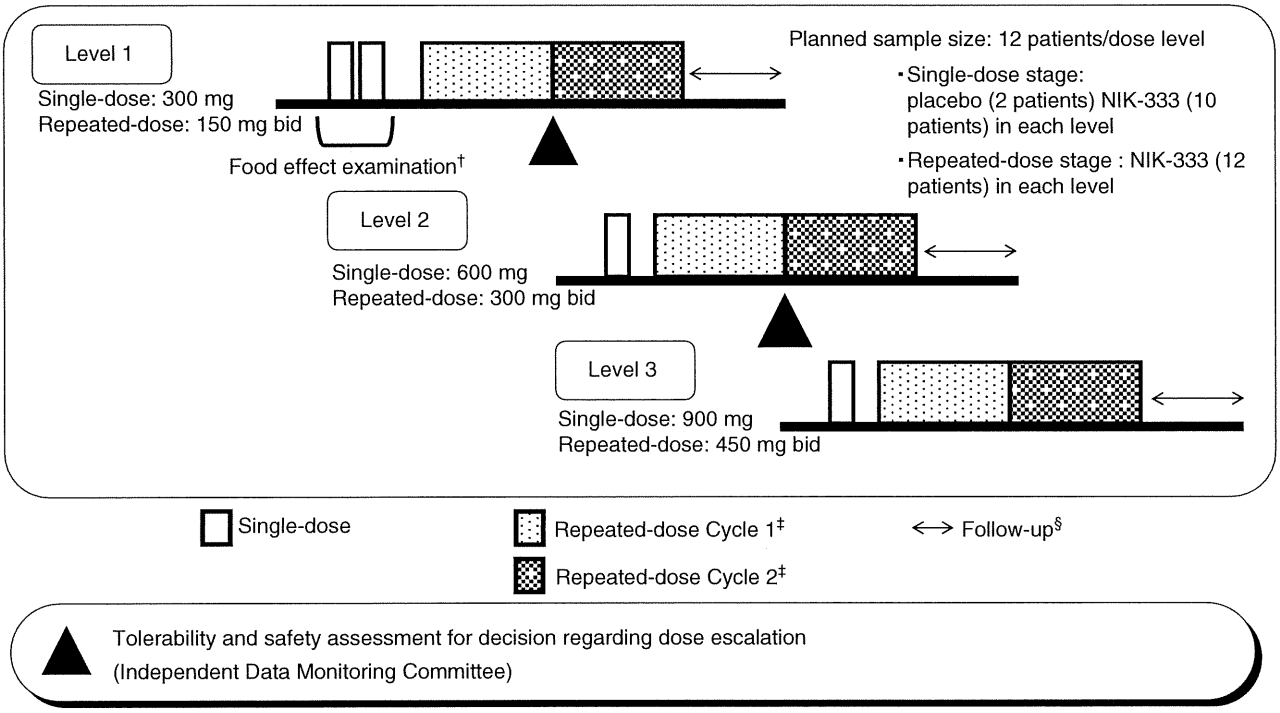


Figure 1 Study design. Three NIK-333 dose levels were investigated, employing a dose escalation design. Starting from dose level 1, tolerability and safety data for the first six patients who completed cycle 1 of the repeated-dose stage in a particular dose level cohort were assessed to arrive at a decision regarding dose escalation to the next dose level. See "Methods" for details. †Single-blind 2-phase cross-over study (level 1 dose cohort only). ‡Twenty-four weeks each. §Follow-up assessments at 12 and 24 weeks after the end of the repeated-dose stage.

in two equally divided doses (i.e. twice daily), after breakfast and after supper. This repeated-dose stage was scheduled in two cycles, cycle 1 (from the start of drug administration to week 24) and cycle 2 (weeks 25–48), in order to assess the tolerability of NIK-333 at the end of each cycle and determine whether or not to proceed to the next stage of the clinical trial. When adverse events meeting the criteria for DLT occurred in three or more of the first six patients who completed cycle 1 in a particular dose level cohort, this dose level was regarded as the MTD, and the clinical trial was aborted without proceeding to the next higher dose level. The tolerability of NIK-333 was also assessed at the end of cycle 2 according to the same criteria.

Administration of NIK-333 was discontinued upon development of any DLT and restarted only after recovery had been confirmed (including transition to a lower grade of severity). If the DLT persisted up to 1 month after drug discontinuation or any other DLT developed after administration of NIK-333 was restated, the patient was withdrawn from the study. Patients developing recurrent HCC or other cancers during the study were also withdrawn.

Follow-up examination after NIK-333 administration

Follow-up physical examination, laboratory investigations, including complete blood cell counts (CBC), serum chemistry, and bone-related examinations, were performed 12 and 24 weeks after the end of repeated-dose NIK-333 administration.

Safety assessment

The safety of NIK-333 treatment was assessed by physical examination and laboratory investigations, including CBC, serum chemistry, urinalysis, electrocardiography, upper gastrointestinal endoscopy, chest radiography, measurement of fibrosis markers (P-III-P, type IV collagen, hyaluronic acid) and bone-related examinations (radiography, bone mineral density analysis by DXA, bone mineral density analysis by digital image

processing [DIP] and measurement of bone metabolism parameters). All adverse events were collected and graded according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 2. The physical examination, CBC, serum chemistry and urinalysis were performed according to the following schedule: at baseline in the single-dose stage; at baseline, and 2 and 4 weeks (once every 4 weeks thereafter) during cycle 1 and once every 4 weeks during cycle 2 of the repeated-dose stage; and once every 12 weeks during the follow-up period. Electrocardiography was performed at baseline and 24 h after NIK-333 administration in the single-dose stage, and at baseline and once every 24 weeks in the repeated-dose stage. Fibrosis markers were measured at baseline in the single-dose stage and once every 12 weeks in the repeated-dose stage. Upper gastrointestinal endoscopy and chest radiography were performed at baseline in the single-dose stage and once every 24 weeks in the repeated-dose stage. Bone-related examinations were performed at baseline in the single-dose stage, and once every 12 weeks (radiography and bone mineral density analysis by DXA) or 24 weeks (bone mineral density analysis by DIP and bone metabolism parameters) in the repeated-dose stage, and once every 12 weeks (radiography and bone mineral density analysis by DXA) or 24 weeks (bone mineral density analysis by DIP and bone metabolism parameters) during the follow-up period.

Recurrence of HCC was assessed by abdominal imaging (computed tomography or ultrasonography) and serum tumor markers (α-fetoprotein, lectin-reactive α-fetoprotein, protein induced by vitamin K absence or antagonist II) were measured at baseline in the single-dose stage, and at baseline and once every 12 weeks in the repeated-dose stage.

Criteria for DLT

The following adverse events for which a causal relationship to the protocol treatment could not be excluded were defined as DLT: (i) grade 4 hematological toxicities other than thrombocytopenia; (ii) platelet count of less than 10 000/μL; (iii) grade 3 and grade 4 non-hematological toxicities, except for changes in the blood glucose level, serum levels of bilirubin, alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ-GTP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and bone mineral density; (iv) grade 4 elevation of blood glucose and serum bilirubin, ALP, γ-GTP, AST and ALT levels; and (v) bone mineral density of less than 70% of YAM.

Pharmacokinetics

Plasma concentrations of unchanged NIK-333 and its lipid forms (i.e. glycerol ester) were determined by liquid chromatography/mass spectrometry (LC-MS)/MS to calculate the area under the blood concentration time curve ($AUC_{0-24\,h}$), $\log(AUC_{0-24\,h})$, maximum drug concentration (C_{max}), $\log(C_{max})$, time to peak drug concentration (t_{max}) and $t_{1/2}$. Urinary concentrations of unchanged NIK-333 were measured using LC with ultraviolet detector (LC-UV).

In the single-dose stage (including the food-effect examination), the plasma concentrations of unchanged NIK-333 and its lipid forms were measured at baseline and 0.5, 1, 2, 4, 6, 8 and 24 h after drug administration.

In the repeated-dose stage, the plasma concentrations of unchanged NIK-333 and its lipid forms were measured using two different sampling schedules. At the start and the end of 24-week NIK-333 administration, blood samples were collected at baseline and 0.5, 1, 2, 4 and 6 h after drug administration. In contrast, samples were collected only at baseline and 2 h after drug administration at Weeks 2, 4, 8, 12, 16, and 20.

During the follow-up period, the plasma concentrations of unchanged NIK-333 and its lipid forms were measured at the end of 48 weeks of the repeated-dose stage, and at the 12- and 24-week of follow-up period.

Statistical analysis

Plasma concentrations of NIK-333 and its metabolite

Plasma concentrations of unchanged NIK-333 and its lipid forms determined in the repeated-dose stage were examined for achievement of the steady state and accumulation by ANOVA using the duration of NIK-333 administration as the variable factor. Plasma pharmacokinetic parameters for unchanged NIK-333 (C_{max} and $AUC_{0-6\,h}$) calculated at the start and week 24 of NIK-333 administration were compared by the paired Student's *t*-test.

Level of statistical significance

In all statistical analyses, $P < 0.05$ (two-tailed) was considered to indicate a statistically significant difference. Analyses were conducted using SAS ver. 8.

RESULTS

Patient characteristics (Table 2 and Fig. 2)

FROM 10 SEPTEMBER 2001 through 2 July 2003, 33 patients in total were enrolled in this study at the

Table 2 Patient background characteristics

	300 mg/day	600 mg/day	900 mg/day
Dose cohort (*n*)	12	12	9
Basic epidemiology			
Age (years)			
Median	59.8	58.9	59.2
Range	50.0–69.0	46.0–74.0	49.0–69.0
Sex (*n*)			
Male	11	12	5
Female	1	0	4
Body mass index (kg/m^2)			
Median	24.3	25.3	23.6
Range	19.2–28.6	21.8–29.6	19.7–26.9
Status of curative treatment			
Previous therapy (*n*)			
Primary	12	7	8
Secondary	0	5	1
Maximum tumor diameter (mm)			
Median	27.8	35.7	48.7
Range	15.0–60.0	15.0–115.0	10.0–156.0
Number of tumor masses (*n*)			
Median	1.1	1.0	1.2
Range	1–2	1–1	1–2
Vascular invasion (*n*)			
Absent	0	2	1
Present	12	10	8
Type of treatment (*n*)			
Surgical resection	8	6	3
Local ablative therapy	4	6	6
Liver function			
Child–Pugh grade (*n*)			
A	12	11	8
B	0	1	1
Interval from curative treatment to start of NIK-333 administration (days)			
Median	610	376	799
Range	128–2180	93–1381	105–1911

National Cancer Center Hospital (Tokyo, Japan). The follow-up survey in the last enrolled patient was completed on 3 September 2004. The studies in the level 1 and level 2 dose cohorts were completed as planned in the protocol, with 12 patients enrolled in each dose cohort. In contrast, the study in the level 3 dose cohort had to be discontinued (both drug administration and patient enrollment were stopped) due to the development of severe treatment-related adverse events, according to the recommendation of the Independent Data Monitoring Committee. Consequently, a total of nine patients were enrolled in the level 3 dose cohort and received NIK-333 for a maximum of 25 weeks. Five (two each in level 1 and level 2, and one in level 3) out of 33 patients were allocated to the placebo group in the single-dose stage.

Safety

Single-dose stage (Table 3)

Grade 1 headache was observed in one (10.0%) out of the 10 patients allocated to the NIK-333 group in the level 2 dose cohort, and grade 1 headache and grade 1 diarrhea were observed in three (37.5%) and one (12.5%), respectively, out of eight patients receiving the

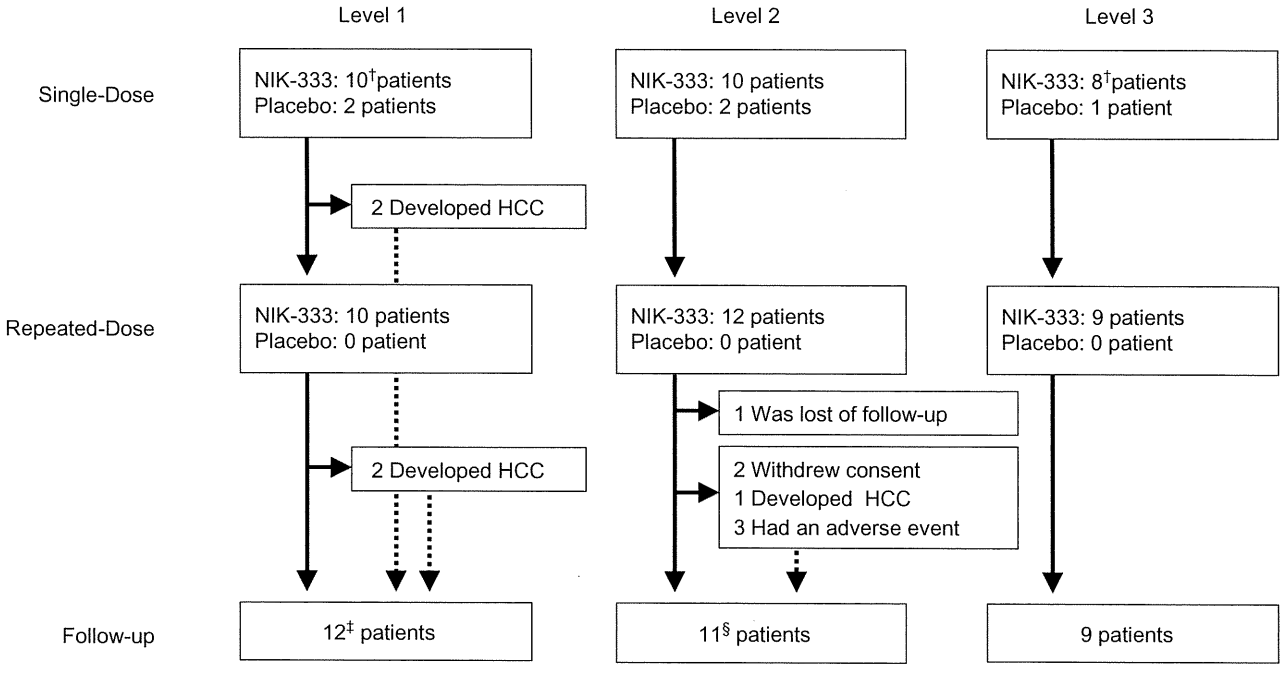


Figure 2 Patients flow. Patients who discontinued the study were enrolled in follow-up survey as possible. †Two patients (patient ID 106 in level 1 and patient ID 305 in level 3) were excluded from the pharmacokinetic (PK) analysis due to concomitant use of medication prohibited by the exclusion criteria. ‡Three patients (patient ID 104, 105 and 106) were excluded from the PK analysis because any blood samples for PK were not corrected due to treatment for recurrent hepatocellular carcinoma (HCC). §One patient (patient ID 206) was excluded from the PK analysis because any blood samples for PK were not corrected due to a withdrawal of consent.

active agent in level 3 dose cohort. These toxicities were all transient and considered to be tolerable, although they occurred more frequently at higher dose levels.

In the placebo group, hot flashes, lactate dehydrogenase elevation and diarrhea were observed in one patient (20.0%) each, although no significant differences in the incidence of treatment-related adverse events between the placebo group and NIK-333 group were observed in each dose cohort (Fisher's exact test).

Repeated-dose stage (Table 4)

In the level 1 and 2 dose cohorts, only three grade 3 treatment-related adverse events, one ALT elevation in the level 1 dose cohort and two γ-GTP elevation in the level 2 dose cohort, were observed. Grade 2 hypertriglyceridemia was observed in one patient in the level 2 dose cohort.

In the level 3 dose cohort, grade 3 hypertension was observed in three out of the nine enrolled patients within 24 weeks of starting the repeated-dose stage (i.e.

Table 3 Treatment-related adverse events in patients receiving the active agent in the single-dose stage

Dose level (mg)	300 ($n = 10 \times 2$)†					600 ($n = 10$)					900 ($n = 8$)					Total ($n = 38$) (%)
Adverse event / grade‡	1	2	3	4	(%)	1	2	3	4	(%)	1	2	3	4	(%)	
Headache	0	0	0	0	0.0	1	0	0	0	10.0	3	0	0	0	37.5	10.5
Diarrhea	0	0	0	0	0.0	0	0	0	0	0.0	1	0	0	0	12.5	2.6

†Total number of events and patients throughout the single-blind two-phase cross-over food-effect study (see Fig. 1). Data obtained in the first and second phases are pooled.
‡Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria version 2.0.

Table 4 Treatment-related adverse events in the repeated-dose stage (incidence >10%)

Dose level (mg/day)	300 ($n = 10$)					600 ($n = 12$)					900 ($n = 9$)					Total ($n = 31$)
Adverse event / grade‡	1	2	3	4	(%)	1	2	3	4	(%)	1	2	3	4	(%)	(%)
Headache	2	0	0	0	20.0	2	0	0	0	16.7	5	0	0	0	55.6	29.0
Hypertension	1	0	0	0	10.0	1	0	0	0	8.3	0	1	3	0	44.4	19.4
Hematuria	0	0	0	0	0.0	0	2	0	0	16.7	3	0	0	0	33.3	16.1
Stomach polyp	2	0	0	0	20.0	0	0	0	0	0.0	0	0	0	0	0.0	6.5
Diarrhea	1	0	0	0	10.0	0	0	0	0	0.0	1	0	0	0	11.1	6.5
Gastroesophageal reflux disease	0	0	0	0	0.0	2	0	0	0	16.7	0	0	0	0	0.0	6.5
Neck stiffness	0	0	0	0	0.0	0	0	0	0	0.0	2	0	0	0	22.2	6.5
Proteinuria	0	0	0	0	0.0	0	0	0	0	0.0	0	2	0	0	22.2	6.5
Abdominal pain	0	0	0	0	0.0	0	0	0	0	0.0	1	0	0	0	11.1	3.2
Dysgeusia	0	0	0	0	0.0	0	0	0	0	0.0	1	0	0	0	11.1	3.2
Fundal hemorrhage	0	0	0	0	0.0	0	0	0	0	0.0	1	0	0	0	11.1	3.2
Stomach displeasure	0	0	0	0	0.0	0	0	0	0	0.0	1	0	0	0	11.1	3.2
Cracked lips	0	0	0	0	0.0	0	0	0	0	0.0	1	0	0	0	11.1	3.2
Epigastralgia	0	0	0	0	0.0	0	0	0	0	0.0	0	1	0	0	11.1	3.2

‡Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria version 2.0.

during cycle 1), which was considered to have a causal relationship to the study drug in all of these patients. Therefore, both study drug administration and patient enrollment were permanently discontinued in this dose cohort. One patient developed grade 2 hypertension and administration of NIK-333 was suspended at his request until permanent discontinuation of the clinical trial. Antihypertensive therapy controlled blood pressure in all patients with grade 3 hypertension, indicating that this treatment-related adverse event can be successfully managed with antihypertensive medications. Fundal hemorrhage occurred in one of the patients with grade 3 hypertension but resolved after this patient was withdrawn from the study, and no specific treatment was necessary.

In addition, the incidence rates of proteinuria and hematuria were higher in the level 3 dose cohort (proteinuria, 22.2%; hematuria, 33.3%) than in the level 1 (proteinuria, 0.0%; hematuria, 0.0%) or level 2 (proteinuria, 0.0%; hematuria, 16.7%) dose cohorts. Both events occurred within 24 weeks of starting the repeated-dose stage (i.e. during cycle 1).

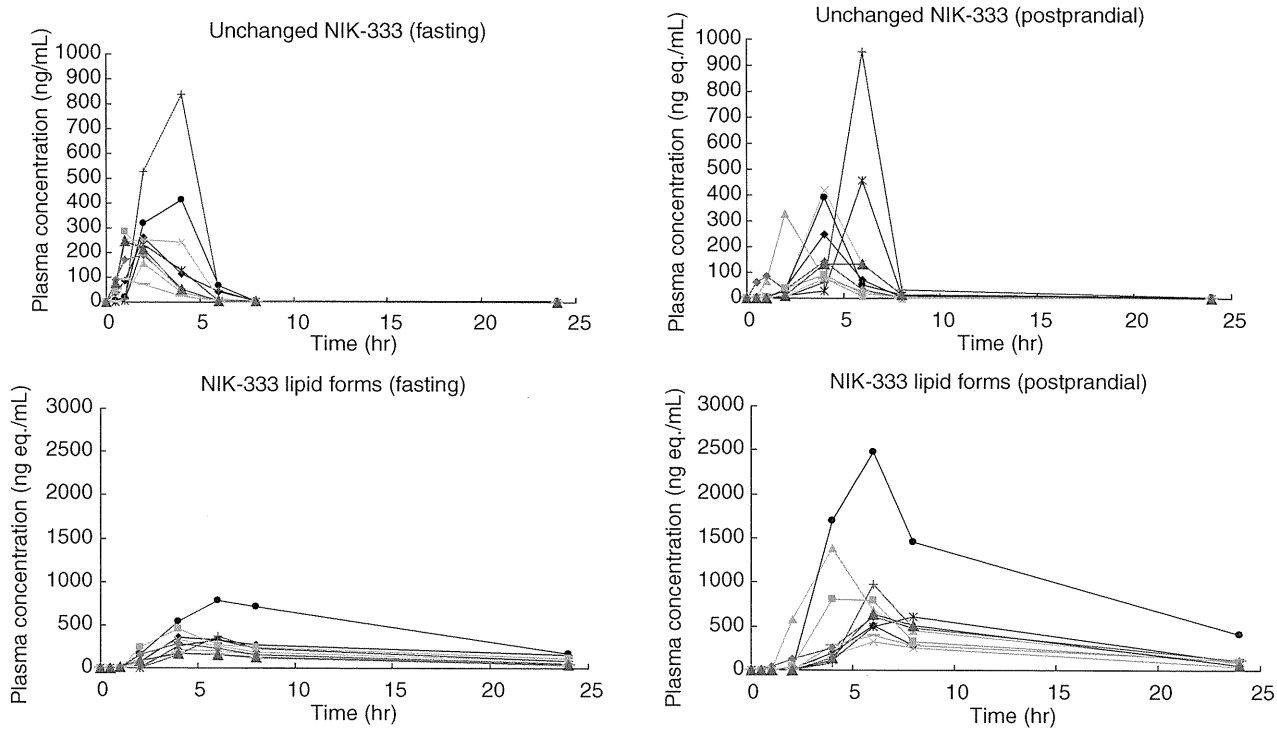
Follow-up survey (Table 5)

No grade 3 treatment-related adverse events were observed in any dose cohorts. Nail changes, a well-known retinoid-related adverse event,[11,12] was noted in two patients in the level 3 dose cohort approximately 12 weeks after the end of NIK-333 administration, however, it resolved by 24 weeks after the end of NIK-333 administration. Changes in the data obtained in the bone-related examinations were considered to be within the range of physiological variability for all dose cohorts.

Table 5 Treatment-related adverse events during the follow-up period

Dose level (mg/day)	300 ($n = 12$)					600 ($n = 11$)					900 ($n = 9$)					Total ($n = 32$)
Adverse event / grade‡	1	2	3	4	(%)	1	2	3	4	(%)	1	2	3	4	(%)	(%)
Nail changes	0	0	0	0	0.0	0	0	0	0	0.0	2	0	0	0	22.2	6.3
Gastritis	1	0	0	0	8.3	0	0	0	0	0.0	0	0	0	0	0.0	3.1
Hyperglycemia	0	0	0	0	0.0	0	1	0	0	9.1	0	0	0	0	0.0	3.1

‡Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria version 2.0.



		C_{max}† (ng/mL)	$AUC_{0\text{-}24hr}$† (ng·hr/mL)	t_{max}† (hr)	$t_{1/2}$† (hr)
Fasting	Unchanged	284.0 ± 216.5	849.5 ± 717.5	1.9 ± 0.9	1.9 ± 2.2
(n = 9)	Lipid forms	295.4 ± 96.8	3518.3 ± 1086.4	4.4 ± 0.9	11.2 ± 3.1
Postprandial	Unchanged	316.1 ± 275.9	841.3 ± 648.3	4.2 ± 1.2	1.3 ± 0.8
(n = 9)	Lipid forms	694.1 ± 326.1	6497.7 ± 1819.0	5.8 ± 1.2	7.2 ± 1.8

† Mean ± S.D.

Figure 3 Pharmacokinetics of NIK-333 in the food-effect examination. A two-phase cross-over single-dose stage was conducted in the level 1 (300 mg) dose cohort ($n = 10$), to determine whether NIK-333 should be taken in a fasting or feeding condition (within 30 min). Plasma concentrations of unchanged NIK-333 and its lipid forms were measured at baseline and 0.5, 1, 2, 4, 6, 8 and 24 h after single-dose administration of NIK-333.

Pharmacokinetics

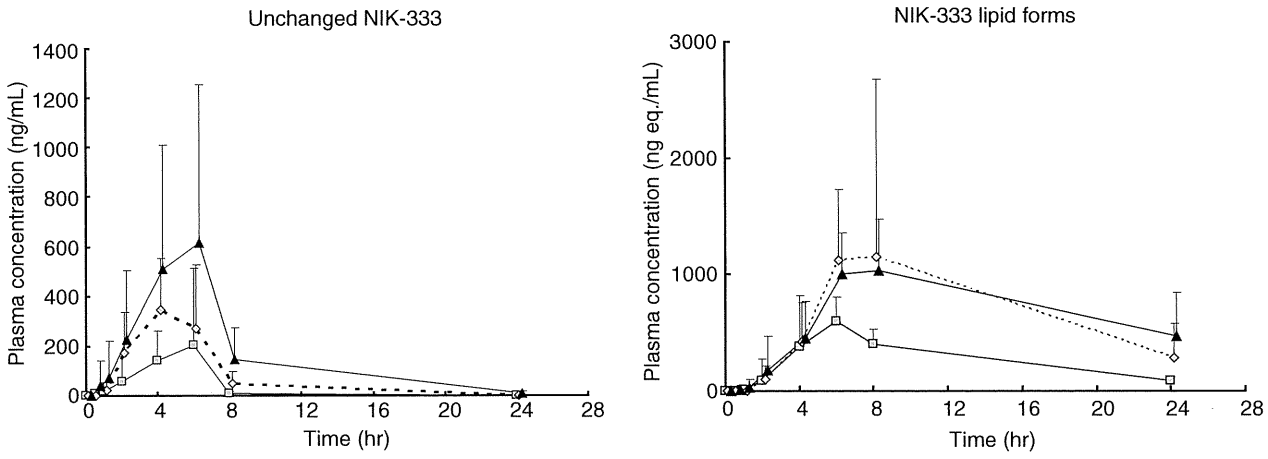
Single-dose stage (including food-effect examination) (Figs 3,4)

The plasma C_{max} and log(C_{max}) calculated for unchanged NIK-333 and its lipid forms following fasting and postprandial administrations were subjected to ANOVA. No significant differences were observed between the fasting and postprandial administration values (unchanged NIK-333: C_{max}, $P = 0.466$; log[C_{max}], $P = 0.982$; and lipid forms of NIK-333: C_{max}, $P = 0.011$; log[C_{max}], $P = 0.004$).

While the plasma concentration of NIK-333 lipid forms tended to saturate at higher dose levels, the plasma concentration of unchanged NIK-333 were increased dose dependently. No urinary excretion of unchanged NIK-333 was observed at any dose level.

Repeated-dose stage (Fig. 5)

No significant variations of the plasma concentrations of unchanged NIK-333 measured either 0 or 2 h after administration were detected by ANOVA at any dose level



		$AUC_{0\text{-}24hr}$ ‡,§ (ng·hr/mL)	C_{max} ‡,§ (ng/mL)	t_{max} ‡ (hr)	$t_{1/2}$ ‡ (hr)
300 mg†	Unchanged	841.3 ± 648.3	316.1 ± 275.9	4.2 ± 1.2	1.3 ± 0.8
(n = 9)	Lipid forms	6497.7 ± 1819.0	694.1 ± 326.1	5.8 ± 1.2	7.2 ± 1.8
600 mg	Unchanged	1873.8 ± 1063.0	468.4 ± 191.0	4.6 ± 1.3	3.4 ± 1.9
(n = 10)	Lipid forms	15883.3 ± 17241.8	1450.5 ± 1466.5	6.4 ± 0.8	8.3 ± 1.9
900 mg	Unchanged	4009.7 ± 1090.9	906.9 ± 579.6	5.3 ± 2.5	3.6 ± 2.5
(n = 7)	Lipid forms	16274.0 ± 6478.0	1239.9 ± 331.6	9.1 ± 6.6	8.9 ± 2.0

Figure 4 Pharmacokinetics of NIK-333 in the single-dose stage. Plasma concentrations of unchanged NIK-333 and its lipid forms were measured at baseline and 0.5, 1, 2, 4, 6, 8 and 24 h after single-dose administration, within 30 min of a meal, of NIK-333 at three different dose levels (300, 600 and 900 mg). †Calculated from the measurements for NIK-333 administration after a meal in the food-effect examination. ‡Mean ± standard deviation. §NIK-333 lipid forms; $AUC_{0\text{-}24hr}$, ng eq.h/mL; C_{max}, ng eq/mL. Single-dose stage: (□) 300 mg dose cohort (level 1); (◇) 600 mg dose cohort (level 2); (▲) 900 mg dose cohort (level 3).

during the 2–24 weeks of the repeated-dose stage; a similar result was obtained by ANOVA for the plasma concentrations of NIK-333 lipid forms. Furthermore, no significant difference in either the mean $AUC_{0\text{-}6\,h}$ (level 1, $P = 0.8936$; level 2, $P = 0.1595$; level 3, $P = 0.8335$) or C_{max} (level 1, $P = 0.6300$; level 2, $P = 0.8341$; level 3, $P = 0.4557$) for unchanged NIK-333 was detected by the paired Student's *t*-test between the start and week 24 of NIK-333 administration within any dose cohort.

Follow-up examination

Investigation of the elimination-phase pharmacokinetics of NIK-333 revealed that at the end of the administration period, unchanged NIK-333 was detected in five out of 28 patients at all dose levels investigated. However, all of their plasma levels (2–6 ng/mL) were close to the detection limit (1 ng/mL), and lower than 1% of the C_{max} calculated for each dose level in the single-dose stage. One of the 10 patients in the level 2 dose cohort, followed up for elimination of NIK-333 serially until the 24-week measurement, exhibited a substantial elevation of the NIK-333 lipid forms (315 ng eq/mL) at 12 weeks after the end of administration in plasma while the concentration of NIK-333 lipid forms at 24 weeks after the end of administration was below the detection limit (25 ng eq/mL).

DISCUSSION

IN THIS CLINICAL trial, we established the safety profile of NIK-333 at each dose level and detailed pharmacokinetics rather than a previous report which reported only average plasma concentration at 1 year after p.o. administration.[3] We did not plan for this study to evaluate the preventive effect of NIK-333, because this study was done with a small sample size and short observation period and NIK-333 is not an anticarcinogenic agent.

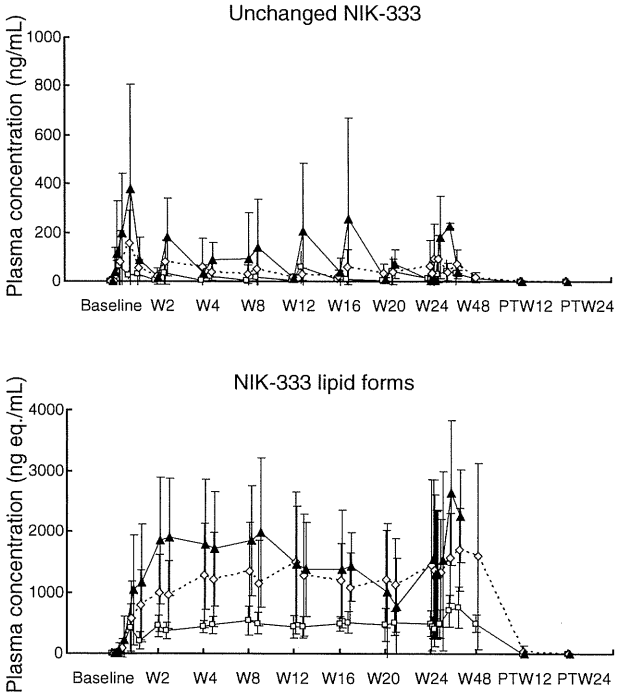


Figure 5 Pharmacokinetics of NIK-333 in the repeated-dose stage and during follow up. W, week; PTW, post-treatment week. See "Methods" for detailed sampling schedules. Repeated dose stage: (□) 300 mg/day dose cohort (level 1); (◇) 600 mg/day dose cohort (level 2); (▲) 900 mg/day dose cohort (level 3).

Because NIK-333 will be used as a chemoprevention agent for patients who have achieved complete cure of HCC and is categorized as a prophylactic agent not an anticarcinogenic agent, it seems necessary for it to have a higher level of safety than an anticarcinogenic agent. Based on this concept and the safety data obtained in this study, the level 3 dose (900 mg/day) was considered to be inappropriate for clinical use as a chemopreventive agent because of the high incidence of grade 2 and 3 hypertension (44.4% [4/9], range of elevation of systolic blood pressure: 14–68 mmHg) in this dose cohort, necessitating clinical trial discontinuation at this dose level. In addition, one patient developed grade 2 hypertension and NIK-333 administration was suspended until discontinuation of the study. In all patients with grade 3 hypertension, discontinuation of NIK-333 and antihypertensive therapy lowered blood pressure. Two patients from the level 3 dose cohort receiving concomitant antihypertensive drugs that had been initiated prior to starting the present study did not develop hypertension during the repeated-dose stage. These findings suggest that hypertension related to NIK-333 administration can be successfully controlled with appropriate antihypertensive therapy. Though clinically meaningful increase in serum creatinine and blood nitrogen were not found, proteinuria and hematuria were observed after starting the repeated-dose stage and seemed to occur more frequently and severely at higher dose levels, and were identified even in patients without hypertension. These findings suggest that NIK-333 might somehow affect renal function independently. Both hypertension and urinary abnormalities were treatment-related adverse events that had not been identified previously in non-clinical studies of NIK-333 and warrant further detailed investigation. From the results of non-clinical study, NIK-333 has several toxicities, thought to be rodent-specific (Murata K., 2002, Martin P.A., 1990, Mayfield R., 1989, unpublished data), for which we set some exclusion criteria (i.e. bone mineral density decrease, liver angioma, esophageal leukoplakia use). However, we did not observe adverse events which seemed to relate to these toxicities. Metabolic abnormalities such as hyperlipidemia are known as a retinoid-related adverse event.[13] In this study, grade 2 hypertriglyceridemia and grade 1 weight loss developed in one patient in the level 2 dose cohort and in one patient in the level 1 dose cohort, respectively. The patients recovered from these adverse events without any treatment. It seems that NIK-333 does not relate to metabolic abnormalities, however, due to small sample size, further investigations are necessary to conclude the relationship between NIK-333 and metabolic abnormalities.

The plasma concentrations of unchanged NIK-333 did not differ significantly depending on whether NIK-333 was taken during fasting or feeding condition. The therapeutic efficacy of NIK-333 is ascribed to the biological activity of unchanged NIK-333. Therefore, it was concluded that NIK-333 should be administrated after meals in the single-dose stage conducted in the level 2 and level 3 dose cohorts, as well as in the repeated-dose stage, because better compliance can be expected if NIK-333 were to be taken after meals. C_{max} of NIK-333 at a single-dose stage of level 1 (300 mg dose group) was 316.1 ± 275.9 ng/mL (mean ± standard deviation). Considering the data from non-clinical studies, the estimated concentration ratio of liver to plasma of NIK-333 was 2–6 (Seki H. *et al.*, 1999, unpublished data). This liver concentration was high enough to induce apoptosis and differentiation in hepatoma-derived cells. These suggest that even with half of this plasma concentration, NIK-333 shows pharmacological effects sufficiently.

Pharmacokinetic analysis demonstrated no apparent accumulation of unchanged NIK-333 during repeated-dose administration of the drug up to week 24, suggesting that the steady state was successfully maintained. Notably, however, significant elevation of the plasma NIK-333 lipid forms (315 ng eq/mL) was detected in one patient in the level 2 dose cohort at 12 weeks after the end of NIK-333 administration. This value was only 45% of the mean C_{max} (694.1 ± 326.1 ng eq/mL) calculated in the level 1 dose cohort during the single-dose stage and the plasma level of NIK-333 lipid forms measured at 24 weeks after the end of NIK-333 administration was below the detection limit, suggesting that the elevation of the plasma lipid forms might not greatly influence the study conclusion regarding the clinical safety of NIK-333.

Obvious inter-patient variability was observed in the pharmacokinetics of NIK-333. During the repeated-dose stage, a rise in the plasma concentration of unchanged NIK-333 or its lipid forms comparable to that in the level 3 dose cohort was detected in some level 2 dose cohort patients. Accordingly, the possibility of treatment-related adverse events (e.g. hypertension) observed in the level 3 dose cohort during NIK-333 administration for 48 weeks also developing at dose level 2 after a longer administration period cannot be ruled out. Even assuming that administration of NIK-333 at dose level 2 would be sufficient to obtain clinical efficacy, careful monitoring of cardiovascular and urinary adverse events are necessary throughout NIK-333 administration.

To ensure the safety of NIK-333 during long-term administration, issues such as criteria for drug discontinuation and possible long-term effects of concomitant therapies still require investigation. In the phase II/III trial, although primarily aimed at confirming the clinical efficacy of NIK-333, it would be important to devise appropriate countermeasures to minimize adverse reactions to this drug in patients, for example, concomitant use of antihypertensives according to treatment guidelines for hypertension and cardiovascular and urological examinations to monitor treatment-related adverse events.

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