oxidized albumin and reduced albumin closely reflect the oxidation reduction state in vivo. Oxidative stress due to aging, nephrotic syndrome, hemodialysis, and diabetes increases the oxidized albumin percentage.[32,33] Human serum albumin preparations that are often used for treatment of hypoalbuminemia in cirrhotic patients include preparations whose major ingredient is oxidized albumin.[34] Effective procedures for converting oxidized albumin to reduced albumin remain unclear. An increased percentage of oxidized albumin may reflect increased scavenging of reactive oxygen species by albumin, indicative of oxidative stress and/or decreased albumin turnover with consequent accumulation of albumin damaged oxidatively.[35,36] The clinical implications of reduced albumin:oxidized albumin ratio in liver disease should be clarified while investigating the pathophysiology and treatment of the disease.

It has been reported that the glycoalbumin level is increased in diabetic patients.[37] However, the method of glycated albumin by a mass spectrometric technique called matrix-assisted laser desorption ionization[38] is based on the assumption that all changes in the molecular mass of albumin in diabetes are due to glycation by glucose. Therefore, the samples determined by a high-performance liquid chromatographic method in our study should be determined simultaneously by the recent new methods.[39] In cirrhotic patients, the percentage of glycated albumin increases with a decrease in total albumin concentration. Glycation of albumin is an irreversible reaction, different from the reversible oxidation reduction reaction of albumin.[40] Unlike oxidized:reduced albumin, glycation of albumin causes an irreversible change in the molecular structure. Glycation of albumin reduces the binding capability of bilirubin to albumin to about half and that of long-chain fatty acids to about one-twentieth.[41] This means that the native function of albumin is attenuated. Therefore, glycated albumin can be considered to be deteriorated albumin. Glycation of albumin in diabetic patients is caused by hyperglycemic stimulation. Interestingly, in this study, the glycoalbumin percentage also was increased in cirrhotic patients without diabetes. Perhaps the hypoalbuminemic status in cirrhotic patients reduces the destruction rate of albumin, which is shown by prolongation of the half-life of albumin,[42] resulting in prolonged stimulation by glucose. This study suggests that this hyperglycemic stimulation is an additional factor because the glycoalbumin percentage in cirrhotic patients accompanied by diabetes was definitely higher than that in patients without diabetes. Reducing sugars, including glucose, bind amino acid residues in protein and form Schiff bases and Amadori products (hemoglobin A_{1c} is a well-known product). These products are gradually changed to irreversible derivatives, called advanced glycation (glycosylation) endproducts (AGEs), over several weeks or several months.[43] However, recent studies[44,45] have suggested that AGEs can form in days by the reaction of α-oxaldehydes such as glyoxal, methylglyoxal, and 3-deoxyglucosone with proteins. Fructosamines also cannot degrade over a few days, although they form over 10 to 15 d, depending on the proteins. Plasma AGE level (Nε-carboxymethyllysine) in cirrhotic patients (not accompanied by renal disorder and/or diabetes) has been reported to increase with the progression of disease and to return to normal after liver transplantation.[46] Increased Nε-carboxymethyllysine levels in cirrhotics may reflect increased oxidative degradation of fructosyllysine to Nε-carboxymethyllysine[47] and decreased turnover of albumin. AGEs produced from glycoalbumin have been considered to be attributable to development of various complications in diabetic patients.[48–49] The increased AGEs in patients with liver disease activate various cells in the liver, such as sinusoidal endothelial cells and Kupffer cells, through AGE receptors and stimulate production of cytokines, including transforming growth factor-β_1, resulting in liver fibrosis and development of complications. Further investigation of the clinical implications of glycoalbumin in the pathophysiology of liver disease is required, as are studies of the kinetics of reduced albumin and oxidized albumin.

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Original Paper

Polyamine Metabolism and Recurrence after Resection for Hepatocellular Carcinoma

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KEY WORDS:
Hepatocellular carcinoma; Polyamine; Ornithine decarboxylase; Liver resection; Tumor-free survival rate; Portal invasion; Intrahepatic metastasis

ABBREVIATIONS:
Ornithine Decarboxylase (ODC); Hepatocellular Carcinoma (HCC); Alpha-Fetoprotein (AFP); Spermidine (SPD); Putrescine (PUT)

ABSTRACT

Background/Aims: Ornithine decarboxylase activity, a rate-limiting enzyme in polyamine biosynthesis, is correlated with the degree of malignancy of hepatocellular carcinoma. We investigated the relationship between polyamine metabolism in hepatocellular carcinoma tissues and the recurrence after resection of hepatocellular carcinoma.

Methodology: The subjects were 49 patients who underwent liver resection for hepatocellular carcinoma. The relationship between ornithine decarboxylase activity and polyamine concentration in hepatocellular carcinoma tissues and the pathologic findings or recurrence after the operation was studied.

Results: The prevalences of moderately or poorly differentiated hepatocellular carcinoma, portal invasion, and intrahepatic metastasis were higher in patients with a high ornithine decarboxylase activity (≥100pmol putrescine/h/mg protein) than in patients with a low ornithine decarboxylase activity (<100pmol putrescine/h/mg protein). The tumor-free survival rate was significantly lower in patients with a high ornithine decarboxylase activity than in patients with a low ornithine decarboxylase activity (p=0.0394). The tumor-free survival rate was significantly lower in patients with a high spermidine concentration (≥80nmol/mg protein) than in patients with a low spermidine concentration (<80nmol/mg protein, p=0.0454).

Conclusions: A high ornithine decarboxylase activity and a high spermidine concentration of hepatocellular carcinoma were risk factors for recurrence after resection of hepatocellular carcinoma.

INTRODUCTION

Ornithine decarboxylase (ODC), a rate-limiting enzyme in polyamine biosynthesis, is important in tumor promotion (1). It has been reported that ODC activity is high in human neoplastic lesions, including breast cancers, and carcinomas in gastrointestinal tract (2-8). We have reported that the polyamine contents and ODC activity in human hepatocellular carcinoma (HCC) tissues are higher than those in the surrounding noncancerous hepatic tissue (9). We also reported that ODC activity is correlated with tumor volume doubling time and that ODC activity was higher in moderately or poorly differentiated HCC than in well-differentiated HCC (10). The prevalences of portal invasion and intrahepatic metastasis were higher in patients with high ODC activities (10). In this study, we investigated the relationship between polyamine metabolism and the recurrence after resection of HCC.

METHODOLOGY

Patients

The subjects were 49 patients who had undergone liver resection for HCC. Their ages ranged from 36 to 70 years, with a mean age of 59.8. Forty patients were men and 9 patients were women.

Operative Specimens

The HCC tissue was collected immediately after each surgical resection. One portion of the tissue was frozen in liquid nitrogen and stored at -80°C until assay. The remaining portions were fixed in 10% neutral formalin and embedded in paraffin for histologic examination. HCC tissue was classified histologically according to Edmondson and Steiner's classification (11), with some modification (12).

Ornithine Decarboxylase Activity

ODC activity was measured as described previously (10,13,14). Frozen hepatic tissue was homogenized in four volumes of 50mmol Tris-HCl (pH 7.4) containing 1mmol/L ethylenediaminetetraacetic acid, 1mmol/L dithiothreitol, and 50μmol/L pyridoxal phosphate. The homogenate was centrifuged at 30,000 g for 30 minutes, and the ODC activity of the supernatant was assayed. In this assay, [^{14}C]putrescine (PUT) formed from [5-^{14}C]ornithine was measured.

Polyamine Concentrations

Hepatic tissue homogenate was mixed with an equal volume of 10% trichloroacetic acid. After centrifugation at 10000 g for 30 min, the concentration of polyamines [PUT, spermidine (SPD), and spermine]

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in the supernatant was analyzed with high-performance liquid chromatography (LC-6A, Shimadzu Techno-Research, Inc., Kyoto) using a fluorescence detector (10,13,14). Polyamines were separated on an STR ODS-II column (4.6x150mm, particle size 5μm, Shimadzu Techno-Research) with solvent A composed of 10mmol/L 1-hexanesulfonic acid (sodium salt) and 100mmol/L sodiumperchloric acid and with solvent B composed of 1:3 mixture of solvent A and methanol. The sample was eluted with 96% solvent A and 4% solvent B for 3 min, followed by a programmed gradient of solvent with a linear gradient curve changing from 4% to 55% solvent B over 22 min at a flow rate of 0.7mL/min.

Statistical Analysis

The student's *t* test was used to evaluate the significance of differences in age and tumor size. Chi-squared test or Fisher's exact test was used to evaluate the significance of differences in categoric data between groups. The tumor-free survival rates were calculated by the Kaplan-Meier method, and the significance of the difference between the curves was evaluated by the log-rank test.

Ethical Considerations

This study complied with the ethical guidelines of the Declaration of Helsinki and was approved by the Ethics Committee of Osaka City University Graduate School of Medicine. All patients gave written informed consent.

RESULTS

ODC activity ranged from 5.2 to 2487pmol PUT/h/mg protein. We divided the patients into two groups according to the levels of ODC activity. One group consisted of 32 patients with a high ODC activity (≥100pmol PUT/h/mg protein, group A) and another group consisted of 17 patients with ODC activity (<100pmol PUT/h/mg protein, group B) because our previous study showed that portal invasion, that is a risk factor for recurrence, was often found in patients with ODC ≥100pmol putrescine/h/mg protein.

The prevalences of moderately or poorly differentiated HCC, portal invasion, and intrahepatic metastasis were higher in group A than in group B (**Table 1**). The tumor-free survival rate was significantly lower in group A than in group B (p=0.0394, **Figure 1**). As compared with the tumor-free survival rate in group B, the rate in group A decreased more rapidly within 4 years after the operation.

Next, we divided the patients into two groups according to the SPD concentration. The median concentration of SPD in all patients was 80nmol/mg protein. One group consisted of 25 patients with a high SPD concentration (≥80nmol/mg protein, group C) and another group consisted of 24 patients with a low SPD concentration (<80nmol/mg protein, group D). There were no significant differences in tumor size, the degree of differentiation of the main tumor, the prevalences of portal invasion and intrahepatic metastasis (**Table 2**). However, the tumor-free survival rate was significantly lower in group C than in group D (p=0.0454, **Figure 2**). As compared with the tumor-free survival rate in group D, the rate in group C decreased more rapidly within 4 years after the operation.

Serum concentration of alpha-fetoprotein (AFP) was positive (≥20ng/mL) in 26 of the 49 patients. There was no significant difference in tumor-free sur-

TABLE 1 Ornithine Decarboxylase Activity and Clinicopathologic Findings of Patients with Hepatocellular Carcinoma

	ODC activity		
	Group A (≥100)	Group B (<100)	
Findings	(n=32)	(n=17)	*p*
Age (years, mean ± SD)	59.9±8.4	59.8±8.8	0.942
Gender (male:female)	27:5	13:4	0.700
Tumor size (cm, mean ±SD)	4.2±2.8	3.6±3.0	0.422
Differentiation of main tumor			
Well-differentiated	0	3	0.0488
Moderately differentiated	28	12	
Poorly differentiated	4	2	
Portal invasion	12	3	0.0959
Intrahepatic metastasis	14	2	0.0283
Cirrhosis	14	11	0.232

ODC activity, ornithine decarboxylase activity (pmol putrescine/h/mg protein).

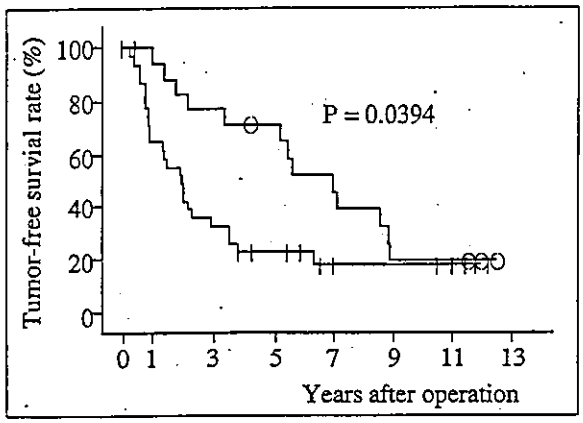

FIGURE 1 Tumor-free survival rates after resection of hepatocellular carcinoma with a high and a low ornithine decarboxylase activity. Open squares, 32 patients who had hepatocellular carcinoma with a high ornithine decarboxylase activity (≥100pmol putrescine/h/mg protein); open circles, 17 patients who had hepatocellular carcinoma with a low ornithine decarboxylase activity (<100pmol putrescine/h/mg protein).

TABLE 2 Spermidine Concentration and Pathologic Findings of Patients with Hepatocellular Carcinoma

	Spermidine concentration		
	Group C (≥80)	Group D (<80)	
Findings	(n=25)	(n=24)	*p*
Tumor size (cm, mean ± SD)	3.8±1.6	4.3±3.7	0.538
Differentiation of main tumor			
Well-differentiated	0	3	0.187
Moderately differentiated	21	18	
Poorly differentiated	4	3	
Portal invasion	9	4	0.196
Intrahepatic metastasis	9	5	0.345

Spermidine concentration (nmol/mg protein).

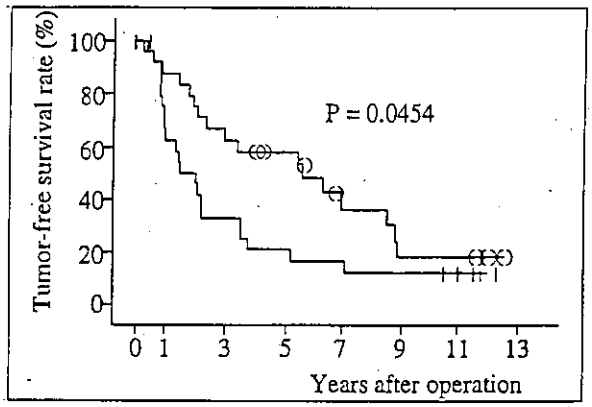

FIGURE 2 Tumor-free survival rates after resection of hepatocellular carcinoma with a high and a low spermidine concentration.
Open squares, 25 patients who had hepatocellular carcinoma with a high spermidine concentration (≥80nmol/mg protein); open circles, 24 patients who had hepatocellular carcinoma with a low spermidine concentration (<80nmol/mg protein).

vival rate between patients with a high serum concentration of AFP (≥20ng/mL) and patients with a low AFP concentration (<20ng/mL) (p=0.968).

DISCUSSION

A relationship between polyamine metabolism and degree of malignancy of HCC evaluated by pathologic examination has been reported previously (9,10) and was confirmed again in this study. In this study, the tumor-free survival rate was significantly lower in patients with a high ODC activity (group A) than in patients with a low ODC activity (group B). Generally, there is a close correlation between increased ODC activity and polyamine concentration and increased cell proliferation (1,13,15). We have reported that ODC activity is correlated with tumor volume doubling time (10). The prevalences of moderately or poorly differentiated HCC, portal invasion, and intrahepatic metastasis were higher in group A than in group B. Thus, HCCs with a high ODC activity have a high degree of malignancy and easily make portal invasion and intrahepatic metastasis, those are risk factors for recurrence, resulting in the high rate of recurrence.

SPD accumulation is important in cell proliferation (15). In this study, the tumor-free survival rate was significantly lower in patients with a high SPD concentration (group C) than in patients with a low SPD concentration (group D). HCCs with a high SPD concentration may also have a high degree of malignancy.

Several previous studies have shown that a high serum concentration of AFP is a risk factor for recurrence. However, the serum concentration of AFP was not a risk factor for recurrence in this study. In this study, the serum concentration of AFP was within the reference range (<20ng/mL) in 23 of the 49 patients. The serum concentration of AFP are often within the reference range, especially in small HCC (16), and dose not always reflect the tumor volume doubling time (10). ODC activity and SPD concentration may reflect more closely the degree of malignancy of HCC.

A high ODC activity and a high SPD concentration of the tumor were risk factors for recurrence after liver resection for HCC. If a tumor has high ODC activity and/or a high SPD concentration, close surveillance for early recurrence is necessary.

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LIVER

Benefit of interferon therapy in hepatocellular carcinoma prevention for individual patients with chronic hepatitis C

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Background: An increase in the incidence of hepatocellular carcinoma (HCC) in Japan since the 1980s suggests an imminent outbreak in other countries where viral spread occurred more recently. Interferon therapy for chronic hepatitis C, in general, has been shown to prevent HCC.
Aims: To determine the scale of benefit in individual patients.
Subjects: Histologically proven chronic hepatitis C patients in the Inhibition of Hepatocarcinogenesis by Interferon Therapy (IHIT) cohort (*Ann Intern Med* 1999;**131**:174), as updated in March 2003.
Methods: The lifetime risk for HCC was calculated based on HCC incidence rates, stratified by sex, age, fibrosis stage, and outcome of interferon therapy. The gain in HCC free survival was defined as the difference between expected HCC free survival with sustained virological response and that without.
Results: The gain in HCC free survival was greater when a patient was younger and fibrosis was more advanced. For example, a 30 year old male with F3 fibrosis gained 12.4 years by attaining sustained response while a patient with F1 fibrosis older than 60 years gained less than one year. For a treatment protocol with a given sustained response rate, prior estimation of the gain can be obtained by multiplying the calculated HCC free survival for responders by the response rate.
Conclusions: The gain in HCC free survival may serve as an indicator of the benefit of interferon therapy in terms of HCC prevention and be useful in the consideration of indication and selection of treatment protocol for individual patients.

Various studies have indicated that a nationwide spread of hepatitis C virus (HCV) took place in Japan in the 1950s and 1960s.[1 2] As a result, there has been a rapid increase in hepatocellular carcinoma (HCC) incidence since 1980, now claiming more than 30 000 deaths each year. Simultaneously, there was a decline in the number of deaths assigned to cirrhosis. The decline may be partly due to advances in HCC diagnosis but the major cause seems to lie in the increasing risk of HCC as patients are getting older. The time lag of 30 years between the peaks of infection spread and HCC incidence corresponds to the observed interval between the time of blood transfusion and carcinogenesis in typical HCC patients.[3] These data strongly suggest an imminent outbreak of HCC incidence in other countries, including the USA, where HCV infection is thought to have been spread more recently.[4 5]

Shortly after the discovery of HCV in 1989,[6 7] interferon therapy was confirmed to be effective against HCV infection.[8–10] We and other groups have shown that interferon therapy significantly reduces the risk of HCC development among chronic hepatitis C patients.[11–13] In our previous study, 50% relative risk reduction, compared with untreated patients, was observed for conventional interferon monotherapy that showed an overall sustained virological response (SVR) rate of 33%, and a relative risk reduction to 20% was revealed among patients who achieved SVR.[13] As the antiviral efficacy of interferon therapy has been improved by recent advances such as combination with ribavirin[14–18] and introduction of pegylated interferons,[19–22] we can expect that the efficacy on HCC prevention has also been strengthened.

We have also shown that cirrhosis gradually resolves once SVR is achieved,[23] suggesting that interferon therapy will also prevent death due to liver failure or variceal rupture. However, HCC is clearly the dominant cause of death in patients with chronic hepatitis C, at least among our cohort in Japan where the average age is over 50 years and most patients abstain from heavy alcohol consumption.[24] Thus we have focused on HCC prevention as the primary object of interferon therapy.

Considering the current status of therapeutics, interferon therapy is clearly recommended only in a selected group of patients.[25] Since the benefit of antiviral therapy differs among individual patients, the indication as well as the choice of regimen should be decided based on the expected benefit for each patient. Quantification of benefit requires reasonable assessment of the lifetime risk of HCC and the expected reduction in it with treatment. In this study, we propose an indicator, the gain in HCC free survival, to quantify the benefit specific to individual patients. The value is calculated based on both life expectancy and individualised risk of HCC, and applicable to distinct protocols with varying efficacy. It may serve as the gold standard for the benefit of antiviral therapy in terms of HCC prevention.

MATERIALS AND METHODS

Incidence rates of HCC

Crude data were obtained from the IHIT (Inhibition of Hepatocarcinogenesis by Interferon Therapy) database,[15 23 24] as updated on 31 March 2003. Every patient underwent liver biopsy in 1990 or later, and liver fibrosis was staged according to the classification system of Desmet and colleagues.[26] Patients had no history of HCC, and were positive for HCV antibody and negative for hepatitis B surface antigen. We excluded those who developed HCC or dropped out of surveillance within one year after liver biopsy, and the start

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; SVR, sustained virological response; NNT, number needed to treat

of observation was set at exactly one year after liver biopsy. Entry into the cohort was closed in 1999. The cohort population analysed in this study consisted of 2392 patients who received interferon monotherapy within one year of liver biopsy, and 395 patients who did not. Among 2392 interferon treated patients, 836 (34.9%) showed SVR, as determined six months after cessation of interferon administration. After undergoing liver biopsy, 90% of patients abstained from alcohol except for infrequent social occasions, and only 2% continued drinking alcohol (>80 g daily).

Patients underwent abdominal ultrasonography every 3–6 months, and contrast enhanced computed tomography was also performed every 6–12 months in patients with advanced fibrosis. A final diagnosis was made based on haemodynamic patterns on contrast enhanced computed tomography, abdominal angiography, or computed tomography during angiography. Ultrasound guided tumour biopsy was performed in ambiguous cases. The SVR group showed 27 events of HCC development during an observation period of 4767 person years; in the non-SVR group, 214 events in 9922 person years; and in the untreated group, 67 events in 2168 person years.

HCC incidence rates, stratified by age, sex, and fibrosis stage at entry, were calculated in each group by the person year method. Risk ratios were analysed using Cox proportional hazard regression. Age, as ranked by 10 years, and fibrosis stage were represented by dummy variables in the analysis. Adjusted HCC incidence rates were calculated so that the sum of squares of differences between the adjusted and observed values, weighted by the number of patients in each category, was minimised while conserving the risk ratios obtained by proportional hazard regression.

HCC free survival

The probability that a patient remains free of HCC at nth year of observation was calculated as:

$$(1-Q1)(1-P1)\times(1-Q2)(1-P2)\times(1-Q3)\times$$
$$(1-P3)\times \ldots \times(1-Qn)(1-Pn)$$

where Qi is the age and sex specific death rate in the general population and Pi is the annual incidence of HCC specific to the patient in the ith year. Age and sex specific death rates were those published by the Ministry of Health, Welfare, and Labour of Japan for the vital statistics surveyed in 2000.[27] The gain in HCC free survival by interferon therapy was defined as the area between the cumulative HCC free survival curves. This model is based on an assumption that fibrosis stage remains constant with time (see model limitations in the results section).

Statistics

Values are expressed as mean (SD) unless otherwise specified. All statistical analyses were performed with SAS Software version 6.12. We used an original program coded in Object Pascal to calculate cumulative HCC free survival.

RESULTS

Incidence rates of HCC

Demographic data of the patients analysed in the current study are summarised in table 1, and observed HCC development and deaths are shown in table 2, illustrating that HCC was the major sequela among the cohort. Crude incidences of HCC did not differ between the untreated group and the non-SVR group in the corresponding category (data not shown). Patients with advanced fibrosis (stages F3 or F4) in the non-SVR group or in the untreated group showed a very high incidence rate. In fact, values obtained were greater than those found in 1999,[15] suggesting that the risk of HCC has increased with time. Fibrosis stage was determined at the time of liver biopsy and had possibly progressed during the observation period. As previously described, HCC incidence rates were substantially lower in the SVR group.

Cox proportional hazard regression analysis revealed that male sex, older age, and advanced fibrosis were associated with a higher risk of HCC, both in the non-SVR groups (table 3) and in the untreated group (data not shown). Multivariate analysis showed that the risk ratio of non-SVR to no treatment was 0.835 (95% confidence interval (CI) 0.625–1.1125; $p = 0.2214$). We previously showed that the risk of HCC was decreased in patients who showed normalised serum alanine aminotransferase levels in spite of continued viraemia after interferon therapy.[24] However, active hepatitis recrudesces not infrequently in a short period[28] and the effect on HCC prevention in those patients appears to decline in the long run. Thus we assumed that interferon therapy without attaining SVR had no effect on HCC prevention. Table 4 shows the incidence rates of HCC, as fitted to the crude data by the least squares method. These values were used in modelling of the estimated HCC free survival of individual patients.

HCC free survival

Using the fitted HCC incidence rates and the age and sex specific death rates, we estimated the lifetime cumulative HCC free survival with or without SVR. Figure 1 shows an example of a 30 year old male patient with chronic hepatitis C with stage F3 fibrosis. The area under the curve indicates the expected HCC free survival and the area between the two curves is the gain in HCC free survival when the patient achieves SVR. The gain, or the area, was calculated to be 12.4 years in this case.

We similarly calculated the gain in HCC free survival under various conditions (see fig 2, table 5). By definition, these values are applicable only after SVR has been achieved. The gain in HCC free survival that can be expected before the virological outcome is known is the product of the value in table 5 and the prior probability of achieving SVR.

The gain in HCC free survival was greater when a patient was younger or fibrosis was more advanced. Judging by the expected gain, indications for treatment are questionable in patients with fibrosis stage F0 or F1 and older than 60 years because they would gain less than one year even if they

Table 1 Demographic data for the patients analysed in the current study

		Interferon treated	
	Untreated	SVR	Non-SVR
No of patients	395	836	1556
Age (y)	55.0 (10.7)	47.7 (11.9)	50.5 (6.4)
Sex (M/F)	204/191	583/253	942/614
Fibrosis stage (F0-1/F2/F3/F4) (n)	128/141/42/84	278/331/173/54	440/568/381/167
(F0-1/F2/F3/F4) (%)	32/36/11/21	33/40/21/6	28/37/24/11

SVR, sustained virological response.

Table 2 Incidence of hepatocellular carcinoma (HCC) and death in the study cohort

	Untreated	Interferon treated	
		SVR	Non-SVR
No of patients	395	836	1556
Follow up (y)	6.5 (2.8)	6.7 (3.0)	7.4 (2.9)
HCC development (n)	67	27	214
Death (n)	33	11	89
With HCC (n)*	22	6	59
Hepatic deaths			
Without HCC (n)	4	1	8
Non-hepatic deaths (n)	7	4	22

*Includes deaths not directly related to HCC in patients who had developed the cancer.
SVR, sustained virological response.

attained SVR. On the other hand, patients with fibrosis stage F3 or F4 and younger than 50 years will gain more than 10 years with SVR, and more than five years even if the probability of attaining SVR is 50%.

Recently, the efficacy of interferon therapy has been improved by the introduction of peginterferon and ribavirin. However, more effective protocols will be accompanied by an increase in cost and incidence of untoward effects. These must be counterbalanced by an increase in expected benefit. While the increase in cost is the same, that in benefit is directly proportional to the values shown in table 5 and differs in each patient. The SVR rate for type 1b genotype high viral load infection was approximately 7% among the current cohort where six months of interferon monotherapy was the main protocol. The combination of peginterferon and ribavirin for 48 weeks, which is still under clinical trials in Japan, is expected to show a response rate of 40% or better for those patients. This difference (33%) corresponds to five years of the gain in HCC free survival in 40 year old patients with fibrosis stage F4 and to approximately one year in 60 year olds with fibrosis stage F2 (one third of the values given in table 5). Although these values are a hypothetical extrapolation, they may be clinically useful in choosing treatment protocols.

Model limitations

The model described in this article is based on several assumptions. Firstly, we assumed that interferon therapy that failed to achieve SVR had no beneficial effect, as described above, and this may result in underestimation of the benefit. However, the difference cannot be large: a 30 year old male with fibrosis stage F4 has a gain of 16.59 years instead of 15.98 years, and an 80 year old male with fibrosis stage F0/1 has a gain of 0.18 years instead of 0.15 years if we based the calculations on the incidence observed in the untreated group.

Secondly, we assumed that the benefit of interferon therapy was limited to HCC prevention. This is certainly an underestimation: successful interferon therapy improves liver function and may prevent death from liver failure. Several studies, failing to find an effect on HCC incidence, still indicated improvement in liver function with interferon therapy.[29 30] However, hepatic death without developing HCC was rare in the current cohort; one patient in the SVR group (variceal rupture (n = 1)), eight in the non-SVR group (liver failure (n = 4), variceal rupture (n = 3), not specified (n = 1)), and four in the untreated group (liver failure (n = 2), variceal rupture (n = 2)) died of a liver related cause without developing HCC, indicating annual mortality rates of 0.02%, 0.08%, and 0.18%, respectively (table 2). These values were small relative to the observed incidence of HCC.

Thirdly, we assumed that fibrosis stage remained constant, with the risk of HCC unchanged except for the increment due to aging. This may not be true: in fact, we previously indicated fibrosis progression in untreated patients and amelioration in interferon responders.[23] However, we did

Table 3 Annual hepatocellular carcinoma (HCC) incidence rates according to age and sex

Age (y)	F0/1	F2	F3	F4
SVR, male				
<39	0.05% (0/65)	0.09% (0/59)	0.16% (0/14)	0.24% (0/4)
40–49	0.05% (0/57)	0.09% (0/66)	0.16% (1/29)	0.24% (0/9)
50–59	0.39% (0/38)	0.69% (3/62)	1.21% (5/51)	1.86% (1/18)
60+	0.70% (3/29)	1.18% (3/38)	2.01% (4/35)	3.20% (1/9)
SVR, female				
<39	0.02% (0/32)	0.05% (0/38)	0.10% (0/7)	0.15% (0/1)
40–49	0.03% (0/25)	0.05% (0/23)	0.10% (0/3)	0.15% (1/1)
50–59	0.23% (0/20)	0.41% (1/33)	0.73% (1/20)	1.12% (1/6)
60+	0.40% (0/6)	0.71% (1/18)	1.25% (0/14)	1.93% (1/6)
Non-SVR, male				
<39	0.05% (0/83)	0.13% (0/72)	0.28% (2/29)	0.56% (0/6)
40–49	0.35% (2/85)	1.00% (4/101)	2.16% (7/46)	4.26% (10/32)
50–59	0.82% (6/82)	2.33% (19/111)	5.06% (26/74)	10.0% (17/33)
60+	1.03% (4/36)	2.93% (13/59)	6.35% (17/64)	12.5% (15/29)
Non-SVR, female				
<39	0.02% (0/37)	0.07% (0/21)	0.14% (0/10)	0.29% (0/2)
40–49	0.18% (0/41)	0.51% (2/44)	1.10% (3/18)	2.17% (0/6)
50–59	0.42% (1/53)	1.19% (8/96)	2.57% (19/80)	5.08% (5/32)
60+	0.52% (1/23)	1.49% (11/64)	3.23% (10/60)	6.37% (12/27)

The percentages indicate the annual incidence rates fitted by the least squares method using the risk ratios shown in table 4. Numbers in parentheses are the observed events/number at risk in each category.
SVR, sustained virological response; F0–F4, fibrosis stage.

Table 4 Risk of hepatocellular carcinoma (HCC) development

Factor	Relative risk (95% CI)	
	SVR	Non-SVR
Male v female	1.66 (0.67–4.13)	1.97 (1.48–2.62)
Age (y)		
<39	1	1
40–49	7.67 (1.69–34.72)	7.61 (1.81–31.93)
50–59	13.20 (2.93–59.53)	17.84 (4.39–72.49)
60+		22.36 (5.48–91.26)
Fibrosis stage		
F0/1	1	1
F2	1.76 (0.47–6.67)	2.86 (1.59–5.13)
F3	3.10 (0.86–11.26)	6.19 (3.50–10.95)
F4	4.78 (1.13–20.18)	12.23 (6.81–21.95)

Relative risks were calculated by Cox proportional hazard regression separately in each group.

not have enough samples to calculate age stratified rates of fibrosis progression. The long term changes in HCC risk in interferon responders have not yet been clearly elucidated. Thus using the incidence rates observed in the seven year observation period was a compromise. All of the assumptions listed here may have underestimated, but not overestimated, the benefit of interferon therapy.

Lastly, we did not analyse the effect of alcohol consumption as there were very few heavy drinkers among the cohort. Alcohol is one of the major risk factors for HCC development and liver failure. The merit of successful interferon therapy may be greater in drinkers if they wish to continue alcohol. However, we recommend abstention to chronic hepatitis C patients whether or not they receive antiviral therapy.

DISCUSSION

To date, large scale cohort studies conducted in Japan, including ours, have unanimously indicated that by far the most important sequela of chronic hepatitis C is HCC development, and that interferon therapy significantly reduces its incidence. In contrast, several studies performed in Western countries found that HCC was less common, and interferon therapy did not have significant effects. The reason for this discrepancy has not been clarified. In this study, we showed that the risk of HCC substantially increased with age when patients of the same sex and fibrosis stage were compared (table 2). The prevalence of HCV infection in Japan is highly skewed to the older generations, and this may partly explain the high incidence of HCC in Japan. If this is the case, HCC incidence will increase substantially in Western countries in the near future, as it did in Japan in the 1980s.

The clinical importance of interferon therapy should be measured in terms of its efficacy in HCC prevention, at least in countries where HCC is the predominant complication of HCV infection. A popular indicator of efficacy of therapy in preventing a disease is the number (of patients) needed to treat (NNT), which is identical to the inverse of absolute risk reduction. Mathematically, NNT for one decrement in HCC development during a lifetime is equivalent to the life expectancy divided by the gain in HCC free survival. Supposing that the SVR rate is 100%, NNT is 3.92 (48.7/12.4; table 5) for a Japanese 30 year old male patient with fibrosis stage F3. This value should be divided by the expected SVR rate if the outcome is not known. As NNT is directly proportional to life expectancy, older patients have smaller values for NNT, indicating "higher efficacy", if the gain in HCC free survival is the same. This may not be intuitive for individualised consideration of indications for treatment.

Several authors have performed cost effective analyses of interferon therapy for chronic hepatitis C based on the Markov model.[31–35] In fact, our current model can be considered as a simplified Markov model where a chronic hepatitis C patient either achieves or does not achieve SVR with interferon therapy, and has the corresponding risk of HCC thereafter. Also, the HCC free survival is equivalent to the gain in quality adjusted life year, where a year before HCC development scores 1 and one after it scores 0. Owing to those simplifications, the model is not dependent on assumptive parameters but on observed data.

In conclusion, by using data obtained in a real cohort, we established an indicator of the benefit of interferon therapy—the gain in HCC free survival. This indicator may be useful in assessing the indications for interferon therapy and in selecting the best treatment protocol for individual patients.

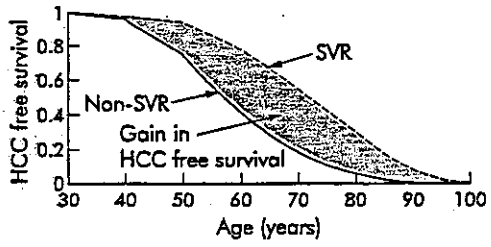

Figure 1 Gain in hepatocellular carcinoma (HCC) free survival by interferon therapy. The case of a 30 year old male patient with fibrosis stage F3. Cumulative HCC free survival curves were determined based on the patient specific HCC incidence rates and age and sex specific death rates in case of sustained virological response (SVR) and non-SVR. The area surrounded by the two curves indicates the gain in HCC free survival obtained by achieving SVR.

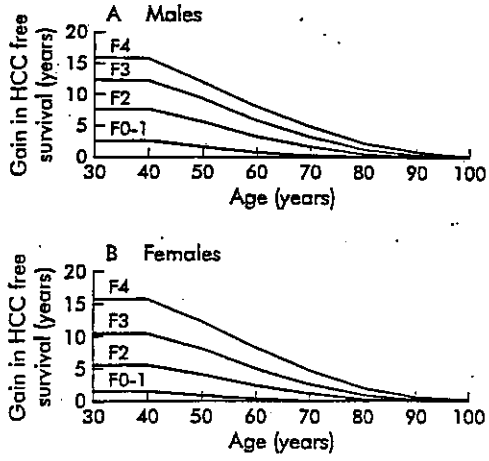

Figure 2 Gain in hepatocellular carcinoma (HCC) free survival by sustained virological response as a function of age and fibrosis stage.

Table 5 Gain in hepatocellular carcinoma (HCC) free survival by sustained virological response as a function of age and fibrosis stage

Age (y)	Life expectancy	F0/1	F2	F3	F4
Males					
30	48.7	2.48	7.66	12.40	15.98
40	39.1	2.52	7.68	12.41	15.96
50	29.9	1.68	5.75	9.45	12.14
60	21.4	0.84	3.38	5.95	8.14
70	14.0	0.40	1.70	3.26	4.98
80	8.0	0.15	0.67	1.40	2.38
Females					
30	55.3	1.45	5.60	10.52	15.73
40	45.5	1.46	5.61	10.51	15.69
50	36.0	0.93	4.24	8.17	12.44
60	26.9	0.44	2.52	5.17	8.39
70	18.2	0.22	1.30	2.81	4.95
80	10.6	0.08	0.52	1.18	2.24

Expressed in years, life expectancy was that in the Japanese general population in 2000. The gain in HCC free survival was the difference in expected cumulative HCC free survival with and without attaining a sustained virological response.

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PRELIMINARY COMMUNICATION

Role of Vitamin K_2 in the Development of Hepatocellular Carcinoma in Women With Viral Cirrhosis of the Liver

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Context Previous findings indicate that vitamin K_2 (menaquinone) may play a role in controlling cell growth.

Objective To determine whether vitamin K_2 has preventive effects on the development of hepatocellular carcinoma in women with viral cirrhosis of the liver.

Design, Setting, and Participants Forty women diagnosed as having viral liver cirrhosis were admitted to a university hospital between 1996 and 1998 and were randomly assigned to the treatment or control group. The original goal of the trial was to assess the long-term effects of vitamin K_2 on bone loss in women with viral liver cirrhosis. However, study participants also satisfied criteria required for examination of the effects of such treatment on the development of hepatocellular carcinoma.

Interventions The treatment group received 45 mg/d of vitamin K_2 (n=21). Participants in the treatment and control groups received symptomatic therapy to treat ascites, if necessary, and dietary advice.

Main Outcome Measure Cumulative proportion of patients with hepatocellular carcinoma.

Results Hepatocellular carcinoma was detected in 2 of the 21 women given vitamin K_2 and 9 of the 19 women in the control group. The cumulative proportion of patients with hepatocellular carcinoma was smaller in the treatment group (log-rank test, $P=.02$). On univariate analysis, the risk ratio for the development of hepatocellular carcinoma in the treatment group compared with the control group was 0.20 (95% confidence interval [CI], 0.04-0.91; $P=.04$). On multivariate analysis with adjustment for age, alanine aminotransferase activity, serum albumin, total bilirubin, platelet count, α-fetoprotein, and history of treatment with interferon alfa, the risk ratio for the development of hepatocellular carcinoma in patients given vitamin K_2 was 0.13 (95% CI, 0.02-0.99; $P=.05$).

Conclusion There is a possible role for vitamin K_2 in the prevention of hepatocellular carcinoma in women with viral cirrhosis.

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We previously reported a 2-year study showing that vitamin K_2 (menaquinone) helps to prevent bone loss in women with viral cirrhosis of the liver.[1] Most of the women agreed to participate in a longer study to clarify the long-term effects of vitamin K_2 on bone loss associated with cirrhosis. The incidence of hepatocellular carcinoma was found to differ between women who received vitamin K_2 and those who did not.

METHODS

The participants in this study were 50 women with viral liver cirrhosis who were admitted to a university hospital between 1996 and 1998. When the results of abdominal dynamic computed tomography and abdominal ultrasonography suggested the presence of hepatocellular carcinoma, abdominal angiography or needle biopsy was performed to confirm the diagnosis. The diagnosis of cirrhosis was based on histological examination of liver specimens obtained by laparoscopy or needle biopsy performed under ultrasonic guidance.

Hepatocellular carcinoma was confirmed in 3 women in the treatment group and 4 in the control group. These 7 women were excluded from further study. The remaining 43 women were randomly assigned using sealed envelopes to a treatment or control group. The treatment group received 45 mg/d of vitamin K_2 (Glakay, Eisai Co, Tokyo, Japan). At the end of the first study, 21 women in the treatment group and 19 in the control group consented to participate in a longer trial. All but 1 woman in each group had hepatitis C

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virus infection; the other 2 women had hepatitis B infection. Seven women, 4 in the control group and 3 in the treated group, had previously received interferon alfa for their hepatitis C virus infections, but hepatitis C virus was not eradicated. No one was given interferon therapy after study entry.

Surveillance for hepatocellular carcinoma was performed according to detailed procedures recommended for follow-up of patients with liver cirrhosis in Japan.[2] Abdominal ultrasonography or abdominal dynamic computed tomography was performed and serum α-fetoprotein levels measured tumors at 3-month intervals. Any abnormality was followed up by tumor biopsy or abdominal angiography to confirm a diagnosis of hepatocellular carcinoma.

Diagnosed cases of hepatocellular carcinoma were classified according to the primary tumor, regional lymph nodes, and distant metastasis (TNM) system of the International Union Against Cancer.[3] Histopathologic diagnosis of hepatocellular carcinoma was performed according to the criteria proposed by Edmondson and Steiner.[4] Compliance with vitamin K_2 in the treatment group was good; no patient had adverse reactions or dropped out of the study. This trial was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee at the Osaka City University Medical School. Written informed consent was obtained from each participant.

Statistical analysis was performed using SAS statistical software (version 8.12, SAS Institute Inc, Cary, NC). The χ^2 test was used to assess homogeneity between the groups. Cumulative incidences were plotted using the Kaplan-Meier method and the statistical significance of differences was analyzed using the log-rank test. Cox regression analysis was used for univariate and multivariate analyses. $P<.05$ was considered significant.

RESULTS

The 2 groups were similar with respect to age, virus type, platelets, alanine aminotransferase, α-fetoprotein, and other clinical findings (TABLE 1). Risk factors for hepatocellular carcinoma[1,6] were also similar between the groups. After the first study commenced, hepatocellular carcinoma was detected in 2 of the 21 patients given vitamin K_2 and in 9 of the 19 patients in the control group (FIGURE 1). The cumulative proportion of women with hepatocellular carcinoma was smaller in the treatment group compared with the control group (log-rank test, $P=.02$; FIGURE 2). The clinical characteristics of the women in whom hepatocellular carcinoma was detected during surveillance are shown in TABLE 2. All newly diagnosed cases of hepatocellular carcinoma were stage I or II according to the International Union Against Cancer classification and were given aggressive anticancer therapy. On univariate analysis, the risk ratio for the development of hepatocellular carcinoma in the treatment group compared with the control group was 0.20 (95% confidence interval, 0.04-0.91; $P=.04$; TABLE 3). On multivariate analysis with adjustment for age, alanine aminotransferase activity, serum albumin, total bilirubin, platelet count, α-fetoprotein, and history of treat-

Table 1. Baseline Characteristics*

	Treatment Group (n = 21)	Control Group (n = 19)	P Value
Age, y	59.8 (8.7)	61.4 (7.1)	.54†
Hepatitis virus, No. (%)			
B	1 (4)	1 (5)	.94‡
C	20 (95)	18 (95)	
Albumin, g/dL	3.9 (0.3)	3.9 (0.3)	.87†
Platelets, $\times 10^9$/mL	147 (54)	121 (52)	.13†
Total bilirubin, mg/dL	0.8 (0.2)	0.9 (0.4)	.47†
Alanine aminotransferase, U/L	81.7 (42.7)	70.4 (33.4)	.36†
α-Fetoprotein, mg/mL	13.4 (17.7)	13.3 (8.7)	.99†
Interferon, No. (%)			
Prior to enrollment§	4 (19)	3 (16)	.79‡
At enrollment	17 (81)	16 (84)	

SI conversion units: To convert bilirubin to μmol/L, multiply by 17.1.
*Values expressed as mean (SD) unless otherwise indicated.
†Values calculated using the Mann-Whitney U test.
‡Values calculated using the χ^2 test.
§Hepatocellular carcinoma developed in 1 of 4 patients in the treatment group and 1 of 3 patients in the control group who received interferon prior to enrollment.

Figure 1. Flow of Participants Through Trial

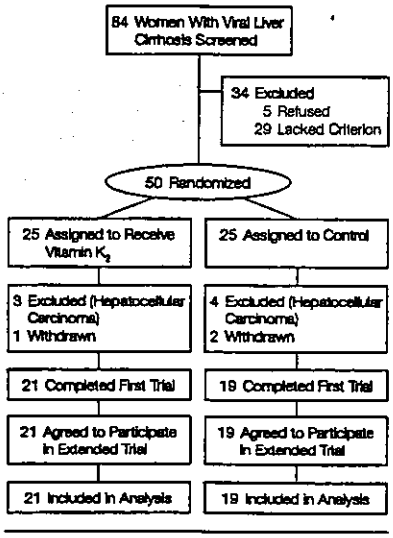

Figure 2. Cumulative Incidence of Hepatocellular Carcinoma Diagnosed in Women Treated With Vitamin K_2

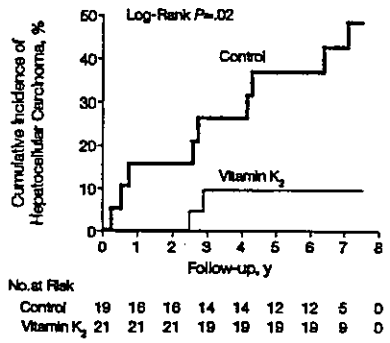

Table 2. Profiles of Women With Hepatocellular Carcinoma

Case No./ Age, y	Group	Type of Hepatitis Virus	No. of Days Diagnosis Occurred After Enrollment	No. of Tumors	Diameter of Largest Tumor, mm	UICC Cancer Stage	Method of Diagnosis	Histological Grade	Type of Therapy
1/70	Control	C	200	1	15	1	Biopsy	1*	PEIT
5/62	Control	C	2333	2	20	2	AAG	Unknown	TAE
6/59	Control	C	282	1	9	1	Biopsy	1*	PEIT
9/70	Control	C	91	1	13	1	Biopsy	1*	PEIT
21/43	Control	C	1516	1	30	2	Biopsy	2*	MCT
25/59	Control	C	1569	2	18	2	AAG	Unknown	TAE
26/67	Control	B	949	1	32	2	AAG	Unknown	TAE
33/57	Control	C	2600	1	30	2	AAG	Unknown	TAE
40/68	Control	C	1002	1	21	2	Biopsy	3*	Operation
4/64	Vitamin K_2	C	907	1	30	2	AAG	Unknown	TAE
27/68	Vitamin K_2	C	1054	1	11	1	Biopsy	1*	PEIT

Abbreviations: AAG, abdominal angiography; MCT, microwave coagulation therapy; PEIT, percutaneous ethanol injection therapy; TAE, transcatheter hepatic arterial embolization; UICC, International Union Against Cancer.
*Based on Edmondson and Steiner classification system (scale of 1-4).[4]

Table 3. Crude Odds Ratios for Development of Hepatocellular Carcinoma

	Odds Ratio (95% Confidence Interval)	*P* Value
Group*	0.20 (0.04-0.91)	.04
Total bilirubin	1.23 (0.33-4.64)	.76
Albumin	3.80 (0.80-18.06)	.09
Platelets	1.55 (0.46-5.19)	.48
Alanine aminotransferase	0.62 (0.17-2.36)	.49
α-Fetoprotein	1.67 (0.36-7.79)	.51
Interferon	1.00 (0.22-4.65)	>.99

*Vitamin K_2 group compared with control group.

Table 4. Adjusted Odds Ratios for Development of Hepatocellular Carcinoma*

	Odds Ratio (95% Confidence Interval)	*P* Value
Group†	0.13 (0.02-0.99)	.05
Total bilirubin	0.29 (0.04-2.04)	.22
Albumin	33.43 (2.36-473.35)	.009
Platelets	2.24 (0.46-10.90)	.32
Alanine aminotransferase	0.39 (0.07-2.16)	.28
α-Fetoprotein	1.69 (0.31-9.34)	.55
Interferon	1.26 (0.20-7.90)	.81

*Values are adjusted for age and all other variables in this table.
†Vitamin K_2 group compared with control group.

ment with interferon alfa, the risk ratio for the development of hepatocellular carcinoma in women given vitamin K_2 was 0.13 (95% confidence interval, 0.02-0.99; $P=.05$; TABLE 4).

COMMENT

Vitamin K is a cofactor for the enzyme γ-glutamyl-carboxylase, which converts glutamate residues into γ-carboxyglutamate. Vitamin K–dependent proteins include prothrombin II and the coagulation factors VII, IX, X, proteins C and S, osteocalcin, surfactant-associated proteins, and bone matrix protein. The vitamin K family of molecules comprises the natural forms vitamin K_1 (phylloquinone) and vitamin K_2 (menaquinones) and the synthetic form of vitamin K_3 (menadione). These naphthoquinone-containing molecules inhibit tumor cell growth in culture, with vitamin K_3 being more potent than either vitamin K_1 or K_2. Vitamin K_2 inhibits growth of human cancer cell lines and induction of differentiation in various human myeloid leukemia cell lines.[7,8] Clinically, vitamin K_2 has successfully treated myelodysplastic syndrome.[9]

A number of findings indicate that vitamin K may play a role in controlling cell growth. Underlying mechanisms possibly involve (1) cycling of oxidation and reduction (as known for vitamin K_3), (2) proteins with growth-inhibitory properties induced by vitamin K, such as prothrombin,[10] (3) previously unidentified pathways involving arylation,[11] (4) or growth arrest genes such as gas 6.[12] Geranylgeraniol, which is a side chain of vitamin K_2, strongly induces apoptosis of tumor cells, suggesting that geranylgeraniol might play an important role in inhibiting cell growth.[13] The mechanisms responsible for the inhibition of cell growth mediated by vitamin K_2 remain unexplained. These or other hypothetical mechanisms may have contributed to the reduced hepatocellular carcinoma incidence among patients receiving vitamin K_2. Indeed, the annual incidence of hepatocellular carcinoma in the control group was 8.8%, which is similar to the incidence of hepatocellular carcinoma (7.9%; 32/107) in cirrhotic patients in Japan[5] compared with 1.6% in the treatment group.

As shown in Table 4, the albumin level showed the highest odds ratio for the development of hepatocellular carcinoma. The serum albumin level is considered an important prognostic factor in liver cirrhosis.[14-17] Low serum albumin levels in patients with liver cirrhosis are associated with disease progression, poor nutritional status, and compromised immunity, which increases the risk of carcinogenesis. The importance of low serum albumin levels as a risk factor for hepatocellular carcinoma should be confirmed in larger studies.

The original goal of our trial was to assess the long-term effects of vitamin K_2 on bone loss in women with viral liver cirrhosis. Our trial had several important limitations when the data were used to assess the value of vitamin K_2 for the primary prevention of hepatocellular carcinoma in patients with liver cirrhosis, resulting from the small study group,

the inclusion of only women, and the participation of only 1 center. However, similar to previously reported randomized controlled studies of cirrhosis in which the primary end point was the development of hepatocellular carcinoma, patients with evidence of hepatocellular carcinoma on highly sensitive imaging studies were excluded, and the 2 study groups were similar with respect to risk factors for hepatocellular carcinoma, such as age, severity of cirrhosis, history of interferon therapy, and type of hepatitis virus infection. The procedures used for the surveillance and diagnosis of hepatocellular carcinoma were also similar to those used in our study. The sensitivity of these procedures for the detection of hepatocellular carcinoma was underscored by the fact that all of the detected cases of hepatocellular carcinoma were stage I or stage II. Our results must also be tempered by the fact that 3 cases of hepatocellular carcinoma were diagnosed in the control group within a year of enrollment. These patients may have harbored occult disease at the time of enrollment. Nonetheless, despite its small size, our study indicates that vitamin K_2 decreases the risk of hepatocellular carcinoma to about 20% compared with the control group, suggesting that vitamin K_2 may delay the onset of hepatocarcinogenesis. Moreover, the safety, relatively low cost, and ease of use of vitamin K_2 led to good compliance with treatment. The results of this preliminary trial are intriguing and suggest that a potential role for vitamin K_2 to prevent hepatocarcinogenesis in patients with liver cirrhosis. These results must be confirmed by multicenter randomized controlled studies with the prevention of hepatocellular carcinoma by vitamin K_2 as the primary end point.

Author Contributions: Dr Shiomi had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Study concept and design: Habu, Shiomi, Kubo, Nishiguchi.
Acquisition of data: Habu, Shiomi, Tamori, Takeda, Nishiguchi.
Analysis and interpretation of data: Habu, Shiomi, Tamaka, Kubo, Nishiguchi.
Drafting of the manuscript: Habu, Shiomi, Tamori, Takeda, Kubo, Nishiguchi.
Critical revision of the manuscript for important intellectual content: Habu, Shiomi, Tamaka, Nishiguchi.
Statistical Analysis: Habu, Tamaka.
Obtained funding: Habu, Shiomi, Tamori, Takeda, Nishiguchi.
Administrative, technical, or material support: Habu, Shiomi, Tamori, Nishiguchi, Kubo.
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Effects of ribavirin combined with interferon-α2b on viral kinetics during first 12 weeks of treatment in patients with hepatitis C virus genotype 1 and high baseline viral loads

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SUMMARY. This study aimed to find how ribavirin increases viral disappearance in patients with hepatitis C virus (HCV) of genotype 1 and high baseline viral loads ($>5.0 \times 10^5$ copies/mL) when given with interferon (IFN). Using the real-time quantitative polymerase chain reaction, we measured serum HCV in 20 patients during the first 12 weeks of therapy with IFN-α2b and ribavirin. Controls were 10 similar patients given IFN-α2b alone. IFN-α2b was given at 6 MU daily for 2 weeks, and then three times weekly. Ribavirin was given at 600 or 800 mg daily. Serum HCV RNA decreased rapidly in the first phase, during the first 24 h of therapy (day 0), and more slowly in the early second phase (days 1–14). The median decrease was by 1.41 and 0.078 log 10/day in these two phases in the combination therapy group, and 0.90 and 0.081 log 10/day in the monotherapy group. The difference between groups in the first phase was not significant ($P = 0.24$), nor was that in the next phase ($P = 0.68$). Later in the second phase, between days 14 and 84, the median decrease was larger in the combination therapy group (0.030 log 10/day) than in the monotherapy group (0.015 log 10/day, $P = 0.035$). In patients with HCV genotype 1 and high viral loads, the effects of ribavirin with IFN-α appeared slowly, after the earliest days of treatment. A long-term favourable outcome of combination therapy may be associated with a rapid viral decline in this later phase of therapy.

Keywords: hepatitis C, interferon, polymerase chain reaction, ribavirin, viral kinetics.

INTRODUCTION

Hepatitis C virus (HCV) infects some 170 million people worldwide and is an important health care problem [1]. Persistent infection with HCV often progresses to chronic hepatitis, liver cirrhosis and hepatocellular carcinoma over the course of several decades. As the report by Hoofnagle *et al.* [2] in 1986 on the effects of interferon (IFN) therapy on chronic hepatitis C, this drug has been the only approved agent for eradication of HCV and perhaps reduction of the incidence of hepatocellular carcinoma [3–5]. Several factors identifiable before therapy are independent predictors of the response to IFN, including the baseline level of serum HCV [6,7] and the HCV genotype [8]. In addition, analysis of the changes in HCV titres in the early part of IFN treatment is useful for prediction of the therapeutic response. After a delay of 7–10 h after IFN administration starts, the amount of viral RNA declines rapidly, with an estimated half-life of 5.0–7.2 h, during the first 1 or 2 days of therapy, and then declines more slowly [9,10]. In patients with HCV genotype 1, viral decline in the early phase of IFN treatment is slower than that in patients with genotype 2 [11], which may explain in part their lower rate of sustained virological response. We reported earlier [12] that the changes in serum levels of HCV genotype 1 during the first 2 weeks of IFN-α treatment can be used for prediction of the long-term outcome of therapy.

Ribavirin is a synthetic guanosine nucleoside analogue that inhibits the replication of various RNA and DNA viruses. In patients with chronic hepatitis C, the combination of IFN-α and ribavirin gives a higher rate of sustained virological response than IFN-α alone [13–17]. The combination has become the standard therapy, especially for patients with HCV genotype 1 and high baseline viral loads. However, the

Abbreviations: HCV, hepatitis C virus; IFN, interferon; TaqMan PCR, real-time quantitative polymerase chain reaction. PEG, polyethyleneglycol.

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synergistic effects of ribavirin when combined with IFN on the changes in HCV levels have not been fully identified. Zeuzem *et al.* reported that viral kinetics do not differ between patients treated with IFN-α alone and those given ribavirin also [18]. One possible reason for this unexpected finding may be that viral kinetics could be analysed in that study only in the first few weeks of therapy. That is, patients with genotype 2 or with low viral loads were included, and the analytical sensitivity of the assay used to measure HCV RNA was limited, so viral RNA became undetectable in most subjects within the first 4 weeks of therapy. Pharmacokinetic studies showed that serum ribavirin concentrations take 4–8 weeks to reach a plateau [19,20]; the synergistic effects of this drug with IFN might become evident slowly, after the earliest phase of treatment. To test this hypothesis, a study of the kinetics of HCV in patients with genotype 1 and high viral loads monitored by a more sensitive quantitative method during a longer term of therapy with IFN alone and in combination with ribavirin is needed.

The aim of this study was to find how ribavirin increases viral decline in patients with chronic HCV infection when given with IFN-α. Using a sensitive real-time quantitative polymerase chain reaction (TaqMan PCR), we monitored serum HCV levels in patients with genotype 1 and high viral loads during the first 12 weeks of combination therapy with IFN-α2b and ribavirin. The results were compared with those in patients with similar baseline characteristics treated with IFN-α2b alone.

MATERIALS AND METHODS

Patients

The subjects were 20 patients with chronic hepatitis C (13 men and seven women; mean age, 55 ± 11 years) who started combination therapy with IFN-α2b and ribavirin at our hospital between March 1999 and December 2002. Ten patients with chronic hepatitis C (six men and four women; mean age, 56 ± 9 years) who were treated with IFN-α2b alone between October 1998 and July 2001 were used as historic controls. The inclusion criteria were as follows: persistent elevation of serum alanine aminotransferase activity for at least 6 months before therapy, presence of genotype 1 of HCV in serum, presence of serum HCV RNA of more than 5.0×10^5 copies/mL by TaqMan PCR, absence of serum hepatitis B surface antigen and signs of other likely causes of chronic liver disease, histological features of chronic hepatitis found in liver biopsy specimens taken within 6 months before the start of therapy, and no evidence of hepatocellular carcinoma on ultrasonography or computed tomography. Serum samples were obtained from the patients before the administration of the drug(s) on the first day of therapy (day 0) and on days 1, 7, 14, 28, and 84, and were stored at −80 °C before being tested. Procedures of the study were in accord with the Helsinki Declaration of 1975 (1983 revision) and were approved by our hospital ethics committee.

Treatment

Patients were treated with recombinant IFN-α2b (Intron A, Schering-Plough, Kenilworth, NJ, USA) by intramuscular injection at the dosage of 6 MU every day for 2 weeks, followed by 6 MU three times a week for 22–46 weeks. Ribavirin (Rebetol; Schering-Plough, Kenilworth, NJ, USA) was given orally twice a day at a total daily dose of 600 mg for the 10 patients who weighed 60 kg or less and 800 mg for the remaining 10 patients who weighed more than 60 kg for 24 weeks. The most common adverse effect of ribavirin is haemolysis. The dose of ribavirin was reduced by 200 mg per day in patients whose haemoglobin concentrations fell below 10 g/dL.

Assays

Routine haematological and biochemical tests were performed by the standard procedures. Genotypes of HCV were identified by direct sequencing of the amplification products generated during the Amplicor Monitor test (Roche Diagnostics, Branchburg, NJ, USA) [21] with an ABI 3700 DNA sequencer (Perkin Elmer Corp./Applied Biosystems, Foster City, CA, USA) [22]. Serum HCV RNA was measured by TaqMan PCR as described by Takeuchi *et al.* [23]. In brief, HCV RNA was extracted from 250 mL of a serum sample, converted to complementary DNA with reverse transcriptase, and amplified by PCR with a TaqMan EZ RT-PCR kit and the ABI Prism 7700 sequence detection system (Perkin Elmer). A TaqMan probe, labelled with fluorescent reporter and quencher dyes, annealed specifically to the template between the primers and then was digested during the PCR, resulting in the emission of fluorescence. Successive PCR cycles exponentially amplified the PCR product and increased the fluorescence intensity. The detection range of the assay was between 2.0×10^2 and 1.0×10^9 copies/mL of HCV RNA. For comparison, a second generation version of the Amplicor Monitor test [21] was used also to measure HCV RNA in serum. The detection range of the assay was between 0.5 and 500 kIU/mL (a standard sample containing 10^5 copies/mL of HCV was assigned a titre of 10^5 IU/mL).

Histology

Liver biopsy was performed for each patient within 6 months before the start of therapy. The histopathological findings were assessed by grading of inflammatory activity and staging of fibrosis by the classification of Desmet *et al.* [24] by an experienced pathologist blinded to the clinical data.

Statistical analysis

Statistical analysis was performed with the Statview SE + Graphics program, version 5.0 (SAS Institute, Cary, NC, USA). Distributions of continuous variables were

Table 1 Baseline characteristics of patients with chronic hepatitis C treated with IFN-α2b with or without ribavirin

Characteristics	Combination therapy group (n= 20)	Monotherapy group (n= 10)	P-value
Age (years)	55 ± 11	56 ± 9	0.96
Sex (M/F)	13/7	6/4	0.93
Previous IFN treatment (+/–)	13/7	6/4	0.93
Haemoglobin (g/dL)	14.3 ± 1.2	14.2 ± 1.5	0.86
ALT (IU/L)	102 (74–135)	94 (73–109)	0.64
HCV RNA (log 10 copies/mL)	7.07 ± 0.35	6.78 ± 0.51	0.11
Grade of inflammation			
Mild	11	7	0.52
Moderate	8	3	
Severe	0	0	
Stage of fibrosis			
Mild	8	6	0.42
Moderate	5	3	
Severe	6	1	

Values represented as mean ± SD for normally distributed variables, and medians (with the interquartile range) for non-normally distributed variables.
Serum HCV RNA was measured by TaqMan PCR.
IFN, interferon; ALT, alanine amino transferase; HCV, hepatitis C virus.

analysed by the Mann–Whitney *U*-test. Differences in proportions were tested by Fisher's exact test. The significance of correlation was evaluated by Spearman's rank analysis. A two-tailed P-value of <0.05 was taken to indicate statistical significance.

RESULTS

Baseline characteristics of patients

The baseline characteristics of patients in the two groups were similar (Table 1). All patients were infected with genotype 1b of HCV, which is the most common kind in this country. In one patient in the combination therapy group, the biopsy sample was too small for evaluation, except for the finding of chronic hepatitis.

Changes in HCV RNA in first 12 weeks of treatment

In the first 12 weeks of treatment, no patient needed reduction in the dose of IFN-α2b. The dose of ribavirin was reduced in one patient at week 10, because the haemoglobin concentration was <10 g/dL. The proportions of patients without HCV RNA detectable by the Amplicor Monitor test and by TaqMan PCR at different times during therapy are shown in Tables 2 and 3, respectively. Of the patients in whom serum HCV RNA decreased to under the detection limit, none had relapse of viraemia during the 12 weeks. Changes in serum HCV RNA as monitored by TaqMan PCR during the first 12 weeks of therapy are shown in Fig. 1. As previously reported, serum HCV levels decreased rapidly during the first 24 h of therapy and more slowly thereafter. For the analysis here, we tentatively defined the period between 0 and 24 h of therapy (day 0) as the first phase, the period between days 1 and 14 as the early second phase, and the period between days 14 and 84 as the late second phase (see Discussion).

Decline of HCV RNA in different phases of treatment

The rate of decrease in serum HCV RNA as monitored by TaqMan PCR in each phase of treatment with IFN-α2b alone

Table 2 Patients without detectable serum HCV RNA by Amplicor Monitor test four times during treatment

Group	n	Numbers (%) on Day 7	Day 14	Day 28	Day 84
Combination therapy	20	0 (0)	3 (15)	8 (40)	13 (65)
Monotherapy	10	0 (0)	0 (0)	0 (0)	6 (60)

Table 3 Patients without detectable serum HCV RNA by TaqMan PCR four times during treatment

Group	n	Numbers (%) on Day 7	Day 14	Day 28	Day 84
Combination therapy	20	0 (0)	1 (5)	3 (15)	12 (60)
Monotherapy	10	0 (0)	0 (0)	0 (0)	4 (40)

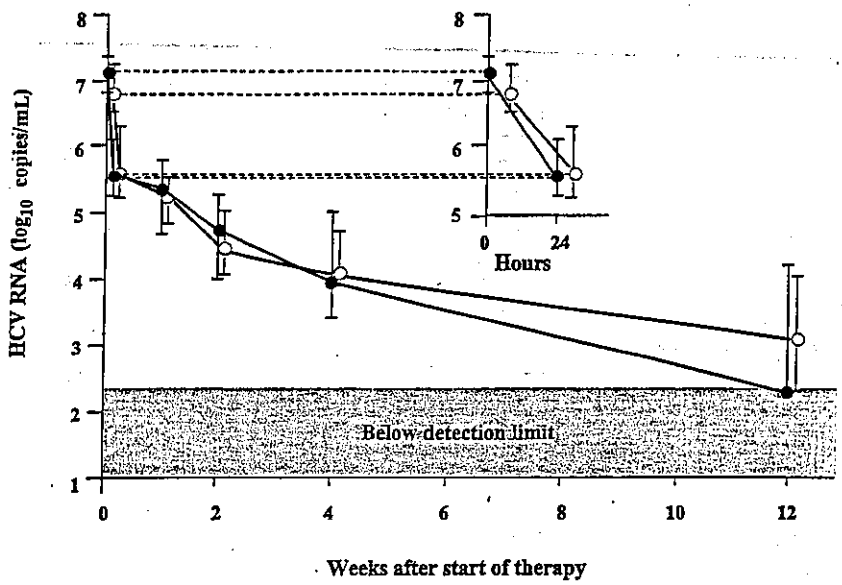

Fig. 1 Changes in serum hepatitis C virus (HCV) RNA during the first 12 weeks of therapy: (●) in 20 patients treated with interferon (IFN)-α2b in combination with 600 or 800 mg of ribavirin daily depending on body weight, and (○) in 10 patients treated with IFN-α2b alone. IFN-α2b was given at the dosage of 6 MU every day for 2 weeks, followed by 6 MU three times a week. Serum HCV RNA was measured by TaqMan PCR. Values are medians, with bars showing the interquartile range. The lower detection limit of TaqMan PCR was 2.0×10^2 copies/mL.

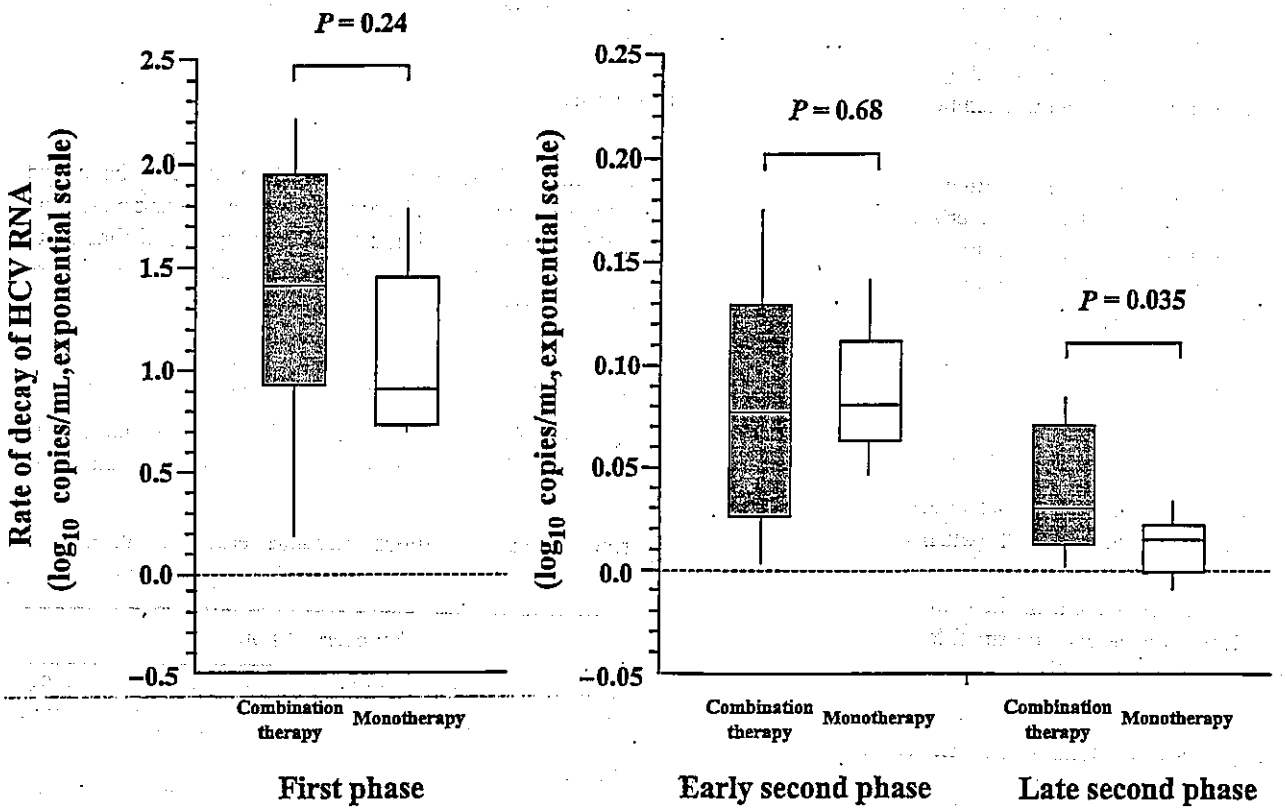

Fig. 2 Rate of decrease in serum hepatitis C virus (HCV) RNA per day in patients treated with interferon (IFN)-α2b alone or in combination with ribavirin: in the first phase between 0 and 24 h of therapy (day 0), in the early second phase between days 1 and 14, and in the late second phase between days 14 and 84. Serum HCV RNA was measured by TaqMan PCR. The boxes show medians and interquartile ranges; the vertical bars show the ranges. In the late second phase between days 14 and 84, the rate of decrease in the combination therapy group was significantly higher than that in the monotherapy group ($P = 0.035$).

or in combination with ribavirin is shown in Fig. 2. The differences in the first 14 days between the two groups were not significant (first phase $P = 0.24$, and second phase $P = 0.68$). In the late second phase (between days 14 and 84, or between days 14 and 28, if HCV RNA was not detected on day 84), the decrease in the combination therapy group was

larger than that in the monotherapy group ($P = 0.035$), when samples below the detection limit of the assay were assigned the viral load of the detection limit and one patient without detectable HCV RNA at day 14 was excluded from analysis.

In patients given both drugs, correlation was not significant between the first phase and early second phase decreases ($r = -0.084$, $P = 0.73$), between the early second phase and late second phase decreases ($r = -0.12$, $P = 0.63$), nor between the late second phase and first phase decreases ($r = 0.29$, $P = 0.23$). In patients given IFN-α2b only, correlation was not significant in any of these comparisons ($r = -0.27$, $P = 0.54$; $r = 0.36$, $P = 0.39$; $r = -0.29$, $P = 0.51$, respectively).

DISCUSSION

Earlier [25], we compared the analytical sensitivity and validity of TaqMan PCR for measurement of HCV RNA with those of other widely used quantitative methods. The sensitivity of TaqMan PCR was the highest, and the method gave accurate results throughout the wide detection range. In this study, the baseline HCV level of all patients was within the detection range of TaqMan PCR, but was more than the upper detection limit (500 kIU/mL) of the Amplicor Monitor test in 18 of the 30 patients. The proportion of patients without detectable HCV RNA during therapy was smaller with TaqMan PCR than with the Amplicor Monitor test. TaqMan PCR was more suitable for the monitoring of changes in HCV RNA levels during antiviral therapy, because of its wide detection range.

We found that the second phase decrease in HCV monitored by TaqMan PCR is correlated with the sustained virological response to IFN-α monotherapy [12]. Neumann *et al.* [9] suggested that the rapid viral decrease in the first phase reflects the dose-dependent effects of IFN on HCV production, and that the slower decrease in the second phase arises from the death of hepatocytes infected with HCV. The rate of HCV-infected cell death may depend on cellular immunity involving cytotoxic T-lymphocytes. Therefore, the results of our previous study may mean that a strong cellular immune response is needed for sustained loss of HCV by treatment with IFN alone.

For two reasons, we divided the second phase into the early second phase (first 2 weeks) and the late second phase (after the first 2 weeks) and evaluated changes in HCV RNA. First, our protocol of IFN administration, which is common in this country, consisted of daily induction therapy for the first 2 weeks and then thrice weekly maintenance therapy. As expected, in both groups, viral decrease in the early second phase (still induction therapy) was larger than that in the late second phase (maintenance therapy). Secondly, it takes several weeks for serum ribavirin concentrations to reach a plateau [19,20]. The exact mechanism of action of ribavirin when combined with IFN is not known [26]. Ribavirin may inhibit HCV RNA-dependent RNA polymerase, the capping structure of viral messenger RNA, and inosine monophosphate dehydrogenase. Other immunomodulatory actions also may contribute to the effects of this drug [27,28]. No matter which action is most important, these pharmacokinetic results suggested that the synergistic effects of ribavirin given with IFN might appear slowly after the earliest phase of treatment. The most striking finding in our study was that in the late second phase, the rate of decrease in HCV in the combination therapy group was larger than that in the monotherapy group. Because the observation period in this study was 12 weeks, we do not know which phase(s) of viral decrease is associated with sustained virological response to combination therapy. However, faster viral decline in the late second phase would contribute to the long-term outcome of treatment.

Changes in serum HCV RNA during the first few weeks of IFN monotherapy are useful in prediction of the outcome [29,30]. Reliable prediction of the response to therapy early would be useful, because IFN is expensive and sometimes has serious side-effects. In this study, however, the viral decrease in the first 2 weeks was similar in the two groups, and was not correlated with the viral decrease afterwards. Perhaps viral decline in the first few days of treatment cannot be used for prediction of the long-term response to combination therapy with IFN-α and ribavirin.

The attachment of a polyethyleneglycol (PEG) moiety to IFN-α produces a biologically active molecule, PEG-IFN-α, with a long half-life and favourable pharmacokinetics. Randomized controlled trials have shown that weekly treatment with PEG-IFN-α plus ribavirin is well tolerated, and gives higher rates of sustained virological response than treatment with unmodified IFN-α plus ribavirin [31,32]. Changes in HCV levels during combination therapy with PEG-IFN-α and ribavirin should be further evaluated in large clinical studies.

In summary, in a group of patients with HCV genotype 1 and high viral loads treated with IFN-α2b plus ribavirin and such a group treated with IFN-α2b alone, differences in the rate of decrease in viral RNA in the first phase, during the first 24 h of therapy, and in the early second phase, between days 1 and 14, were not significant. Later in the second phase, between days 14 and 84, the rate of decrease in the combination therapy group was greater than that in the monotherapy group. Our results suggest that a long-term favourable response to therapy with IFN-α2b and ribavirin for chronic hepatitis C is associated with rapid viral disappearance in this later phase.

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