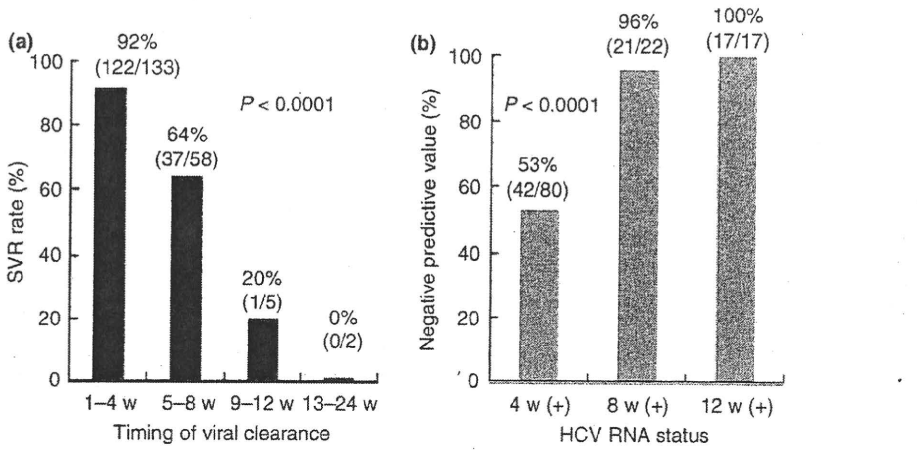

Fig. 1 (a) SVR rates according to timing of viral clearance. The number above each bar shows the percentage, and the numbers inside parentheses show the number of patients showing responses over the total number in the subgroup. The timing of viral clearance was time-dependently correlated with SVR ($P < 0.0001$). (b) Negative predictive values according to time of HCV RNA positivity. The number above each bar shows the percentage, and the numbers inside parentheses show the number of patients showing responses over the total number in the subgroup. The time of HCV RNA positivity was time-dependently correlated with NPV ($P < 0.0001$).

week 12 and 0% (0/2) from week 13 until week 24. The Mantel–Haenszel chi-square test showed that SVR rates were diminished with a delay in the timing of viral clearance becoming late ($P < 0.0001$). Significantly, more patients who attained RVR achieved final SVR (92%, 122/133) than patients who failed to attain RVR (48%, 38/80; $P < 0.0001$).

Next, we examined the negative predictive value (NPV) for the proportion of patients with treatment failure among those with HCV RNA persistence at week 4, 8 and 12 (Fig. 1b). NPV was 53% at week 4, 96% at week 8 and 100% at week 12. Only one of the 22 patients with positive HCV RNA at week 8 reached SVR.

Predictors of sustained virological response

Both pretreatment and treatment factors that could be associated with the response to Peg-IFN and ribavirin combination therapy were compared between patients with and without SVR in Table 2. This univariate analysis showed that age ($P = 0.029$), baseline HCV RNA level ($P = 0.033$), past IFN treatment history ($P = 0.028$), platelets counts ($P = 0.020$) and having RVR ($P < 0.0001$) contributed to achievement of SVR. Factors that were significantly associated with SVR by univariate analysis were then analysed by multivariate logistic regression analysis. SVR was attained independent of high platelet counts [odds ratio (OR) 1.070, 95% confidence interval (CI) 1.003–1.140, $P = 0.040$] and having RVR (OR 11.526, 95% CI 5.317–24.984, $P < 0.0001$; Table 3). As for drug doses, the mean dose of Peg-IFN alpha-2b was 1.32 ± 0.27 μg/kg/week in patients with SVR and 1.27 ± 0.29 μg/kg/week in those without SVR ($P = 0.130$), while that of ribavirin was 10.2 ± 1.9 and 10.2 ± 2.0 mg/kg/day ($P = 0.949$), respectively. Thus, neither Peg-IFN nor ribavirin drug exposure during the full treatment period affected attainment of SVR.

Predictors of rapid virological response

To delineate features that might help identify patients most likely to reach RVR, we also analysed these factors because having RVR turned out to be one of the most powerful predictors of SVR attainment. By univariate and multivariate logistic-regression analyses, RVR was attained independent of younger age (OR 0.648, 95% CI 0.494–0.850, $P = 0.002$) and lower baseline HCV RNA level (OR 0.964, 95% CI 0.944–0.984, $P < 0.0001$; Tables 4 & 5). The mean dose of Peg-IFN alpha-2b during the first 4 weeks was 1.31 ± 0.27 μg/kg/week in patients with RVR and 1.31 ± 0.29 μg/kg/week in those without RVR ($P = 0.259$), that of ribavirin was 10.1 ± 1.8 mg/kg/day and 10.3 ± 2.1 mg/kg/day ($P = 0.637$), respectively. Thus, neither Peg-IFN nor ribavirin drug exposure during the first 4 weeks had an impact on attainment of RVR.

Virological response according to drug exposure and the timing of viral clearance

Impact of drug exposure on sustained virological response

To more closely evaluate the impact of drug exposure on virological response, we classified the average doses of both drugs into four categories (Peg-IFN alpha-2b: up to 0.9 μg/kg/week, from 0.9 to >1.2 μg/kg/week, from 1.2 to >1.5 μg/kg/week, from 1.5 μg/kg/week; ribavirin: up to

Table 2 Factors associated with SVR among patients who completed the treatment – univariate analysis

Factor	SVR (n = 160)	Non-SVR (n = 53)	P-value
Age (years)*	52.4 ± 12.6	56.9 ± 10.2	0.029
Sex (male/female)	66 / 94	26 / 27	0.202
Body weight (kg)*	59.5 ± 11.5	59.9 ± 12.5	0.896
Body mass index (kg/m^2)*	22.8 ± 3.1	22.8 ± 3.5	0.817
HCV RNA (KIU/mL)†	1170	1600	0.033
Past IFN therapy (naive/experienced)‡	116/41	31/22	0.028
Fibrosis (F 0–2/3–4)§	106/10	30/5	0.247
Activity (A 0–1/2–3)§	62/54	20/15	0.847
White blood cells (/mm^3)*	5260 ± 1680	4720 ± 1500	0.078
Neutrophils (/mm^3)*	2740 ± 1270	2420 ± 1020	0.186
Red blood cells (×10^4/mm^3)*	435 ± 44	437 ± 55	0.820
Haemoglobin (g/dL)*	13.9 ± 1.3	14.0 ± 1.5	0.441
Platelets (×10^4/mm^3)*	19.0 ± 6.0	16.5 ± 6.2	0.020
ALT (IU/L)*	86 ± 89	64 ± 45	0.514
γ-GTP (U/L)*	54 ± 67	58 ± 59	0.512
Creatinine (mg/dL)*	0.7 ± 0.1	0.7 ± 0.1	0.457
Mean Peg-IFN dose (μg/kg/week)*	1.32 ± 0.27	1.27 ± 0.29	0.130
Mean ribavirin dose (mg/kg/day)*	10.2 ± 1.9	10.2 ± 2.0	0.949
RVR (yes/no)	122/11	38/42	<0.0001

IFN, interferon; HCV, hepatitis C virus; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; CI, confidence interval. *Values expressed as mean ± sd, †values expressed as median, ‡interferon treatment history was not known for three patients, §data for 62 patients are missing.

Table 3 Factors associated with SVR among patients who completed the treatment – multivariate analysis

Factor	Category	Odds ratio	95% CI	P-value
Age (years)	By 10	–	–	NS
HCV RNA (KIU/mL)	By 100 KIU/mL	–	–	NS
Platelets (×10^4/mm^3)	By 1 × 10^4/mm^3	1.068	1.002–1.139	0.045
Past IFN therapy	Naïve/experienced	–	–	NS
RVR	Yes/no	11.251	5.184–24.419	<0.0001

IFN, interferon; HCV, hepatitis C virus; CI, confidence interval.

8 mg/kg/day, from 8 to >10 mg/kg/day, from 10 to >12 mg/kg/day, from 12 mg/kg/day). SVR rates relative to the mean drug doses during the full treatment period and the timing of HCV RNA clearance are shown in Table 6. As also shown in Fig. 1a, the respective rates for SVR according to the timing of viral clearance were 92% in patients clear of HCV RNA until week 4, 64% from week 5 until week 8 and 14% from week 9 until week 24. On the contrary, according to mean drug doses, the respective rates for SVR were 89% (24/27), 73% (11/15), 79% (85/107) and 82% (40/49) in patients who received Peg-IFN up to 0.9 μg/kg/week, from 0.9 to >1.2 μg/kg/week, from 1.2 to >1.5 μg/kg/week and from 1.5 μg/kg/week, respectively, and 80% (24/30), 80% (40/50), 82% (68/83) and 79% (27/34) in patients who received ribavirin up to 8 mg/kg/day, from 8 to >10 mg/kg/day, from 10 to >12 mg/kg/day and from 12 mg/kg/day, respectively. If the category of the timing of viral clearance was the same, the respective rates for SVR attainment according to the mean doses of both Peg-IFN and ribavirin were similar. Furthermore, multivariate analysis by the Mantel–Haenszel chi-square test showed that neither the mean dose of Peg-IFN (P = 0.795) nor ribavirin (P = 0.649) affected SVR rates after stratification of the timing of viral clearance. Among the patients with RVR, SVR rates were as high as 88–100% regardless of Peg-IFN alpha-2b medication, and the least medicated group (<0.9 μg/kg/week, the mean dose with SD was 0.77 ± 0.10 μg/kg/week, 0.50–0.89) showed 100% of SVR rate (19/19). Similarly, SVR rates were as high as 91–94% regardless of ribavirin medication among the patients with RVR, and 17 of 18 patients (94%) in the least medicated group (<8 mg/kg/day, the mean dose with SD was 6.9 ± 0.90 mg/kg/day, 5.0–7.9)

Table 4 Factors associated with RVR among patients who completed the treatment – univariate analysis

Factor	RVR (n = 133)	Non-RVR (n = 80)	P-value
Age (years)*	51.9 ± 12.3	56.3 ± 11.3	0.010
Sex (male/female)	60/73	32/48	0.279
Body weight (kg)*	60.2 ± 11.6	58.6 ± 11.9	0.276
Body mass index (kg/m^2)*	22.9 ± 3.2	22.6 ± 3.1	0.369
HCV RNA (KIU/mL)†	1050	1800	0.001
Past IFN therapy (naive/experienced)‡	97/34	50/29	0.068
Fibrosis (F 0–2/3–4)§	86/8	50/7	0.315
Activity (A 0–1/2–3)§	51/43	31/26	1.000
White blood cells (per mm^3)*	5300 ± 1760	4850 ± 1400	0.205
Neutrophils (per mm^3)*	2740 ± 1290	2530 ± 1090	0.340
Red blood cells (×10^4/mm^3)*	440 ± 45	432 ± 49	0.628
Haemoglobin (g/dL)*	13.9 ± 1.4	13.9 ± 1.4	0.975
Platelets (×10^4/mm^3)*	18.9 ± 6.1	17.5 ± 6.1	0.170
ALT (IU/L)*	87 ± 93	69 ± 52	0.630
γ-GTP (U/L)*	57 ± 71	53 ± 53	0.658
Creatinine (mg/dL)*	0.7 ± 0.1	0.7 ± 0.1	0.203
Mean Peg-IFN dose (μg/kg/week)*	1.31 ± 0.27	1.31 ± 0.29	0.259
Mean ribavirin dose (mg/kg/day)*	10.1 ± 1.8	10.3 ± 2.1	0.637

IFN, interferon; HCV, hepatitis C virus; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; CI, confidence interval. *Values expressed as mean ± SD, †values expressed as median, ‡interferon treatment history was not known for three patients, §data for 62 patients are missing.

Table 5 Factors associated with RVR among patients who completed the treatment – multivariate analysis

Factor	Category	Odds ratio	95% CI	P-value
Age (years)	By 10	0.648	0.494–0.850	0.002
HCV RNA (KIU/mL)	By 100 KIU/mL	0.964	0.944–0.984	<0.0001

HCV, hepatitis C virus; CI, confidence interval.

achieved SVR. In addition, we examined the drug impact on SVR in the patients with the least medication of both drugs (<0.9 μg/kg/week of Peg-IFN and <8 mg/kg/day of ribavirin). Nine patients were categorized into this group and six of these patients achieved SVR (67%); patients with RVR had a significantly higher SVR rate (100%, 5/5) than patients without RVR (25%, 1/4; P = 0.048). Thus, SVR attainment was dependent on time, not on drug dose.

DISCUSSION

In the present study, we found that having RVR and high platelet counts were statistically associated with reaching SVR according to multivariate analysis. The timing of viral clearance was closely related to the treatment effect in patients with genotype 2, similar to the case for those with genotype 1. Ninety-two per cent of SVR was observed for patients with RVR and, conversely, 96% of the patients with HCV RNA positivity at week 8 showed non-SVR. The predictability of SVR based on EVR, defined as a decline of at least 2-log from the baseline of the HCV RNA level at week 12, has been assessed, and genotype 1 patients who have failed to reach EVR are recommended to discontinue the treatment after 12 weeks, because the likelihood of SVR is 0–3% in the absence of EVR [5,13]. On the basis of our examination of patients with genotype 2, not EVR, but 8-week monitoring of the HCV RNA level can be used.

As a significant factor for SVR, not liver fibrosis, but the platelet count was selected. Everson *et al.* [14] reported that patients with low platelet counts (≤12.5 × 10^4/mm^3) achieved lower SVR rates than patients with normal platelet counts (>12.5 × 10^4/mm^3) even in the case of patients with the same category of liver fibrosis treated by Peg-IFN plus ribavirin combination therapy. Thus, independent of liver fibrosis, thrombocytopenia itself seems to participate in treatment failure, although the mechanism remains unknown.

Our study also demonstrated that younger age (OR 0.648, 95% CI 0.494–0.850, P = 0.002) and lower HCV RNA level (OR 0.964, 95% CI 0.944–0.984, P < 0.0001) were statistically associated with reaching an RVR. Zeuzem *et al.* [7] previously reported that pretreatment viral load was not

Table 6 SVR rates according to Peg-IFN alpha-2b and ribavirin exposure and the timing of viral clearance among patients with virological response during the treatment

Timing of viral clearance (week)	Peg-IFN dose (μg/kg/week)				Ribavirin dose (mg/kg/day)				Total
	<0.9	0.9–1.2	1.2–1.5	1.5≤	<8	8–10	10–12	12≤	
1–4	100% (19/19)	91% (10/11)	92% (65/71)	88% (28/32)	94% (17/18)	92% (33/36)	91% (51/56)	91% (20/22)	92% (122/133)
5–8	63% (5/8)	33% (1/3)	64% (19/30)	71% (12/17)	58% (7/12)	54% (7/13)	74% (17/23)	60% (6/10)	64% (37/58)
9–24	–	0% (0/1)	17% (1/6)	–	–	0% (0/1)	0% (0/4)	50% (1/2)	14% (1/7)
Total	89% (24/27)	73% (11/15)	79% (85/107)	82% (40/49)	80% (24/30)	80% (40/50)	82% (68/83)	79% (27/34)	81% (160/198)

*$P = 0.795$ for comparison of the four Peg-IFN groups after stratification of the timing of viral clearance. **$P = 0.649$ for comparison of the four ribavirin groups after stratification of the timing of viral clearance.

associated with reaching RVR in genotype 2 patients. In contrast, Dalgard *et al.* [15] reported that independent predictors of RVR in genotype 2 or 3 patients were male gender, younger age (≤40 years) and low viral load (≤400/KIU/mL). The influence of viral load on reaching RVR remains controversial in the Peg-IFN and ribavirin combination therapy in genotype 2 patients, but patients with lower viral load seem favoured to reach HCV RNA levels below the detection limit, that is, to attain RVR, if the virological response is the same.

Recently, because of substantial adverse effects and costs associated with this therapy, studies have been carried out to determine the possibility of further reducing the total amount of drug medication without compromising antiviral efficacy in HCV genotype 2 and 3 patients. There seem to be two ways to achieve. One is by shortening the treatment duration, and the other is by decreasing the doses of the treatment drugs. With respect to the former, several studies on genotype 2 patients have been reported. At first, some studies of small numbers of subjects demonstrated that cumulatively analysed genotype 2 and 3 patients had high SVR rates up to 12 to 16 weeks of therapy (82–94%), similar to patients subjected to 24-week therapy (76–95%) [16–19]. However, further prospective investigation of large numbers of subjects revealed that shortening the treatment duration was associated with an increase in the rate of relapse and that significantly higher relapse rates led to lower SVR rates (71–81.1%), even among those with RVR [15,20,21]. The latest study by Mangia *et al.* [22] showed that shortened therapy after RVR was acceptable only for patients who had no signs of advanced liver fibrosis and low BMI. Considering the results of these trials, shortened therapy is regarded as optional treatment for selected patients displaying favourable baseline characteristics. Therefore, shortening treatment duration from 24 weeks should not be generally recommended for patients who are infected genotype 2 or 3 and can tolerate 24-week Peg-IFN and ribavirin combination therapy.

Another attempt to improve the treatment tolerability for genotype 2 or 3 patients has focused on dose reduction of treatment drugs. Weiland *et al.* [23] examined low-dose Peg-IFN alpha-2a (135 μg weekly) with a weight-based standard-dose of ribavirin (11 mg/kg daily) for genotype 2 and 3 patients. They demonstrated that SVR rates of 86% were achieved, which is equal to those in previous representative randomized controlled studies of standard dose Peg-IFN therapy (76–84%) [4–6]. In contrast, Ferenci *et al.* [24] examined the efficacy of standard-dose Peg-IFN alpha-2a (180 μg weekly) with low-dose ribavirin (400 mg daily) in comparison with standard-dose Peg-IFN alpha-2a (180 μg weekly) and ribavirin (800 mg daily) for genotype 2 and 3 patients, and demonstrated that there was no difference between the two treatment groups with respect to SVR rates (64% with 400 mg/day compared with 69% with 800 mg/day) and relapse rates (20% with 400 mg/day compared

with 17% with 800 mg/day). These studies showed that either drug dose can be reduced for genotype 2 and 3 patients without compromising antiviral efficacy. In the present study, neither Peg-IFN nor ribavirin drug exposure participated in reaching RVR and SVR. In particular, more than 90% of patients having RVR achieved SVR regardless of the drug exposure level, as long as the mean Peg-IFN dose was over 0.5 μg/kg/week and ribavirin was over 5.0 mg/kg/day. The results of our study suggested that genotype 2 patients may receive reduced levels of both drug doses on the condition that they can complete the full 24-week course of combination therapy. Randomized, prospective trials that reduced both Peg-IFN and ribavirin should be conducted for CHC patients to clarify this.

In the present study, while the treatment outcome was independent of the individual ribavirin exposure in patients who had completed the 24-week treatment, the most common reason to withdraw the treatment was decreased haemoglobin because of ribavirin medication. Based on the results of randomized controlled trials [6], using a ribavirin dose of 800 mg/day is recommended for genotype 2/3 patients [1–3]. However, several studies have shown that some patients cannot tolerate even this suboptimal ribavirin dose. This is a serious problem for patients with the risk of anaemia, especially elderly patients. The ageing of patients is progressing around the world, requiring improvement in treatment tolerability. Recently, Andriulli *et al.* [25] examined the effect of ribavirin in a 12-week course of therapy on CHC genotype 2 patients with RVR in two groups, one continuing with ribavirin and the other receiving Peg-IFN alpha-2a alone after week 6. The relapse rates were higher (46% *vs* 17%; $P < 0.001$) and overall SVR rates were lower (54 *vs* 82%; $P < 0.001$) in patients who stopped receiving ribavirin at week 6. Thus, ribavirin medication throughout the treatment period is necessary to raise the SVR rate even in genotype 2 or 3 patients with RVR. In the present study, the ribavirin dose could be reduced without loss of efficacy for genotype 2 patients, as long as the patients were treated for 24 weeks. Therefore, in the patients with the risk of anaemia, it would be better to reduce the dose of ribavirin before anaemia arises rather than being forced to discontinue the combination therapy because of anaemia caused by ribavirin medication. We previously reported that in CHC patients treated by IFN or Peg-IFN in ribavirin combination therapy, a decline of haemoglobin concentration by 2 g/dL at the end of 2 weeks from the start of the treatment can be used to identify patients likely to develop severe anaemia [26,27]. This kind of predictive factor for the progression to severe anaemia can be of much help in reducing ribavirin with appropriate timing.

Our study has some limitations. First, it is a retrospective study, and we could not obtain complete information for all patients. However, this is the first study of Peg-IFN and ribavirin combination therapy in which the drug dose of Peg-IFN and ribavirin taken by each patient was assessed independently for HCV genotype 2 patients. Our results can be taken as an evidence offering suggestions for the treatment of CHC genotype 2 patients. Second, this cohort included patients with different histories of past IFN treatment. Patients who had failed to recover with previous IFN-based treatment were likely to experience treatment failure again [28]. Therefore, we examined the predictors of treatment response separately according to treatment history, and confirmed that in both naïve and treatment-experienced patients, the mean dose of Peg-IFN and ribavirin showed no correlation with SVR or RVR in both groups.

In conclusion, our study demonstrates that RVR is an important treatment predictor and more than 90% of patients having RVR achieve SVR with combination therapy of Peg-IFN and ribavirin for genotype 2 infected CHC patients regardless of the drug exposure. Further prospective, randomized studies are necessary to assess whether the standard or a reduced dose of each drug can produce equivalent outcomes.

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Reduced risk of hepatocellular carcinoma after interferon therapy in aged patients with chronic hepatitis C is limited to sustained virological responders

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SUMMARY. This study was undertaken to investigate the effect of interferon (IFN) monotherapy on the risk of hepatocellular carcinoma (HCC) in aged-patients with chronic hepatitis C. Seven hundred and twenty-five patients with histologically proven chronic hepatitis C were enrolled in this retrospective cohort study: 531 received IFN monotherapy for 6 months between 1992 and 1995, and 157 were collected as a historical control. The effect of IFN therapy on the development of HCC was compared between the patients with chronic hepatitis C under 60 years old (non-aged group, $n = 531$) and those 60 and over (aged group, $n = 194$). A stepwise Cox proportional-hazards regression analysis in the non-aged group revealed that IFN therapy (risk ratio 0.52, 95% CI 0.33–0.81, $P = 0.004$), older age ($P = 0.001$), and higher histological stage ($P < 0.001$) were independent factors associated with the development of HCC. In the aged-group, only higher histological stage ($P = 0.002$) and male gender ($P = 0.011$), but not IFN therapy (risk ratio 0.77, 95% CI 0.42–1.40, $P = 0.386$), were identified as independent risk factors for HCC, although HCC was significantly reduced when sustained virological response (SVR) was obtained (risk ratio 0.23, 95% CI 0.08–0.64, $P = 0.005$). In conclusion, inhibitory effect of IFN on development of HCC in the patients with chronic hepatitis C aged 60 and over was limited to the patients achieving SVR when treated with 6 months-IFN monotherapy.

Keywords: aged patients, chronic hepatitis C, hepatocellular carcinoma, interferon, sustained virological response.

INTRODUCTION

In Japan, based on the epidemiological surveillance as well as the study on molecular tracing of hepatitis C virus (HCV), HCV infection is considered to spread from the 1920s and to expand more after World War II [1–5]. The data of first-time blood donor candidates in Osaka demonstrated that the prevalence of anti-HCV antibodies among the candidates born in 1925–1935 was 7–10%, which was much higher than the prevalence of anti-HCV antibodies among the younger population [6]. Accordingly, chronic hepatitis C patients have become aged in Japan and HCV-related hepatocellular carcinoma (HCC) patients have also been shown to be old with a peak around age 70 and tended to decrease [1,3,5]. More importantly, the main cause of death in the patients with chronic hepatitis C has been reported to be HCC [7–10].

In the 1990s, interferon (IFN) therapy was used for the treatment of the patients with chronic hepatitis C worldwide and it has been shown by many studies including our reports that IFN therapy reduced the risk of HCC in patients with chronic hepatitis C [7,11–17]. This inhibitory effect of IFN therapy on hepatocarcinogenesis is notable when sustained virological response (SVR) was obtained, although SVR rate of IFN monotherapy was not very high. It has been also

Abbreviations: IFN, interferon; HCC, hepatocellular carcinoma; SVR, sustained virological response; HCV, hepatitis C virus; non-SVR, nonsustained virological response.

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reported that HCC development was significantly reduced in the patient achieving SVR as compared with those without SVR in chronic hepatitis C patients treated with IFN and ribavirin [18].

For the treatment of the patients with chronic hepatitis C, a combination of peginterferon and ribavirin has become a standard therapy, which has a high SVR rate [19–21]. However, the combination treatment has several adverse effects such as haemolytic anaemia which may not be tolerable for aged patients with chronic hepatitis C. On the other hand, aging is a significant risk factor for HCC in chronic hepatitis C patients. Accordingly, it is an important issue whether IFN monotherapy could reduce incidence of HCC in aged patients with chronic hepatitis C. Recently, Arase *et al.* [22] reported that long-term IFN monotherapy using low-dose of natural IFN-α was effective in preventing hepatocarcinogenesis in aged patients with chronic hepatitis C. In contrast, the hepatitis C antiviral long-term treatment against cirrhosis (HALT-C) Trial has shown that maintenance peginterferon therapy for 3.5 years did not reduce the incidence of HCC and the rate of disease progression in chronic hepatitis C patients with bridging fibrosis or cirrhosis who failed to respond to the combination therapy of peginterfeon-α2a and ribavirin [23,24].

We conducted a long-term multicenter retrospective cohort study to clarify the effect of 6-month IFN monotherapy on the incidence of HCC in aged patients with chronic hepatitis C.

MATERIAL AND METHODS

Patients

This study was conducted at Osaka University Hospital and six university-affiliated hospitals. IFN-treated patients consisted of 568 consecutive patients with chronic hepatitis C who had undergone liver biopsy 1 week to 2 months before IFN therapy and received either human lymphoblastoid IFN, recombinant IFN-α2a or recombinant IFN-α2b for 6 months between 1992 and 1995. The control group consisted of 158 consecutive patients with chronic hepatitis or cirrhosis who had undergone liver biopsy between January 1986 and December 1989, when IFN therapy had not been available in Japan. All the patients were positive for anti-HCV. The inclusion criteria in this study were as follows: (1) histological diagnosis of chronic hepatitis or cirrhosis; (2) no history of clinical signs at entry into the study of complications of cirrhosis, i.e. ascites, jaundice, encephalopathy, or variceal bleeding; (3) no previous IFN therapy; (4) no evidence of HCC at entry into the study as assessed by ultrasonography and/or computed tomography; (5) absence of serum hepatitis B surface antigen; (6) absence of co-existing liver diseases such as autoimmune hepatitis or primary biliary cirrhosis and (7) absence of excessive alcohol consumption (>80 g/day).

Sustained virological response was defined as persistent HCV RNA negativity during IFN therapy and follow-up. Patients showing positive HCV RNA after IFN therapy were classified as nonsustained virological response (non-SVR). In the patients with non-SVR, patients whose ALT levels decreased to the normal range and remained normal during IFN therapy were classified as transient biochemical response and patients without a decrease of ALT levels of the normal range during the therapy were classified as zbiochemical nonresponse.

Hepatitis C virus antibody was measured by first-, second-, or third-generation enzyme-linked immunosorbent assays (Ortho Diagnostics, Tokyo, Japan). Serum HCV RNA was measured by reverse transcription polymerase chain reaction or complementary DNA assay [25].

Follow-up

The starting date of follow-up of the patients was defined as the date of liver biopsy. Abdominal ultrasonography or computed tomography and biochemical examinations including α-fetoprotein were performed every 3–6 months during follow-up equally in the IFN-treated and control patients. The diagnosis of HCC was confirmed by needle biopsy, by surgically resected tumour specimens, or by typical radiological findings on hepatic angiography or dynamic computed tomography. In the patients residing in Osaka whose follow-up data were not obtained, the Osaka Cancer Registry was used to determine whether HCC had occurred and the data were available until the end of 2002 in this study [13,26]. Accordingly, we decided to use the date of the development of HCC or the end of 2002 as the end of follow-up. As the longest observation period of the patients in the IFN group was 11 years, only the follow-up data for the first 11 years were considered in the control group. The study protocol was in accordance with the Helsinki Declaration of 1975 (revised in 1983) and approved by the Ethical Committee of the Ikeda Municipal Hospital.

Histological evaluation

The sections were stained with haematoxylin–eosin and Azan-Mallory and histology of liver biopsy specimens was scored by two authors in a blinded manner using two scoring methods as described before [13]. Briefly, fibrosis score of Desmet *et al.* was used for the assessment of histological staging and a total score of histological activity (components 1–3) using the Knodell histological activity index was used for the assessment of histological grading [13,27,28].

Statistical analysis

Patients who did not complete the treatment protocol were included for the analysis on an intention-to-treat basis. The chi-square test and Student's *t*-test were used to compare the

baseline characteristics. The Kaplan–Meier method was used to calculate the cumulative incidence of HCC, and the log-rank test was used to compare the cumulative incidence of HCC between the groups. To estimate independent risk factors for the development of HCC, a stepwise Cox proportional-hazards regression analysis was used. For the analysis, IFN therapy, age, gender, and histological staging and activity scores were used as variables. A P value <0.05 was considered statistically significant. Data are presented as the mean ± SD and were analysed using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Table 1 shows the baseline characteristics of the aged (60 years old and over) and non-aged (under 60 years old) groups. Both the histological stage and activity were significantly higher in the aged group than in the non-aged group. The proportion of male patients of the non-aged group was significantly higher than that of the aged group. In Table 2, baseline characteristics of controls and IFN-treated patients in the aged and non-age groups were compared. In the non-aged group, age at entry, proportion of male gender, histological activity score, serum ALT level and platelet count did not differ between the control and IFN-treated patients. However, histological stage of IFN-treated patients was less advanced as compared with that of the control patients. In the age-group, age at entry, proportion of male gender, histological stage and activity, serum ALT level and platelet count did not differ between the control and IFN-treated patients.

During the follow-up period, HCC was found in 35 controls and 44 IFN-treated patients among the non-aged group and in 14 controls and 48 IFN-treated patients among the aged group. The median tumour sizes of HCC in controls and IFN-treated patients at the time of discovery on ultrasonography or computed tomography were 22 mm (range, 10–55 mm) and 19 mm (range, 8–52 mm) respectively ($P \geq 0.2$). In the non-aged group, the cumulative incidence of HCC estimated by the Kaplan–Meier Method of IFN-treated patients was significantly lower than that of control patients (log-rank test, $P < 0.001$, Fig. 1a), whereas there was no difference in the cumulative incidence of HCC between controls and IFN-treated patients in the aged group (log-rank test, $P = 0.498$, Fig. 1b). The cumulative incidence of HCC of SVR and non-SVR patients and controls of the aged and non-aged groups are shown in Fig. 2. The 10-year incidences of HCC for controls, non-SVR and SVR patients in the non-aged group were 30.1%, 15.8%, 4.5% respectively (log-rank test, $P < 0.001$, Fig. 2a). Also, the 10-year incidences of HCC for controls, non-SVR and SVR patients in the aged group were 39.1%, 38.9%, 12.7% respectively (log-rank test, $P = 0.015$, Fig. 2b).

In Table 3, risk ratios for the development of HCC calculated by a stepwise Cox regression analysis in the aged and non-aged patients with chronic hepatitis C according to virological and biochemical responses to IFN are summarized. In the 410 IFN-treated patients of non-aged group, 134 patients (32.7%) achieved SVR and the remaining 276 showed non-SVR (Table 3). Of this 276 patients showing non-SVR, 163 showed transient biochemical response and 113 showed biochemical nonresponse during the IFN treatment. On the other hand, 41 (25.9%) of 158 IFN-treated patients of the aged group obtained SVR and the other 117 did not obtain SVR (Table 3). Of the 117 non-SVR patients, 57 showed transient biochemical response and 60

Table 1 Baseline characteristics of aged and non-aged patients with chronic hepatitis C

	Non-aged group (n = 531)	Aged group (n = 194)	P value
Control group (n)/IFN group (n)	121/410	36/158	0.262
Age	48.1 ± 9.7	63.7 ± 3.3	<0.001
Gender			
Male	353	108	0.009
Female	178	86	
Histological stage*			
F0, 1	186	37	0.001
F2	157	69	
F3	141	69	
F4	47	19	
Histological activity†			
<10	329	104	0.049
≧ 10	202	90	
ALT (IU/L)	117 ± 86	104 ± 60	0.053
Platelete count ($10^4/\mu$L)	15.4 ± 5.6	14.4 ± 5.6	0.040

*According to Desmet *et al.*[27] †Based on components 1–3 of the Knodell histological activity.

Table 2 Baseline characteristics of controls and IFN-treated patients in aged and non-aged groups

	Non-aged group			Aged group		
	Controls	IFN-treated	*P* value	Controls	IFN-treated	*P* value
n	121	410		36	158	
Age	48.4 ± 10.5	48.0 ± 9.4	0.736	64.6 ± 3.6	63.5 ± 3.2	0.059
Gender						
Male	75	278	0.273	22	86	0.579
Female	46	86		14	72	
Histologic stage*						
F0,1	27	159	<0.001	8	29	0.933
F2	28	129		12	57	
F3	47	94		12	57	
F4	19	28		4	15	
Histologic activity†						
<10	72	257	0.525	20	84	0.854
≧ 10	49	153		16	74	
ALT (IU/L)	127 ± 80	114 ± 88	0.132	110 ± 85	103 ± 53	0.523
Platelete count ($10^4/\mu L$)	15.2 ± 6.1	15.4 ± 5.4	0.766	15.0 ± 5.4	14.3 ± 5.7	0.486
HCV RNA load						
High	ND‡	166		ND‡	54	
Low	ND‡	116		ND‡	30	
HCV RNA serotype						
1	ND‡	231		ND‡	90	
2	ND‡	102		ND‡	32	

*According to Desmet *et al.* [27] †Based on components 1–3 of the Knodell histologic activity. ‡Not done.

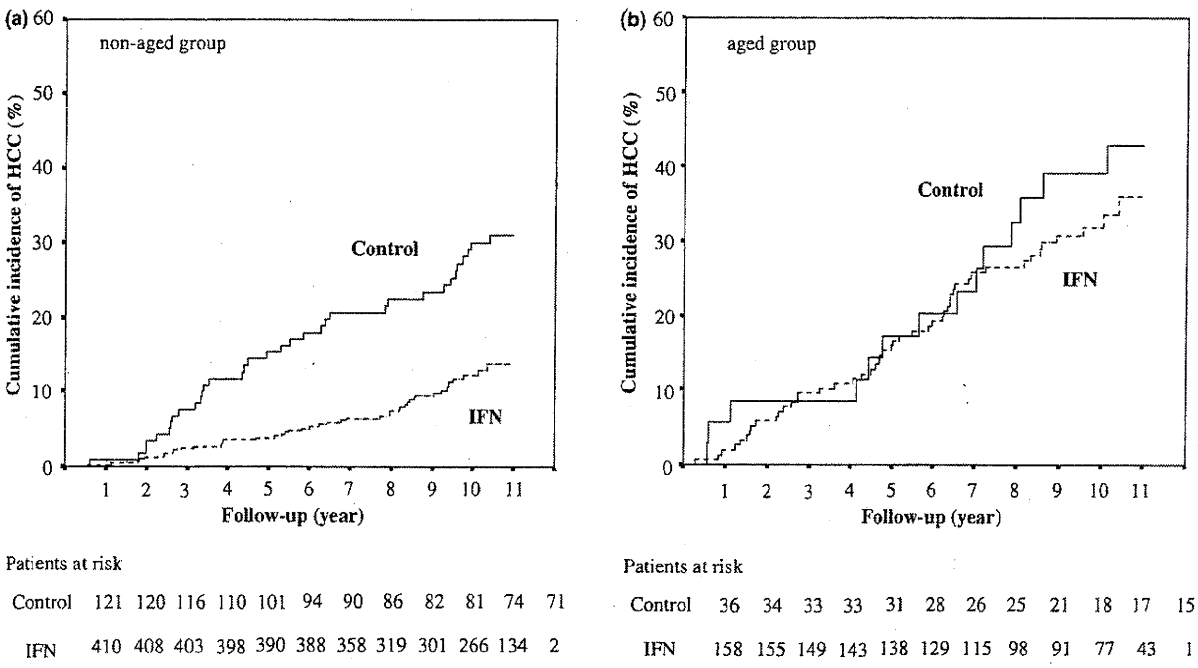
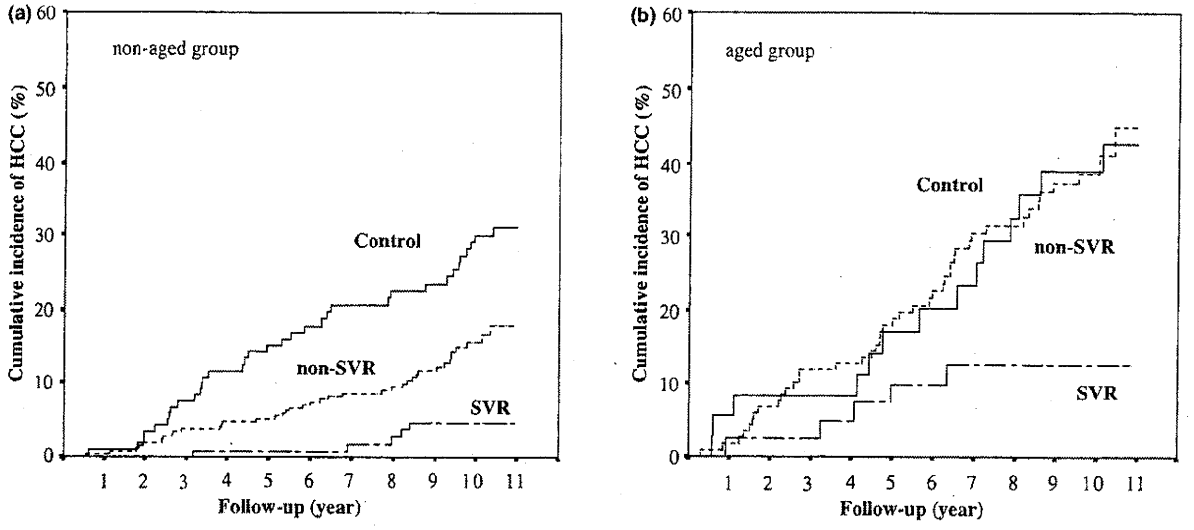


Fig. 1 Cumulative incidence of hepatocellular carcinoma in IFN-treated (dotted line) and control (solid line) patients of the non-aged group (a) and the aged group (b). A log-rank test of the two curves showed a significant difference in the non-aged group ($P < 0.001$), whereas no significant difference was observed in the aged group ($P = 0.498$).



Patients at risk

Control	121	120	116	110	101	94	90	86	82	81	74	71
Non-SVR	276	274	269	264	259	257	239	212	199	179	85	2
SVR	134	134	134	134	131	131	119	107	102	87	49	0

Patients at risk

Control	36	34	33	33	31	28	26	25	21	18	17	15
Non-SVR	117	115	115	103	100	93	82	67	60	50	26	1
SVR	41	40	40	40	38	36	33	31	31	27	17	0

Fig. 2 (a) Cumulative incidence of hepatocellular carcinoma categorized by sustained virological response (dashed line), nonsustained virological response (dotted line), and controls (solid line) of the non-aged group (a) and the aged group (b). A log-rank test of the three curves showed a significant difference between these groups (non-aged group, $P < 0.001$; aged group, $P = 0.015$).

showed biochemical nonresponse. In the non-aged group, stepwise Cox regression analysis identified IFN therapy (risk ratio 0.52, 95% CI 0.33–0.81, $P = 0.004$), older age (risk ratio 1.07, 95% CI 1.03–1.10, $P = 0.001$), and higher histological stage (score 3 or 4) (risk ratio 4.03, 95% CI 2.41–6.76, $P < 0.001$) as independent risk factors associated with the development of HCC. In the non-aged group, the development of HCC was strongly suppressed when SVR was achieved (risk ratio 0.20, 95% CI 0.08–0.50, $P < 0.001$) (Table 3). In the patients with transient biochemical response of the non-SVR group among the non-aged group, HCC development was also significantly reduced (risk ratio 0.47, 95% CI 0.26–0.86, $P = 0.015$). In the aged group, stepwise Cox regression analysis revealed that only higher histological stage (score 3 or 4) (risk ratio 2.27, 95% CI 1.36–3.78, $P = 0.002$) and male gender (risk ratio 2.00, 95% CI 1.17–3.41, $P = 0.011$) were independent factors responsible for the development of HCC (Table 3). Although IFN therapy was not identified as an independent variable for HCC, the risk of HCC was significantly decreased in the patients with SVR in the aged group as shown in the Table 3 (risk ratio 0.23, 95% CI 0.08–0.64, $P = 0.005$). In the

Table 3 Risk ratios for hepatocellular carcinoma in aged and non-aged patients with chronic hepatitis C according to virological and biochemical responses to interferon*

	Non-aged group (n = 531)				Aged group (n = 194)			
	n	Risk ratio	95% CI	P value	n	Risk ratio	95% CI	P value
Control group	121	1.00			36	1.00		
IFN group	410	0.52	0.33–0.81	0.004	158	0.77	0.42–1.40	0.388
Sustained virological response	134	0.20	0.08–0.50	0.001	41	0.23	0.08–0.64	0.005
Nonsustained virological response	276	0.65	0.41–1.03	0.068	117	1.07	0.58–1.97	0.821
Transient biochemical response†	163	0.47	0.26–0.86	0.015	57	0.67	0.32–1.43	0.303
Biochemical nonresponse†	113	0.86	0.51–1.47	0.584	60	1.46	0.77–2.78	0.245

*A stepwise Cox regression analysis was carried out by using interferon therapy, age, gender, and histologic stage and histologic activity scores as variables. †Nonsustained virological response was classified into transient biochemical response and biochemical nonresponse according to the ALT response during the interferon treatment.

patients with transient biochemical response of the non-SVR group of aged patients. HCC development was not reduced (risk ratio 0.67, 95% CI 0.32–1.43, $P = 0.303$, Table 3) in contrast to the patients showing transient biochemical response in the non-aged group.

As the cumulative incidence of HCC calculated by the Kaplan–Meier Method of the patients with SVR in the aged group was much higher than that in the non-aged group, we also carried out Cox proportional-hazards regression analysis to estimate risk factors responsible for HCC development in the 175 patients achieving SVR. As a result, older age (risk ratio 1.09, 95% CI 1.01–1.18, $P = 0.025$) and higher histological activity before IFN therapy started (10 or more of the total score of components 1–3 in Knodell's histological activity index) (risk ratio 4.16, 95% CI 1.07–16.25, $P = 0.040$) were identified as risk factors associated with HCC among the patients with SVR.

DISCUSSION

In this long-term retrospective cohort study, an inhibitory effect of 6 months-IFN monotherapy in early 1990s on the cumulative incidence of HCC were compared between the patients with histologically proven chronic hepatitis C under 60 years old (non-aged group) and those 60 years old and over (aged group). Because of retrospective analysis, there were some differences in baseline characteristics between the two groups. In the aged group, the histological stage and activity as well as the proportion of male patients were significantly higher than in the non-aged group. Also, SVR rate in the aged group was lower than that in the non-aged group. To avoid the influence of these biases, we performed Cox proportional-hazards regression analysis to see whether IFN monotherapy reduced the risk of HCC in the aged and non-aged groups. Then, we found that IFN therapy for 6 months significantly reduced the risk of HCC (risk ratio 0.52) in the non-aged group, whereas this inhibitory effect of IFN monotherapy on HCC development was recognized only in the patients achieving SVR among the aged-patients.

It is difficult to explain why IFN had no inhibitory effect on HCC development in the aged patients, whereas IFN had significant inhibitory effect in the non-aged patients of this study. Many clinical studies have demonstrated that aging was an independent risk factor associated with HCV-related HCC other than advanced histological staging and male gender [7,11–17,29]. However, molecular mechanism of the impact of aging on haepetocarcinogenesis has not been elucidated. Moriya *et al.* reported that lipid hydroperoxide products accumulated in the liver without inflammation and may play a role in the development of HCC in HCV core gene transgenic mice [30,31]. A long-term infection of HCV may lead to HCC through some molecular alterations.

Recently, there have been two controversial reports from the United States and Japan as to the long-term effect of low-dose IFN therapy on the incidence of HCC in chronic hepatitis C [22,24]. The report from Japan was a non-randomized retrospective study and observed beneficial effect of long-term natural IFN-α therapy on hepatocarcinogenesis in aged chronic hepatitis C patients [22]. The HALT-C Trial from the United States, a large prospective randomized study, reported that treatment with peginterfeon-α2a at a dose of 90 μg weekly for 3.5 years did not prevent HCC development in the patients with bridging fibrosis or cirrhosis who did not obtain SVR by combination therapy of peginterferon and ribavirin [24]. The result was consistent with our data in the aged patients. However, the annual incidence of HCC of the HALT-C Trial, about 1%, was much lower than that in the aged group in this study, about 4%. Accordingly, a randomized prospective study to determine the effect of long-term IFN or peginterferon therapy on the incidence of HCC in chronic hepatitis C, especially in the aged patients, may be needed in Japan.

This study has a limitation, because we used historical controls as control patients. A lead-time bias may have occurred. Detection of HCC by the screening program could be less effective in controls than IFN-treated patients. In that case, we might underestimate the effect of IFN on the cumulative incidence of HCC. However, such underestimation may be unlikely as the tumour sizes at the time of detection were not different between the control and IFN-treated patients.

The 10-year incidence of HCC for SVR patients of the aged group (12.7%) was much higher than that of non-aged group (4.5%) in our study. Makiyama *et al.* [32] studied the risk factors for developing HCC after obtaining sustained biochemical response to IFN therapy in chronic hepatitis C and reported that older age, male gender and advanced fibrosis were associated with HCC. Consistent with their results, we found that older age was an independent risk factor for HCC in the patients with SVR, suggesting a high potential of developing HCC even after eradication of HCV RNA in the aged patients. Another possibility is that malignant foci, which could not be detected by imaging modalities, had already existed before IFN therapy. Our finding indicates that even in the patients showing SVR, a follow-up examination to investigate HCC should be carried out for at least 10 years, particularly in the aged patients.

In conclusion, IFN monotherapy reduced the risk of HCC in the patients with chronic hepatitis C under 60 years old. In contrast, this inhibitory effect of IFN on hepatocacinogenesis was limited to patients showing SVR in the aged-patients when treated with 6 months-IFN monotherapy. These results suggest that combination therapy of peginterferon and ribavirin is recommended even in the aged patients with chronic hepatitis C to obtain better preventive effect of IFN on HCC development. For reasons of relatively high cumulative incidence of HCC in the aged chronic hepatitis C patients with SVR to IFN therapy, they should be followed carefully even after eradication of HCV by IFN therapy.

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RAPID COMMUNICATION

STAT3 signaling within hepatocytes is required for anemia of inflammation in vivo

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Abstract
Background Anemia of inflammation, commonly observed in patients with chronic diseases, is associated with decreased serum iron. Hepcidin, mainly produced by hepatocytes in a STAT3- and/or SMAD-dependent manner, is involved in iron homeostasis. What remains to be established is whether or not the hepatic IL-6/STAT3 signal has a role in anemia of inflammation in vivo.
Methods Turpentine oil was subcutaneously injected into wild-type mice or hepatocyte-specific STAT3-deficient mice (L-STAT3KO) to induce inflammation.
Results Turpentine injection increased serum IL-6 levels. It activated liver STAT3 in wild-type mice, but not in L-STAT3KO mice. In chronic inflammation, wild-type mice showed decreased serum iron levels and anemia with up-regulation of hepcidin levels in the liver. In contrast, L-STAT3KO mice showed no increase in hepatic hepcidin levels or anemia.
Conclusions Liver STAT3 is critically involved in the development of anemia of inflammation via the expression of hepcidin. The liver regulates anemia of inflammation through STAT3 signaling.

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Keywords Hepcidin · Anemia · STAT3 · Liver · Iron

Introduction

Hepcidin, a peptide hormone produced by hepatocytes, mediates interactions between the immune system and iron metabolism. As the key regulator of transmembrane iron transport, hepcidin controls the absorption of iron in the intestine, the mobilization of iron from hepatic stores, and iron recycling by macrophages. Hepcidin production is up-regulated by excess body iron and inflammation [1, 2] and down-regulated by anemia [2].

During inflammation, IL-6 rapidly induces hepcidin synthesis and corresponding hypoferremia [3]. The discovery that hepcidin expression is directly regulated by inflammatory cytokines has linked hepcidin to anemia of inflammation [4, 5], which is commonly observed in patients with chronic diseases and is associated with decreased serum iron and iron-laden bone marrow macrophages [6]. Anemia of inflammation is characterized by increased uptake and retention of iron by cells of the reticuloendothelial system, leading to low serum iron levels despite adequate iron stores. Under inflammatory circumstances, hepatic STAT3 is the key transcription factor responsible for IL-6-induced activation of hepcidin gene expression [7]. The liver is a very important organ for hepcidin expression, but there has been no report of the liver regulating anemia of inflammation.

In the present study, we used hepatocyte-specific STAT3-deficient mice (L-STAT3 KO) and examined the significance of STAT3 signaling within hepatocytes in anemia of inflammation. We found in vivo evidence for the necessity of STAT3 for anemia of inflammation.

Materials and methods

Animals

Five-week-old BALB/cA female mice purchased from CLEA Japan, Inc. (Tokyo, Japan) were used for the experimental iron overload. Mice carrying a STAT3 gene with 2 *loxP* sequences flanking exon 22 and a STAT3 null allele (STAT3 fl/−) have been described previously [8]. To generate mice with hepatocyte-specific STAT3 deficiency, we crossed STAT3 fl/− mice and Alb-Cre transgenic mice [9], which express the Cre recombinase gene under regulation of the albumin gene promoter. We crossed Alb-Cre STAT3 fl/fl mice and STAT3 fl/−. The resulting Alb-Cre STAT3 fl/− mice and Alb-Cre STAT3 fl/fl mice were used as L-STAT3 KO mice. Gender-matched STAT3 fl/− mice and STAT3 fl/fl mice obtained from the same litter were used as control mice. Although the data are not shown, there was no difference in hepcidin expression between STAT3 fl/fl mice and STAT3 fl/− mice upon turpentine injection. All animals were housed under specific pathogen-free conditions and treated with humane care under approval from the Animal Care and Use Committee of Osaka University Medical School.

Inflammation

Chronic inflammation was produced by subcutaneous injection of turpentine oil (0.1 ml/20 g of body weight; Nacalai Tesque, Kyoto, Japan) into the intrascapular fat pad at weekly intervals for 2 weeks (three injections per week). Control mice were similarly injected with an equivalent volume of sterile saline solution.

Hematological analysis and measurement of serum cytokine of mice

Blood samples were obtained 72 h after chronic turpentine injection and collected in heparinized tubes. Blood cell counts and erythrocyte parameters were determined using an automatic blood analyzer. Iron levels were measured using Quickauto-Neo Fe (SHINO-TEST Corp., Kanagawa, Japan). The level of IL-6 in the serum was measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits.

Real-time reverse-transcription PCR

cDNA, equivalent to 50 ng RNA, was used as a template for real-time reverse-transcription PCR (RT-PCR) using an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Hepcidin messenger RNA (mRNA) expression was measured using TaqMan Gene Expression Assays (Assay ID Mm00519025_m1) and was corrected with the quantified expression level of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA measured using TaqManGene Expression Assays (Assay ID Mm99999915_g1). All samples were assayed in triplicate.

Western blot analysis

We isolated liver protein from L-STAT3 KO mice and control littermates and performed Western blot analysis as previously described [10].

Statistics

Data are expressed as mean ± SD and compared using the Mann–Whitney test. Statistical significance was set at $P < 0.05$.

Results

During the inflammatory process, hepcidin gene expression is dramatically induced, resulting in anemia with iron deficiency

Subcutaneous injection of turpentine is a classical and widely accepted model of inflammation in mice [2, 3]. This treatment induces a variety of inflammatory cytokines such as IL-6 and TNF alfa. Previous research has revealed a predominant role of IL-6 in pathogenesis during turpentine-induced inflammation [10]. When wild-type mice were intra-subcutaneously injected with turpentine, hepatic STAT3 was activated (Fig. 1a). Hepcidin mRNA expression in the liver was increased after a single turpentine injection to Balb/cA mice (Fig. 1b), which resulted in up-regulation of the hepcidin gene during inflammatory states. As shown in Fig. 1c, a repeated injection of turpentine for 2 weeks resulted in a decrease of serum iron and an increase of unsaturated iron-binding capacity (UIBC). This chronic treatment resulted in anemia with reduction of key blood parameters, i.e., red blood cell, hemoglobin, and hematocrit (Fig. 1d).

Anemia of inflammation does not occur in L-STAT3 KO mice

Next we treated L-STAT3 KO mice in the same way. Turpentine oil raised serum IL-6 in both L-STAT3 KO mice and control littermates without any significant difference, but with tendency was higher in L-STAT3 KO mice (Fig. 2a). Hepatic STAT3 was activated by turpentine injection in control mice but not in L-STAT3 KO mice

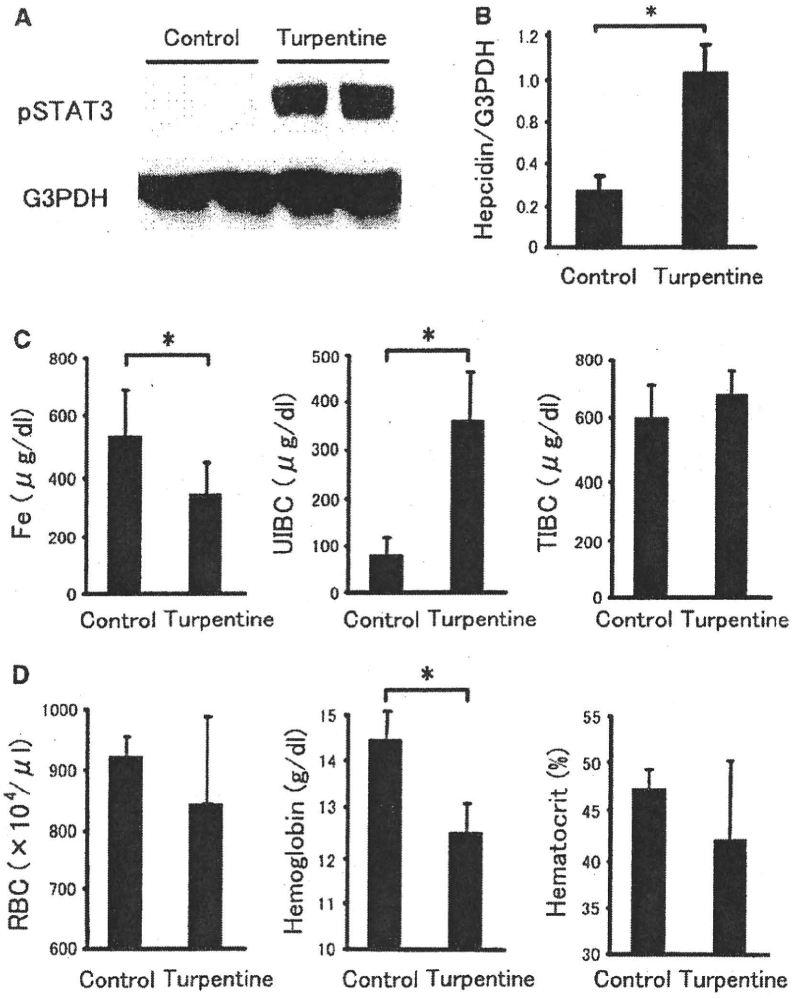

Fig. 1 Liver STAT3 activation, hepcidin mRNA expression, and hematological indices in turpentine-treated Balb/cA mice. **a** Liver lysates from mice after turpentine single injection (16 h) were analyzed by Western blot with an antibody to phosphorylation STAT3 and with an antibody to G3PDH as the loading control. **b** Relative changes in hepcidin mRNA level in the liver after single injection of turpentine (24 h); $n = 12$ for control mice and $n = 8$ for turpentine mice; $^*P < 0.05$. **c** Iron parameters in mice following chronic turpentine treatment; $n = 7$ for each group; $^*P < 0.05$. **d** Hematological parameters in mice following chronic turpentine treatment; $n = 4$ for control mice and $n = 6$ turpentine mice; $^*P < 0.05$

(Fig. 2b). Hepcidin mRNA expression in the liver was increased after turpentine injection, but was not observed in L-STAT3 KO mice (Fig. 2c). To check whether hepatocyte-STAT3 is required for anemia of inflammation, we measured the blood parameters of both mice. As shown in Fig. 2d, anemia after chronic treatment was observed in control mice, but not in L-STAT3 KO mice. Additionally, there was no significant difference in other blood parameters, i.e., white blood cells and platelets (Fig. 2e).

Discussion

In this research, we demonstrated that hepatic STAT3 regulates anemia of inflammation via hepcidin expression in vivo. Hepcidin expression can be up-regulated by high iron levels or during acute phase inflammatory responses. Each pathway of hepcidin expression has been reported in the BMP/SMAD pathway and the IL-6/STAT3 pathway. Hepcidin expression is thought to be due to the interaction between SMAD and STAT3 [12]. For example, the effect of IL-6 on hepcidin expression depends on SMAD4, as evidenced by the failure of IL-6 to increase hepcidin expression in mice with hepatocellular knockout of SMAD4 [12]. However, we found that hepatic STAT3 was not necessary for hepcidin expression through the SMAD pathway in the presence of iron excess (data not shown).

With respect to hepcidin expression, the BMP/SMAD pathway has been well studied [13]. Many reports state that the IL-6/STAT3 pathway regulates the expression of hepcidin in inflammation [7], but none comment on the IL-6/STAT3 pathway regulating anemia of inflammation. We therefore examined whether the liver could control anemia of inflammation via the IL-6/STAT3 pathway. In this experiment, we used L-STAT3 KO mice, which have the ability to intercept the inflammatory signals. Hepatic STAT3 is necessary for hepcidin expression during systemic inflammation, and thus the IL-6/STAT3 pathway in the liver can be expected to be involved with the immune system. It is well known that during chronic disease the

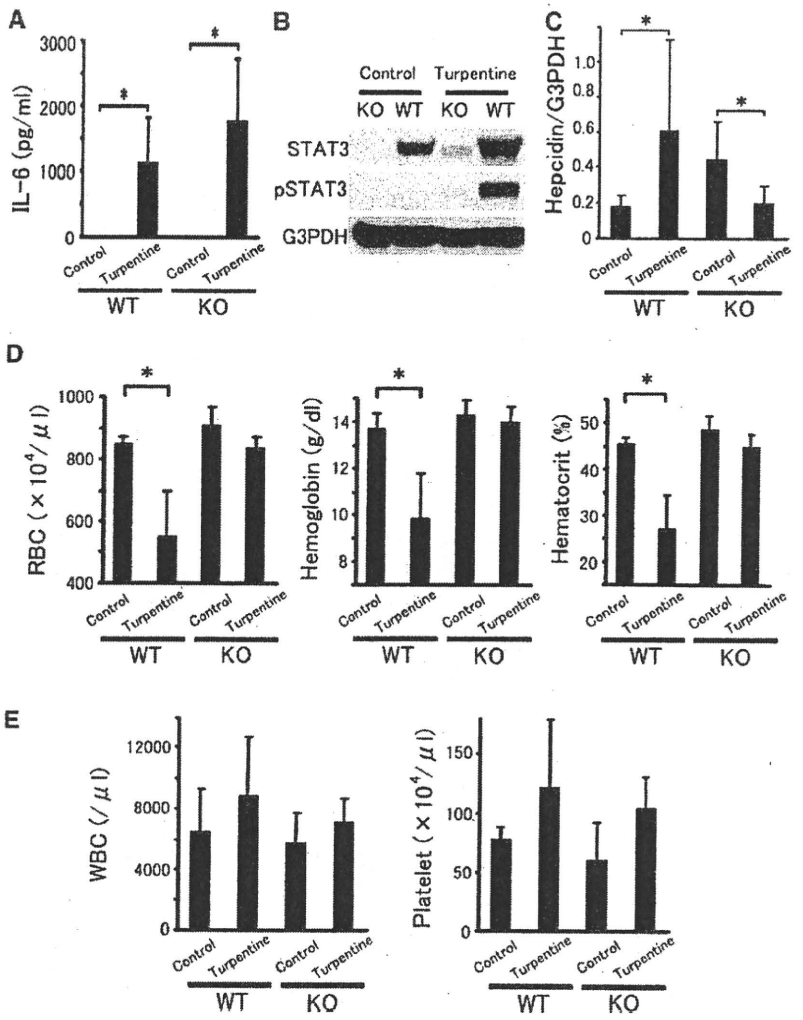

Fig. 2 Serum IL-6 levels, liver STAT3 activation, hepcidin mRNA expression, and hematological indices after turpentine injection in L-STAT3 KO mice and wild-type mice. **a** Serum IL-6 of L-STAT3 KO mice ($n = 10$) and control littermates ($n = 12$) after single injection of turpentine (24 h) or physiological saline; $n = 4$ for each group; $^*P < 0.05$. **b** Liver lysates from mice given a single injection of turpentine were analyzed by Western blot with an antibody to STAT3 and phosphorylation STAT3, and with an antibody to G3PDH as the loading control (24 h). **c** Relative changes in hepcidin mRNA level after chronic treatment in both L-STAT3 KO mice ($n = 10$) and control littermates ($n = 12$). As control group, L-STAT3 KO mice and control littermates were injected with physiological saline; $n = 4$ for each group; $^*P < 0.05$. **d** Red blood cells, hemoglobin and hematocrit in mice following chronic turpentine treatment in L-STAT3 KO mice ($n = 4$) and control littermates ($n = 6$). As control group, L-STAT3 KO mice and wild-type mice were injected with physiological saline; $n = 4$ for each group; $^*P < 0.05$. **e** White blood cells and platelets in mice following chronic turpentine treatment in L-STAT3 KO mice ($n = 4$) and control littermates ($n = 6$). As control group, L-STAT3 KO mice and wild-type mice were injected with physiological saline; $n = 4$ for each group

circulating levels of IL-6 are increased, resulting in an up-regulation of hepcidin activity and subsequent decreases in serum iron levels that may eventually lead to anemia [3]. Moreover, research has shown that STAT3 signaling within hepatocytes controls attenuation of the systemic inflammatory response and lethality during sepsis [11]. On the other hand, under the influence of elevated hepcidin concentrations, the macrophages, hepatocytes, and enterocytes retain iron that would otherwise be released into the plasma. This induced state of hypoferremia contributes to host immunity as invading microorganisms would find only a limited amount of plasma iron if the iron had been shifted from the circulation into cellular stores. For example, macrophages limit the intracellular growth of bacteria during iron depletion [14]. However, hypoferremia also limits the availability of iron for erythropoiesis, thereby contributing to the anemia associated with infection and inflammation [15]. The result of infection/inflammatory-mediated up-regulation in hepcidin levels is iron sequestration in macrophages and decreases in the absorption of

iron from the small intestine [4], eventually resulting in anemia. This is thought to be the relationship between the IL-6/STAT3 pathway in the liver and the host defense system that works against infecting microorganisms. In other words, liver STAT3 may control host immunity by causing anemia with iron deficiency. In summary, the present data indicate that anemia of inflammation is regulated by liver STAT3, which may be involved with the systemic immune system.

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Conflict of interest statement No conflicts of interest exist.

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ORIGINAL ARTICLE

EphA2-derived peptide vaccine with amphiphilic poly(γ-glutamic acid) nanoparticles elicits an anti-tumor effect against mouse liver tumor

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Abstract The prognosis of liver cancer remains poor, but recent advances in nanotechnology offer promising possibilities for cancer treatment. Novel adjuvant, amphiphilic nanoparticles (NPs) composed of L-phenylalanine (Phe)-conjugated poly(γ-glutamic acid) (γ-PGA-Phe NPs) having excellent capacity for carrying peptides, were found to have the potential for use as a peptide vaccine against tumor models overexpressing artificial antigens, such as ovalbumin (OVA). However, the anti-tumor potential of γ-PGA-Phe NPs vaccines using much less immunogenic tumor-associated antigen (TAA)-derived peptide needs to be clarified. In this study, we evaluated the effectiveness of immunization with EphA2, recently identified TAA, derived peptide-immobilized γ-PGA-Phe NPs (Eph-NPs) against mouse liver tumor of MC38 cells (EphA2-positive colon cancer cells). Immunization of normal mice with Eph-NPs resulted in generation of EphA2-specific type-1 CD8+ T cells. Immunization with Eph-NPs tended to provide a degree of anti-MC38 liver tumor protection more than that observed for immunization with the mixture of EphA2-derived peptide and complete Freund's adjuvant (Eph + CFA). Neither Eph-NPs nor Eph + CFA vaccines inhibited tumor growth of BL6, EphA2-negative melanoma cells. Splenocytes isolated from MC38-bearing mice treated with Eph-NPs showed strong and specific cytotoxic activity against MC38 cells. Immunization with Eph + CFA induced liver damage as evidenced by elevation of serum alanine aminotransferase, while Eph-NPs vaccination did not exhibit any toxic damage to the liver. These results demonstrated that immunization with Eph-NPs displayed anti-tumor effects against liver tumor by generating acquired immunity equivalent to the toxic adjuvant CFA, suggesting that safe γ-PGA-Phe NPs could be applied clinically for the vaccine treatment of liver cancer.

Keywords Peptide vaccine · EphA2-derived peptide · Acquired immunity · Liver tumor

Abbreviations

IFA	Incomplete Freund's adjuvant
NPs	Nanoparticles
γ-PGA	Poly(γ-glutamic acid)
Phe	L-Phenylalanine
CFA	Complete Freund's adjuvant
PBS	Phosphate buffered saline
i.p.	Intraperitoneal
ALT	Alanine aminotransferase
DCs	Dendritic cells

Introduction

Immunotherapies using peptide vaccine combined with immunologic adjuvants, such as incomplete Freund's

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adjuvant (IFA), saponin QS-21, and several cytokines, could enhance the anti-tumor immune response after immunization [1, 2]. To date, these therapies have been clinically applied to patients with several types of cancer and have shown limited anti-tumor effects [3–7]. This is because dose-limiting toxicities of the adjuvant were often observed or the adjuvant effects of the peptide vaccine were too weak to induce a sufficient anti-tumor effect. At present, only aluminum salt has been approved as an immunological adjuvant for clinical use; it appears to have weak activity as an adjuvant [8]. Thus, a new strategy using strong and safe immunologic adjuvant is needed to improve their clinical efficacy in cancer treatment. Recently, advances in nanotechnology have offered promise for application in medical science. Some investigators have reported testing various kinds of nanoparticles (NPs) using efficient antigen-carriers for their biological potential [9–11]. We previously demonstrated the efficacy of immunotherapies using HIV-capturing non-biodegradable polystyrene NPs in an animal model [12–15]. However, non-biodegradable polystyrene NPs would not be applicable in clinical situations as vaccine material due to their safety issues. To improve NP-based vaccines, we have successfully generated biodegradable NPs composed of poly(γ-glutamic acid) (γ-PGA) and hydrophobic amino acid, L-phenylalanine (Phe) [16]. γ-PGA is a naturally occurring poly(amino acid) that is synthesized by certain strains of *Bacillus*. The polymer is made of D- and L-glutamic acid units linked through the α-amino and the γ-carboxylic acid groups, respectively. γ-PGA is water soluble, biodegradable and edible. Therefore, the potential applications of γ-PGA and its derivatives have been of interest in a broad range of fields, including the medical field [17–19]. γ-PGA-Phe NPs can be degraded by γ-glutamyl transpeptidase [20], which is widely distributed in the entire body, and various molecules such as proteins and peptides can be immobilized on the surface or encapsulated into γ-PGA-Phe NPs [21]. We demonstrated that γ-PGA-Phe NPs have an excellent capacity for carrying various proteins and peptides into antigen-presenting cells such as dendritic cells (DCs) and macrophages [22]. However, previous reports were studies that examined the potential of vaccines with γ-PGA-Phe NPs using artificial antigens, such as OVA, which are much more immunogenic than tumor-associated self-antigens. The anti-tumor potential of tumor-associated antigen (TAA)-derived peptide vaccine must be examined in order to establish peptide vaccine therapy using γ-PGA-Phe NPs.

The liver is the most common site of distal metastasis for tumors developing in distal organs, such as the colon, stomach and pancreas, and the physiological status of this organ correlates with the survival of patients with advanced disease, even if the primary tumor site has been resected curatively [23, 24]. We demonstrated that the recently identified TAA EphA2 is overexpressed in colon cancer tissues and that EphA2-derived peptide pulsed DCs showed the high potential as a cancer vaccine in a mouse tumor model [25, 26], suggesting that EphA2-derived peptide could be applicable to evaluate the potential of peptide vaccines with γ-PGA-Phe NPs.

In the present study, we demonstrated that immunization with EphA2-derived peptide-immobilized γ-PGA-Phe nanoparticles (Eph-NPs) displayed anti-tumor effects against EphA2-expressing liver tumor by eliciting EphA2 antigen-specific acquired immunity equivalent to peptide vaccine using the strongest but very toxic adjuvant, complete Freund's adjuvant (CFA). These results indicate that peptide vaccine using γ-PGA-Phe NPs could be a promising candidate for a vaccine adjuvant against liver cancer.

Materials and methods

Mice

Female C57BL/6 mice were purchased from Clea Japan Inc. (Tokyo, Japan) and were used at 6–8 weeks of age. They were housed under conditions of controlled temperature and light with free access to food and water at the Institute of Experimental Animal Science, Osaka University Graduate School of Medicine. All animals received humane care and our study protocol complied with the institution's guidelines.

Cell lines

MC38 as EphA2-positive cell, a mouse colon carcinoma cell derived from C57BL6/J mice, was generously provided by Dr. Kazumasa Hiroishi (Showa University School of Medicine, Tokyo) [25]. BL6 as EphA2 negative cell, a melanoma cell line, and YAC-1, a sensitive cell line to NK cells were purchased from American Type Culture Collection (Rockville, MD) [25]. These cell lines were maintained in Complete Medium (RPMI medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in 5% CO_2.

Preparation of peptide-immobilized γ-PGA-Phe NPs

Nanoparticles composed of γ-PGA-Phe were prepared as previously described [27]. To prepare EphA2-derived peptide-immobilized NPs (Eph-NPs), a carboxyl group of the γ-PGA-Phe NPs (10 mg/ml) was first activated by water-soluble carbodiimide (1 mg/ml in 20 mM phosphate buffer, pH 5.8) for 20 min. The NPs (5 mg) obtained by centrifugation were mixed with 1 ml EphA2-derived peptide (0.5 mg/ml) in phosphate buffered saline (PBS)