Table 1 Classification of polymorphisms identified in the IFN-signalling related genes

Gene	Number of base pairs screened	SNP	Ins/del	Total polymorphisms	Distribution by gene structure					
					5′FL	5′UTR	CDS (sSNP, nsSNP)	Intron	3′UTR	3′FL
IFNAR1	7522	8	2	10	2	1	0 (0, 0)	5	2	0
IFNAR2	4849	6	0	6	0	0	1 (0, 1)	4	1	0
JAK1	11312	18	2	20	0	0	2 (2, 0)	18	0	0
TYK2	8270	19	0	19	0	0	8 (3, 5)	10	1	0
STAT1	10647	20	3	23	0	0	3 (3, 0)	18	1	1
STAT2	8646	13	1	14	0	0	1 (0, 1)	11	2	0
IRF9	3171	3	0	3	0	0	0 (0, 0)	2	1	0
RIG-I	8819	26	1	27	0	1	4 (1, 3)	19	3	0
IPS-1	5105	11	1	12	0	0	3 (1, 2)	2	7	0
IRF3	3968	8	2	10	0	3	2 (1, 1)	4	0	1
IRF7	2589	8	1	9	1	0	4 (2, 2)	4	0	0
IRF2	5668	13	1	14	0	0	2 (1, 1)	10	1	1
Total	74898	153	14	167	3	5	30 (14, 16)	107	19	3

SNP, single nucleotide polymorphism; ins, insertion; del, deletion; UTR, untranslated region; FL, flanking region; CDS, coding region; sSNP, synonymous SNP; nsSNP, nonsynonymous SNP.

Table 2 One hundred and sixty-seven polymorphisms in the IFN-signalling related genes

IFNAR1 (10 polymorphisms)	**−347**	−6*	51	10595	**10848**	10927	24135	24469*
	33483*	**33741**						
IFNAR2 (6 polymorphisms)	14693	14983	22299*	**22687**	33267*	34057*		
JAK1 (20 polymorphisms)	91	365	12755	13212	13242*	21305	30599	30856*
	34934*	34999	35312	38993	39038	**40725**	40870	40871
	41498	42571*	46465	51217*				
TYK2 (19 polymorphisms)	2243*	12345*	12529	14003*	**14006**	14145*	15192*	15452
	15560	18074	18164*	18279	21293*	26247*	26378*	**26525**
	26561*	26854	29721*					
STAT1 (23 polymorphisms)	283	821*	**4270**	5384	6630*	6751	16036	16151
	16539	23416	24514	**27161***	27452	28838	30625	34532*
	35386*	35574*	37058	37178	39478	44152*	**45397**	
STAT2 (14 polymorphisms)	88	3706	3765*	**4757***	**4901***	9465	9488	9634
	9819	10543	11441*	16088*	18063	18306*		
IRF9 (3 polymorphisms)	621*	**1129***	4265*					
RIG-I (27 polymorphisms)	**90**	177	354	391*	408	**33794***	33971	**35083**
	35263	37764	38008*	38086	41043	46072	49075*	53235
	58363*	58590*	58615*	59861*	60046*	60133*	60139	**66873**
	69596	69667	70306					
IPS1 (12 polymorphisms)	10717	10748	**10952***	**15495**	**15538**	18908	**19354**	19653
	19836*	**20479***	20921*	20927				
IRF3 (10 polymorphisms)	**95**	175	188	244	418*	1389	2320	**2652**
	6206	6304*						
IRF7 (9 polymorphisms)	−198	390*	**457**	**789**	1335	1598*	2488	2686
	2829*							
IRF2 (14 polymorphisms)	45305	45371*	45420	55441*	56210	**66675**	75602*	**83546**
	83649*	83700*	83749*	**85509**	86327	87066		

Gene number is expressed as the nucleotide position from the first nucleotide of the transcriptional start codon. Polymorphisms in boldface are selected as tag SNP markers.
*Newly discovered polymorphisms.

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Table 3 Demographic, virological and clinical features of patients with chronic hepatitis C treated by IFN plus ribavirin combination therapy

Variable	SVR (n = 98)*	Non-SVR (n = 105)*	P-value
Sex			
Male	64	50	**0.011**
Female	34	55	
Age (years)†	56.0 (24–72)	58.5 (27–74)	**0.023**
Weight (kg)†	61.8 (43–91)	61.9 (41–94)	0.821
Pre-treatment ALT (IU/L)†	69 (17–285)	57 (16–304)	0.170
Interferon history‡			
Naive	58 (51.8%)	54 (48.2%)	0.311
Relapse	30 (47.6%)	33 (52.4%)	
Nonresponse	10 (35.7%)	18 (64.3%)	
HCV genotype			
1	50 (34.0%)	97 (66.0%)	**0.00000002**
2, 3	48 (85.7%)	8 (14.3%)	
HCV RNA titre (kIU/mL)			
<100	15 (93.7%)	1 (6.3%)	**0.004**
100–500	35 (49.3%)	36 (50.7%)	
500–850	22 (44.9%)	27 (55.1%)	
850≤	26 (38.8%)	41 (61.2%)	
Fibrosis score			
0	5 (62.5%)	3 (37.5%)	**0.0005**
1	38 (59.4%)	26 (40.6%)	
2	19 (45.2%)	23 (54.8%)	
3	5 (20.0%)	20 (80.0%)	
4	2 (28.6%)	5 (71.6%)	

SVR, sustained virologic response. P-values in boldface are significant.
*SVR and non-SVR were evaluated in patients who had completed therapy for 24 or 48 weeks.
†Values are median (range).
‡One hundred six patients had received previous treatment with IFN-α monotherapy for 24 weeks, but failed to respond or relapsed.

HCV genotypes 2 and 3, 48 (85.7%) had SVR, whereas 50 of 147 (34.0%) patients with HCV genotype 1 had SVR, indicating that HCV genotype 1 was significantly associated with non-SVR (P = 0.00000002). In addition, a lower viral load before treatment (P = 0.004), male sex (P = 0.011), young age (P = 0.023), and lower degree of liver fibrosis (P = 0.0005) were significantly associated with SVR.

SNP genotyping analyses revealed that the frequencies of all 35 polymorphisms detected in the 240 hepatitis C patients were not significantly different from those in healthy volunteers. The success scores of the Taqman assay were 96.4–100% and those of direct sequencing were 95.8–100%. Univariate analyses of 35 polymorphisms revealed that a *TYK2* exon8 15560-G/T polymorphism (rs2304256) was significantly associated with virologic response to IFN-based therapy [P = 0.050, OR = 0.66 (0.44–0.99)] (Table S5).

In contrast to the univariate analysis, however, multiple logistic regression analyses demonstrated that the rs2304256 was not significant (P = 0.675) (Table 4). As a host factor, only a lower degree of liver fibrosis before therapy (P = 0.007) was significantly associated with SVR in the multiple logistic regression model. On the other hand, HCV genotypes 2 and 3 (P = 0.00005) and a lower viral load before therapy (P = 0.027) were both significantly associated with SVR.

Genetic polymorphisms associated with the adverse effects of IFN-based therapy

A total of 132 of 240 (55.0%) patients required either a discontinuation or a dose reduction of IFN or RBV due to the following adverse events: anaemia (n = 50), neutropenia or leucocytopenia (n = 32), thrombocytopenia (n = 17), depression (n = 7), and other causes (malaise, alopecia, and abdominal discomfort). The relationship between baseline characteristics and occurrence of haematologic adverse effects of the IFN plus RBV combination therapy is summarized in Table S6.

To identify the host genetic polymorphisms associated with the haematologic adverse effects of IFN plus RBV therapy, we focused on decreases in blood cell counts during the therapy and analysed the association with the SNPs in IFN signalling pathway-related genes. Consistent with previous reports [30,31], leucocyte, neutrophil, and platelet counts and haemoglobin levels usually declined in the initial 2–4 weeks of treatment, then stabilized during treatment, and returned to baseline levels within 12 weeks from the end of treatment in patients receiving IFN plus RBV therapy (Fig. 1). Therefore, we evaluated the decreases in leucocyte, neutrophil, and platelet counts and haemoglobin level at 4 weeks of treatment. We first examined the predictive factors for neutropenia. In 240 patients, absolute neutrophil counts decreased by an average of 39.3% from baseline during the first 4 weeks of treatment. Univariate analyses of 32 polymorphisms and clinical features showed that two SNPs, an *IFNAR1* intron2 10848-A/G polymorphism (rs2243594), and a *STAT2* intron5 4757-G/T, were associated with neutropenia caused by IFN-based therapy [P = 0.038, P = 0.020] (Table 5, Table S7). Furthermore, multivariate linear regression analysis confirmed that both polymorphisms were significantly associated with the neutropenia (P = 0.013, P = 0.009). Next, we examined the predictive factors for leucocytopenia. Absolute leucocyte counts decreased by an average of 29.9% from baseline within the first 4 weeks of treatment. Univariate analyses indicated that an *IFNAR1* intron2 10848-A/G polymorphism (rs2243594), an *IRF2* intron6 66675-C/T polymorphism (rs2241500), and female sex were associated with leucocytopenia (P = 0.048, P = 0.026, P = 0.016,

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Table 4 Univariate and multiple logistic regression analyses of SNPs and clinical factors associated with the efficacy of IFN plus ribavirin combination therapy

	Univariate analysis		Multiple logistic regression analysis	
Variable	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)
SNPs				
TYK2 15660-G/T	**0.050**	0.66 (0.44–0.99)	0.675	0.48 (0.14–1.67)
Clinical variables				
Sex	**0.011**	2.07 (1.17–3.66)	0.082	2.09 (0.90–4.84)
Age	**0.023**	0.16 (0.04–0.65)	0.347	0.69 (0.11–4.22)
HCV genotype	**0.00000002**	11.6 (5.09–26.6)	**0.00005**	7.35 (2.54–21.2)
Viral load	**0.004**	0.25 (0.10–0.62)	**0.027**	0.22 (0.06–0.88)
Fibrosis stage	**0.0005**	12.0 (2.63–54.8)	**0.007**	10.3 (1.72–62.3)

P-values in boldface are significant. SNP, single nucleotide polymorphism.

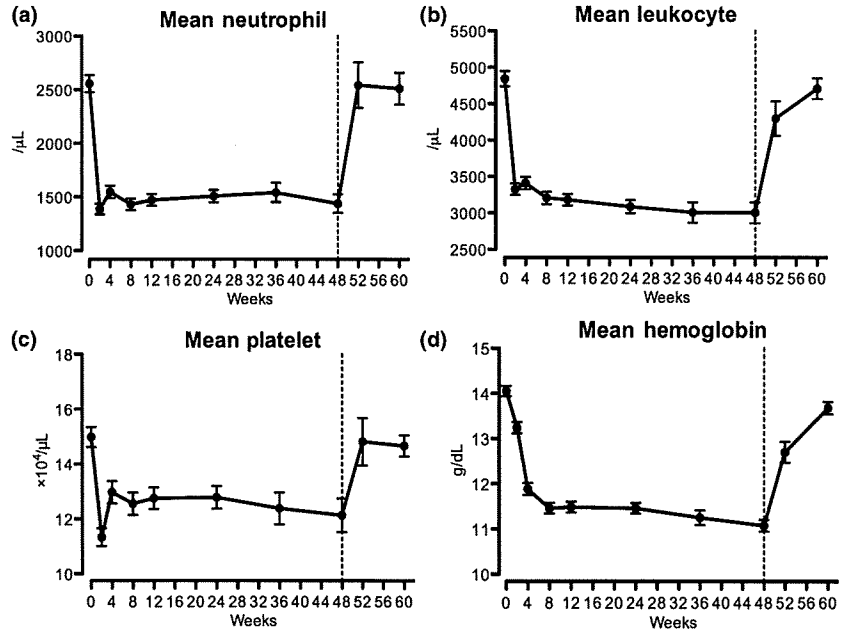

Fig. 1 Change in mean neutrophil (a), leucocyte (b), and platelet counts (c), and haemoglobin levels (d) during and after IFN plus RBV therapy. The results are shown as mean ± SEM.

respectively). Multivariate analysis, however, indicated that none of the factors, including *IFNAR1* rs2243594, *IRF2* rs2241500 and sex, were significant. Third, we examined the predictive factors for thrombocytopenia. Absolute platelet counts decreased by an average of 12.5% from baseline during the first 4 weeks of treatment. Univariate analyses showed that only an *IRF7* exon2 789-G/A (rs1061501) was associated with thrombocytopenia ($P = 0.031$). Finally, we examined the predictive factors for anaemia. Absolute haemoglobin concentration decreased by an average of 15.8% of baseline within the first 4 weeks of treatment. Univariate analyses revealed that anaemia was associated with older age ($P = 0.0004$), but not with any of the polymorphisms.

We examined the genotype results (variant allele carrier) of an *IFNAR1* intron2 10848-A/G polymorphism (rs2243594), a *STAT2* intron5 4757-G/T polymorphism, and an *IRF7* exon2 789-G/A polymorphism (rs1061501) for their association with various clinical and histologic features among 240 patients (Table S8). None of the factors, however, were associated with the SNPs identified.

DISCUSSION

In this study, we evaluated the influence of genetic polymorphisms on adverse effects and efficacy of IFN plus RBV combination therapy. Although several studies have evaluated the influence of host genetic polymorphisms on virologic response to IFN-based therapy, no studies have looked at possible association of adverse effects of the IFN-based therapy and host genetic polymorphisms. We report for the first time that certain SNPs in the IFN signalling pathway-related genes were associated with haematologic adverse effects in chronic hepatitis C patients undergoing IFN-based therapy.

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Table 5 Univariate and multiple linear regression analyses of SNPs and clinical factors associated with leucocytopenia, neutropenia and thrombocytopenia

Variable	Unit of B coefficient	Univariate analysis			Multiple analysis		
		P-value	*B* coefficient	SE *B*	*P*-value	*B* coefficient	SE *B*
Neutropenia							
IFNAR1 10848-A/G	%	**0.038**	6.94	3.31	**0.013**	6.43	2.57
STAT2 4757-G/T	%	**0.020**	−14.3	6.09	**0.011**	−13.8	5.41
Leucocytopenia							
IFNAR1 10848-A/G	%	**0.048**	4.14	2.08	0.109	1.62	1.61
IRF2 66675-C/T	%	**0.026**	3.44	1.53	0.054	3.00	1.54
Sex	%	**0.016**	7.79	3.20	0.134	3.30	2.20
Thrombocytopenia							
IRF7 789-G/A	%	**0.031**	4.15	1.92	ND	ND	ND
Anaemia							
Age	%/year	**0.0004**	0.28	0.08	ND	ND	ND

P-values in boldface are significant. SE, standard error. ND, not done because only one factor was significant in the univariate analysis.

The representative side effect of IFN-based combination therapy with RBV that causes poor therapeutic tolerance is haematologic toxicity, such as anaemia, neutropenia, and thrombocytopenia [4,32]. In fact, several studies reported that less than half the patients with hepatitis C were able to complete IFN plus RBV combination therapy at the assigned dose of both drugs, causing reduced therapeutic efficacy [5,6]. One thing to be noted is that the decrease in neutrophil and platelet counts induced by IFN-based therapy varies among patients, and thus it is difficult to predict the risk of haematologic toxicities in chronic hepatitis C patients receiving IFN-based therapy. The molecular mechanism of IFN-induced haematologic toxicities, however, is unknown. Several studies suggested the possibility that IFN treatment causes bone marrow suppression [33,34]. In agreement with this hypothesis, it was shown that a significant drop in platelet count after the initiation of IFN therapy is accompanied by a moderate increase in thrombopoietin levels in the failing liver, which may be insufficient to counteract the myelosuppressive action of IFN [35]. Another study suggested that IFN-mediated cytopenia may be due to rapid sequestration of platelets and leucocytes in the capillary beds of the liver and spleen [36]. Our current findings suggest that some of the IFN signalling pathway-related genes are involved in the decrease in neutrophil and platelet counts in response to IFN treatment. Interestingly, a recent study demonstrated that an intrinsic program for apoptosis controls platelet survival and dictates life span [37]. They revealed that platelets are genetically programmed to die by apoptosis and the antagonistic balance between antiapoptotic and proapoptotic molecules determines platelet life span. It is well known that IFN signalling induces the expression of multiple IFN-stimulated genes including molecules with proapoptotic or antiapoptotic function, such as tumour necrosis factor-related apoptosis-inducing ligand Fas, and X-linked inhibitor of apoptosis-associated factor 1 [38]. Thus, it is possible that IFNAR1, STAT2, and IRF7 contribute to the occurrence of neutropenia and thrombocytopenia by regulating the magnitude of IFN signalling involved in the apoptotic pathway in the haematopoietic cells in patients receiving IFN-based treatment.

In this study, three SNPs were associated with cytopenia in chronic hepatitis C patients receiving IFN plus RBV combination therapy. Among them, rs1061501 in the *IRF7* gene was located in the exon region but is a synonymous SNP. Recently, Kimchi-Sarfaty *et al.* demonstrated [39] that a synonymous SNP that did not affect amino acid sequence was capable of changing the function of the resultant protein. Indeed, the presence of a rare codon marked by a synonymous SNP in the *Multidrug Resistance 1* gene affects the timing of cotranslational folding and thereby alters the structure of substrate. Thus, it is possible that the synonymous rs1061501 contributes to a functional change in the IRF7 protein. On the other hand, rs2243594 in the *IFNAR1* gene and the SNP in the *STAT2* gene associated with neutropenia were located in an intronic region. In general, intronic SNPs provide little evidence for changes in protein structure or function, but an intronic mutation in the *p53* gene could have functional consequences by regulating gene expression, suggesting that the effect is mediated by a nonsynonymous and disruptive coding change in linkage disequilibrium with the associated intronic SNP or by a change in RNA splicing, editing, or expression [40]. Thus, it is possible that two intronic SNPs associated with neutropenia contribute to functional changes in the IFNAR1 and STAT2 proteins.

In contrast to the adverse effects of IFN plus RBV combination therapy, none of the host genetic polymorphisms in the IFN signalling pathway-related genes analysed were

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associated with therapeutic efficacy. The results indicated that viral factors, including viral genotype and pre-treatment viral load, and histological fibrosis grade were likely to have critical roles in treatment response. Consistent with many previous reports [41–43], we found that HCV genotypes 2 and 3, low viral load, and early fibrosis stage predict a favourable virologic response to IFN plus RBV combination therapy. On the other hand, it was reported that several SNPs in certain genes are associated with efficacy in IFN-based therapy [8–14,16,17]. Many of these previous studies, however, evaluated the association between the SNP and the treatment response using only univariate and not multivariate analyses that included viral factors. In fact, in our univariate analysis, one *TYK2* SNP (rs2304256) showed a possible association with therapeutic efficacy. Multivariate analysis, however, revealed that this SNP was not significant. Taken together, these findings suggest that the viral factors and host histological grade of liver fibrosis are important predictors of the treatment response in chronic hepatitis C infection. Although no significant association was observed between the efficacy and the IFN signalling pathway-related genes examined, it is possible that polymorphisms of other genes might play a role in the treatment response to IFN-based therapy.

In conclusion, we demonstrated that the SNPs in the *IFNAR1* and *STAT2* genes were associated with neutropenia and the SNP in the *IRF7* gene was associated with thrombocytopenia in chronic hepatitis C patients receiving IFN plus RBV combination therapy. In contrast, the virologic factors and histological grade of liver fibrosis are important predictors for virologic response to the IFN-based therapy, whereas no host genetic polymorphisms in IFN signalling pathway-related genes analysed affected the therapeutic efficacy. Further analyses are required to clarify the mechanisms of how those polymorphisms affect the biologic function of the IFN signalling and contribute to the occurrence of haematological adverse effects in IFN-treated patients.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Oligonucleotide sequences for primers and probes used for Taqman SNP genotyping assay.

Table S2 Assay ID of primers and probes used for Taqman SNP genotyping assay.

Table S3 Oligonucleotide sequences for primers used for PCR amplification and sequencing.

Table S4 List of discovered polymorphisms in 12 IFN-signalling related genes.

Table S5 Genotype frequency in the genotyped 35 polymorphisms of the IFN-signalling related genes.

Table S6 Demographic, virological and clinical features of patients with chronic hepatitis C treated by IFN plus ribavirin combination therapy.

Table S7 Linear regression analyses of 35 SNPs and clinical factors associated with haematologic adverse effects.

Table S8 Demographic, and clinical features according to three polymorphisms significantly associated with IFN-induced neutropenia and thrombocytopenia.

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Alimentary Pharmacology & Therapeutics

Clinical trial: extended treatment duration of peginterferon-alpha2b plus ribavirin for 72 and 96 weeks in hepatitis C genotype 1-infected late responders

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SUMMARY

Background
The benefits of prolonging peginterferon and ribavirin after 48 weeks of treatment to maximize sustained virological responses (SVR) in hepatitis C virus (HCV) genotype 1-infected patients remain to be understood.

Aim
To investigate whether extended treatment longer than 72 weeks may be superior to 72-week treatment.

Methods
A total of 120 treatment-naïve or retreated patients with HCV genotype 1 were treated with peginterferon-alpha-2b (1.5 μg/kg/week) plus weight-based ribavirin. We had 34 late responders, in whom HCV RNA first became undetectable at week 12–48, and randomized them into three groups receiving standard-dose peginterferon-alpha-2b plus low-dose ribavirin (200 mg/day) for extended 24 weeks (group A), receiving low-dose peginterferon-alpha-2b (0.75 μg/kg/week) plus low-dose ribavirin for extended 48 weeks (group B) or no extended treatment (group C), and evaluated the outcome according to their virological response.

Results
Multivariate analysis showed that the treatment for 96 weeks was identified as a significant, independent factor associated with SVR in HCV genotype 1-infected late responders in comparison with group A [odds ratio (OR), 10.002; $P = 0.080$] and group C (OR, 17.748; $P = 0.025$).

Conclusion
Extending the treatment duration from 48 weeks to 96 weeks improves SVR rates in genotype 1-infected patients with late virological response to peginterferon-alpha-2b and ribavirin.

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INTRODUCTION

The hepatitis C virus (HCV) is a common cause of cirrhosis and hepatocellular carcinoma. For the management of HCV genotype 1 infection, 48 weeks of therapy with pegylated interferon plus ribavirin is recommended.[1] Although the introduction of pegylated interferon in combination with ribavirin in recent years greatly improved the treatment outcome of HCV infection, the treatment outcome of HCV type 1-infected patients remains unsatisfactory and sustained virological responses (SVR) can be obtained in only approximately 45%.[2–4]

Hepatitis C virus kinetics during the early phase of treatment is recognized as a predictor of the final therapeutic outcome. Assessment of early virological response (EVR) correlates closely with the likelihood of the ultimate eradication of HCV in patients treated with ribavirin in combination with interferon[5] or pegylated interferon.[6–8] After 48 weeks of treatment, the likelihood of SVR was approximately 90% in patients who achieved undetectable serum HCV RNA at week 4 of treatment in subjects infected with HCV genotype 1, whereas patients with less than 2-log decrease in HCV RNA levels by week 12 of treatment had virtually no chance of developing SVR.[6, 7] On the basis of these findings, discontinuation of treatment in nonresponders at this time was recommended to avoid unnecessary therapy.[1, 9] However, high relapse rates in slow responders may indicate that treatment was not administered for a sufficient duration in patients with slow virological response.

An analysis based on a mathematic model from a phase III randomized trial of peginterferon-alpha-2a and ribavirin, Drusano and Preston suggested that the rate of SVR in patients infected with HCV genotype 1 directly correlates with the duration of treatment once HCV RNA has been cleared from serum.[10] As the average time to clear serum HCV RNA was over 30 weeks, the authors concluded that 48-week duration of therapy was inadequate for most patients with genotype 1. Indiscriminate extension of treatment in patients with HCV genotype 1 is not beneficial. It has been currently reported that there is a subgroup of genotype 1-infected patients, the so-called 'slow responders', who benefit from extending the treatment duration from 48 weeks to 72 weeks that significantly improves SVR rates.[11–14] Therefore, prolonged treatment has the potential to improve cure rates, although it will increase the cost of treatment and may increase the probability that a patient will experience adverse events. However, prolonged duration and optimal doses of pegylated interferon or ribavirin after 48 weeks of treatment to maximize SVR still remain to be understood. We aimed to investigate whether extended treatment longer than 72 weeks using the dose reduction of pegylated interferon after 48 weeks of treatment may be superior to the 72-week treatment using the standard dose of pegylated interferon. To tolerate such a long treatment, we tapered doses of pegylated interferon and/or ribavirin substantially after 48 weeks of treatment.

In hepatitis C genotype 1 patients, a slow virological responder was commonly defined as a patient with at least a 2-log decrement in baseline serum HCV RNA, albeit detectable viraemia at 12 weeks and undetectable serum HCV RNA at 24 weeks.[13] However, Mangia *et al.* reported that SVR rates of HCV genotype 1 patients who first achieved undetectable HCV RNA at week 12 were 38.1% and 63.4% in 48 weeks and 72 weeks treatment respectively.[14] In a multicentre study in Japan, SVR rate of HCV genotype 1b patients in whom HCV RNA became negative for the first time at week 12 was 41.2% in 48 weeks treatment, although SVR rate of patients in whom HCV RNA became negative within 8 weeks was over 80% (personal communication to Dr Kuboki).[15] These studies indicate that extended treatment duration is recommended in patients with undetectable HCV RNA at week 12 to improve cure rates.

Following these concepts, we randomized HCV genotype 1-infected late responders, in whom HCV RNA was positive at 8 weeks of treatment and negative for the first time during 12–48 weeks of treatment, into groups receiving standard-dose peginterferon-alpha-2b (1.5 μg/kg/week) plus low-dose ribavirin (200 mg/day) for additional 24 weeks (total 72 weeks) or receiving low-dose peginterferon-alpha-2b (0.75 μg/kg/week) plus low-dose ribavirin (200 mg/day) for additional 48 weeks (total 96 weeks) and evaluated the outcome according to their virological response.

METHODS

Patients

The purpose of this study was to assess prospectively the efficacy of extended treatment duration of peginterferon-alpha-2b plus ribavirin in HCV genotype

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1-infected late responders. Adult patients of both genders aged more than 18 years testing positive for anti-HCV, with consistent detection of HCV RNA above 100 000 IU/mL by reverse-transcription polymerase chain reaction [RT-PCR; Amplicor HCV (version 2), Roche Diagnostics, Branchburg, NJ, USA] and elevated serum alanine aminotransferase (ALT) levels were eligible for enrollment. Patients were excluded if they had decompensated liver diseases, other causes of liver disease, hepatitis B infection, haemoglobin values <13 g/dL, white blood cell count <4000/μL, thrombocytopenia <100 000 /μL, neoplastic, severe cardiac, neurological, autoimmune or thyroid disease. Also excluded were patients with alcohol or drug abuse, women who were pregnant or considering pregnancy in the next 18 months or men whose partners were considering pregnancy in the next 18 months. A late responder was defined as a patient with HCV RNA positive at 8 weeks of treatment and negative for the first time during 12–48 weeks of treatment. Written informed consent was obtained from all patients, and an institutional review board at each participating centre approved the study protocol.

Study design

This study was conducted between December 2004 and December 2005 at eight centres (two university hospitals and six general hospitals) in Japan. In this partially randomized, open-label, parallel-group, multicentre study, one hundred twenty treatment-naïve or retreated patients who met the criteria for entry were enrolled and received treatment with subcutaneous peginterferon-alpha-2b (1.5 μg/kg/week) (Peg-Intron; Schering Corp., Kenilworth, NJ, USA) and oral ribavirin (600–1000 mg/day based on weight: ≤60 kg, 600 mg; 61–80 kg, 800 mg; and >80 kg, 1000 mg) (Schering Corp.) for 48 weeks. Thirty-seven of 120 patients had been treated previously with conventional interferon or conventional interferon plus ribavirin for 24 weeks ('relapsers' and 'nonresponders' were included), but had not been treated previously with pegylated interferon and ribavirin. Pegylated interferon and ribavirin dose modifications followed standard criteria and procedures. The late responders whose serum HCV RNA became undetectable during 12–48 weeks after treatment were randomized to 1 of the 3 treatment groups: extended therapy for an additional 24 weeks with standard-dose peginterferon-alpha2b (1.5 μg/kg/week) plus low-dose ribavirin (200 mg/day) (total treatment duration of 72 weeks; group A); extended therapy for an additional 48 weeks with low-dose peginterferon-alpha-2b (0.75 μg/kg/week) plus low-dose ribavirin (200 mg/day) (total treatment duration of 96 weeks; group B); and not-extended therapy (total treatment duration of 48 weeks; group C).

Liver biopsies, which were not mandatory for the patients to be enrolled, were performed in 100 patients within 6 months before study entry, and histological changes were recorded according to the criteria of Desmet *et al.*,[16] with the grading of activity and the staging of fibrosis being defined as A0 (no histological activity), A1 (mild activity), A2 (moderate activity), A3 (severe activity), and as F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis) and F4 (cirrhosis) respectively.

On the basis of SVR rate of 35% in the 48 weeks treatment (Group C in our study)[14, 15] and predicted improvement of the rate of 50% or higher in Group A or B (SVR rate of 85%), we calculated the required sample size of 14 for each group with α-error of 0.05 and ß-error of 0.80.

Measurement of HCV RNA

Serum samples were collected in each institution and centrally stored at −80 °C. Anti-HCV was tested by third-generation enzyme-linked immunoassay (Abbott Laboratories, North Chicago, IL, USA). Quantification of serum HCV RNA was performed by a single central laboratory (SRL Laboratory Co., Tokyo, Japan) to avoid variability between available assays using RT-PCR (Amplicor HCV Monitor test [version 2], Roche Diagnostics, Branchburg, NJ, USA) with a lower limit of detection of 600 IU/mL. Serum HCV qualitative test (detection limit 50 IU/mL; Amplicor HCV kit [version 2], Roche Diagnostics) was assessed at every 4 weeks after treatment. HCV genotyping was performed by RT-PCR using genotype-specific primers[17] in a single central laboratory (SRL Laboratory Co.) using a modification of a method described by Ohno *et al.*[18]

Determination of nucleotide and deduced amino-acid sequences of the IFN-sensitivity-determining-region (ISDR) and core region

RNA coding for ISDR in the NS5A region was amplified by nested RT-PCR. For direct sequencing of the NS5A (2209–2248) region, after the first-round PCR,

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the second round of nested RT-PCR was performed using an external sense primer and internal antisense primer.[19] The second-round PCR products were purified and directly sequenced using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer Applied Biosystem, Warrington, UK) in a 310 DNA sequencer (ABI 3100 Genetic Analyzer, Perkin Elmer Applied Biosystems). Electropherograms were analysed using Sequence Navigator software (Perkin Elmer Applied Biosystems). The deduced amino acid sequences of ISDR were compared with the sequence of the prototype isolate of HCV-J. Detection of amino acid substitutions of aa 70 and aa 91 in core region of HCV genotype 1b was performed using mutation-specific primer as an alternative to the direct sequencing method.[20] The major protein type was determined based on the relative intensity of the bands for wild (aa 70, arginine; aa 91, leucine) and mutant (aa 70, glutamine/histidine; aa 91, methionine) in agarose gel electrophoresis. All of the above procedures were performed centrally by SRL Laboratory.

Efficacy end points

The primary aim of the study was to assess the effect of extended treatment duration of peginterferon-alpha-2b plus ribavirin on sustained virological response (SVR) for patients with late virological response defined as HCV-RNA positive at week 8, but negative at weeks 12–48. SVR was defined as the sustained disappearance of serum HCV RNA for 24 weeks after the end of treatment. Treatment failure was categorized as relapse (reappearance of HCV RNA during the follow-up period after an end of treatment response), nonresponse (HCV RNA positive at week 48) or discontinuation (treatment withdrawn for any reason).

The secondary endpoint was the evaluation of discontinuation. It was thought important to decrease the numbers of patients with discontinuation to achieve higher SVR.

Statistical analysis

The efficacy analysis was conducted on an intention-to-treat basis. All patients who received at least one dose of study medication were included in the intention-to-treat population. The baseline characteristics of patients randomized to groups A, B and C were compared using Fisher's exact test for categorical data and Kruskal–Wallis test for continuous variables. Univariate and stepwise multivariate logistic regression analyses were used to determine independent predictive factors that were associated with SVR. Correlations were tested using Pearson's rank correlation coefficient.

RESULTS

Patient profiles

The median age of the enrolled population of 120 patients from eight centres was 60 years, 61% were men, and 98% were infected with genotype 1b. The trial participant flow is shown in Figure 1. Of 120 patients with genotype 1 infection treated with peginterferon-alpha-2b and ribavirin during that study period, 39 patients (33%) were late responders to therapy and met inclusion criteria. However, only 35 patients participated and were randomized, because four late responders declined to participate in the study. Of 120 enrolled patients, 25 patients (21%) stopped treatment within 48 weeks.

Thirty-five late responders, all of whom were genotype 1b, were assigned to group A (n = 12), group B (n = 10) or group C (n = 13). However, one patient of group B who was found to be HCV RNA negative at week 8 and did not meet inclusion criteria was excluded for this analysis. Baseline demographic, biochemical and virological characteristics of patients did not differ among three groups (Table 1). Time when patients first achieved undetectable HCV RNA did not differ among three groups.

Outcomes of patients

At week 48 of treatment, HCV RNA was undetectable in all of 34 patients in groups A, B and C. At the end of therapy, HCV RNA was undetectable in 92%, 100% or 100% of patients from each group A, B or C respectively (Figure 2). At the end of the follow-up period, virological response was sustained in 58% (7/12) of patients in group A, 89% (8/9) of patients in group B and 38% (5/13) of patients in group C (Figure 2). Surprisingly, one patient in group B who first became HCV negative at week 28 of treatment achieved SVR. As shown in Figure 2, relapse rate was lesser in patients treated for 96 weeks (11%) than in those treated for 72 weeks (42%, P = 0.178) or 48 weeks (62%, P = 0.031). Moreover, we have assessed the treatment outcome of patients who had detectable HCV RNA at

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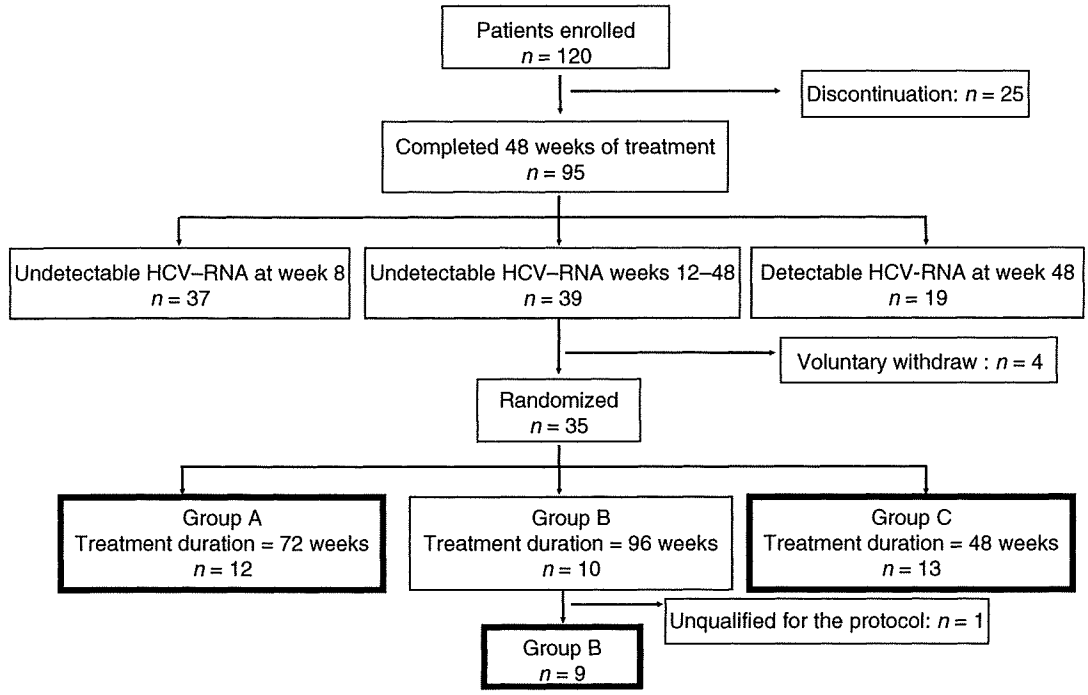

Figure 1. Flow of participants throughout the study.

week 12, but undetectable HCV RNA at week 48. Although patient numbers among the treatment subgroup were limited, virological response was sustained in 33% (2/6) of patients in group A, 67% (2/3) of patients in group B and 0% (0/5) of patients in group C.

During the extended treatment for patients in groups A and B, only one patient in group A discontinued ribavirin intake, but none except the patient in both groups needed dose reductions of peginterferon-alpha-2b or ribavirin. In addition, haemoglobin levels increased again in groups A and B after 48-week treatment probably because of the dose reduction of ribavirin during the extended treatment (Figure 3). The rate of SVR among HCV genotype 1-infected patients was significantly higher in patients treated for 96 weeks than in those treated for 48 weeks ($P = 0.034$, Table 2 and Figure 2), although the difference between the rates of SVR in group A and group C was not significant (Table 2). The rate of SVR of patients in group B (89%) was comparable to that of patients achieving early virological response whose HCV RNA was negative at week 8 [78% (29/37)].

Several baseline and on-treatment predictors of SVR (group, age, activity grade, total cholesterol), which P values were lower than 0.2 using Fisher's exact test, were examined by logistic regression analysis. The stepwise multivariate logistic regression analyses for four variables showed that group and age were independent predictive factors of SVR. The treatment for 96 weeks was identified as a significant, independent factor associated with SVR in HCV genotype 1-infected late responders [group B vs. group A; odds ratio (OR), 10.002; confidence interval (CI), 0.757–132.148; $P = 0.080$, group B vs. group C; OR, 17.748; 95% CI, 1.427–220.746; $P = 0.025$].

Sustained virological responses in the total study population

Sustained virological responses was obtained in 52 of 120 (43%) of the total intention-to-treat population and 52 of 117 (44%) of those with 24-week follow-up data. SVR was obtained in 2 of 25 (8%) of patients with treatment discontinuation. For the improvement of SVR in the total population, it must be important to decrease the number of patients with treatment discontinuation. Interestingly, we found that the number of patients enrolled per hospital was significantly associated with the reduced ratio of patients with treatment discontinuation (Figure 4).

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Table 1. Characteristics of patients at baseline

Treatment duration	Group A (N = 12) 72 weeks	Group B (N = 9) 96 weeks	Group C (N = 13) 48 weeks	P-value
Gender				
Male	8 (67%)	5 (56%)	8 (62%)	0.908
Female	4 (33%)	4 (44%)	5 (38%)	
Age (year)	54 (35–73)	60 (48–70)	62 (35–71)	0.657
Serum ALT (IU/L)*	52 (26–255)	61 (40–108)	64 (17–171)	0.437
HCV RNA (KIU/mL)				
<1500	4 (33%)	5 (56%)	3 (23%)	0.317
≥1500	8 (67%)	4 (44%)	10 (77%)	
Number of mutations in ISDR				
0	5 (45%)	6 (67%)	9 (75%)	0.282
1–3	5 (45%)	3 (33%)	3 (25%)	
4-	1 (9%)	0 (0%)	0 (0%)	
Core 70 mutation				
W	9 (75%)	8 (89%)	9 (75%)	0.708
M	3 (25%)	1 (11%)	3 (25%)	
Core 91 mutation				
W	9 (75%)	6 (67%)	10 (83%)	0.595
M	3 (25%)	3 (33%)	2 (17%)	
Fibrotic stage				
0–1	2 (22%)	3 (33%)	5 (38%)	0.559
2–4	7 (77%)	6 (67%)	8 (62%)	
Activity grade				
0–1	3 (33%)	4 (44%)	4 (31%)	0.893
2–3	6 (67%)	5 (56%)	9 (69%)	
Loss of HCV RNA (week)				
12	6	6	8	0.653
16	2	0	4	
20	0	0	0	
24	3	1	1	
28	1	1	0	
32	0	1	0	

HCV, hepatitis C virus; ISDR, interferon-sensitivity-determining-region.
*Normal range of ALT: 7–40 IU/L.
Fisher's exact test was used for categorical data to compare differences, and continuous variables were compared by Kruskal-Wallis test.
Hepatic histology was not evaluated in three patients in group A, because liver biopsy was not performed.
Lack of completeness was due to incomplete sampling.

DISCUSSION

A 48-week treatment with pegylated interferon plus ribavirin has now become the standard of care for patients with HCV genotype 1. The duration of antiviral therapy is one of the most important factors influencing treatment outcome, especially in HCV genotype 1-infected patients.[11–14] Berg *et al.* investigated the efficacy of 48 weeks vs. 72 weeks of treatment with peginterferon-alfa-2a plus ribavirin in treatment-naïve patients with HCV type 1 infection. In this study, prolongation of the therapeutic regimen for up to 72 weeks does not lead to higher SVR rates in the intention-to treat population, but patients who still are HCV-RNA positive at week 12 show significantly higher SVR rates when treated for 72 weeks instead of 48 weeks.[11] Sánchez-Tapias *et al.* have recently demonstrated that extension of treatment with peginterferon-alfa-2a plus ribavirin from 48 to 72 weeks significantly increases the rate of SVR in patients with detectable viraemia at week 4 of treatment.[12] Pearlman *et al.* have demonstrated that extending the treatment

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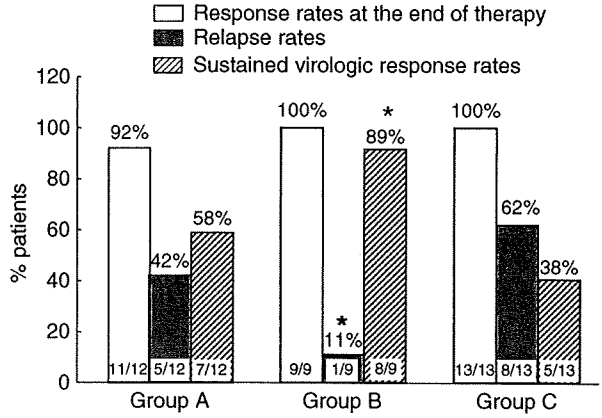

Figure 2. Frequency of virological response rates at the end of therapy and virological relapse rates in groups A, B and C. These rates are shown as a percentage and the number of patients with virological response or virological relapse in relation to the total number of patients examined is shown at the bottom of each column.
*$P < 0.05$ compared with group C.

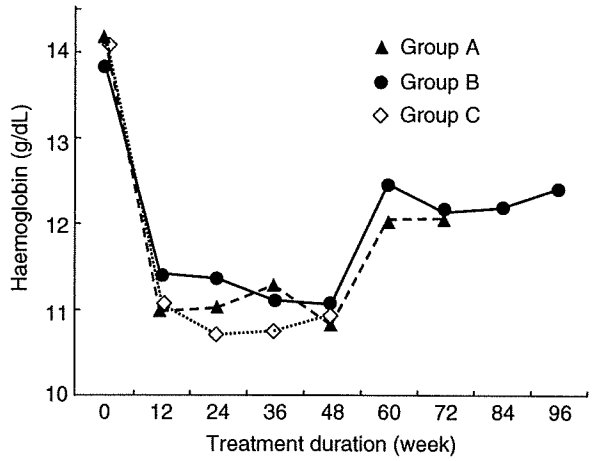

Figure 3. Time course of haemoglobin levels in groups A, B and C during therapy. The patients in groups A and B were given low-dose ribavirin (200 mg/day) beyond 48 weeks.

duration from 48 weeks to 72 weeks in genotype 1-infected patients with slow virological response to peginterferon- alfa2b plus ribavirin, which was defined by achieving at least a 2-log decrement in HCV RNA from baseline, yet having detectable HCV RNA at 12 weeks and undetectable HCV RNA at 24 weeks, significantly improves SVR rates.[13]

However, all of the aforementioned studies extended the treatment duration to 72 weeks to improve SVR rates in slow responders with HCV genotype 1. Furthermore, it is unclear if the standard doses of peginterferons and ribavirin continued to be used after week 48, although these patients achieved undetectable HCV RNA before 24 weeks. It was reported that treatment discontinuation was more frequent in patients treated for 72 weeks than those for 48 weeks.[13] In the prediction model developed by Drusano and Preston, it was concluded that type 1-infected patients required the continuous absence of detectable HCV RNA in serum for 36 weeks to attain 90% probabilities of SVR,[10] suggesting the importance of treatment duration when serum HCV RNA is continuously negative. In the present study, we used the minimum dose of ribavirin (200 mg/day) beyond 48 weeks in late responders who first became HCV RNA undetectable after 12 weeks and compared the efficacy and safety of additional 24 weeks of treatment (total 72 weeks) with the standard dose of peginterferon- alfa2b with those of additional 48 weeks of treatment (total 96 weeks) with the half dose of peginterferon- alfa2b.

Our data showed that SVR rates were higher in the 96-week group as compared with the 72-week group as well as the 48-week group (89% vs. 58% or 38%, respectively). The SVR rates seem to be higher than those previously reported.[11–13] The differences in the SVR rates could be because of our criteria of late responders that include patients with a first virological response at week 12. Only one of 21 patients in group A and B became HCV RNA positive during the extended treatment after 48 weeks of treatment, suggesting that the intentional dose reductions of peginterferon-alpha-2b and ribavirin between weeks 48 and 96 did not cause adverse effects on viral load. Only one of 21 patients discontinued ribavirin intake, but the others did not need dose reductions of peginterferon-alpha-2b or ribavirin and therapy discontinuation during the extended treatment, indicating that the intentional dose reductions of peginterferon-alpha-2b and ribavirin between weeks 48 and 96 were safe for patients with chronic hepatitis C genotype 1. Moreover, the intentional dose reductions during the last part of the treatment improved haemoglobin levels (Figure 3), which might result in tolerating a long treatment.

Among patients who discontinued treatment up to week 48, the rate of SVR was 8% (intention-to-treat analysis), which is much lower than that with patients treated for at least 48-weeks [53% (50/95)]. These data highlight the relevance of encouraging adherence to therapy.[21] Interestingly, the number of patients

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Table 2. Predictors of sustained virological response to combination therapy

Factor	Definition	OR (95% CI)	*P*-value
Univariate logistic regression analysis			
Group	B vs. C	12.800 (1.208–135.579)	0.034
	B vs. A	5.714 (0.532–61.409)	0.150
	A vs. C	2.240 (0.451–11.114)	0.324
Gender	Female	2.045 (0.477–8.773)	0.336
Age	<60 years	3.344 (0.802–13.941)	0.098
Previous IFN course	Naïve	1.750 (0.420–7.288)	0.442
Serum ALT*	≥63 (IU/L)	2.500 (0.584–10.696)	0.217
Total cholesterol	≥170 (mg/dL)	4.480 (0.986–20.354)	0.052
HCV RNA	<1500 KIU/mL	3.000 (0.636–14.150)	0.165
ISDR mutation	W	1.072 (0.250–4.591)	0.926
Core 70 mutation	W	1.200 (0.221–6.520)	0.833
Core 91 mutation	W	0.900 (0.175–4.630)	0.900
Fibrotic stage	2–4	1.625 (0.355–7.434)	0.532
Activity grade	2–3	2.229 (0.497–9.997)	0.295
Stepwise multivariate logistic regression analysis			
Group	B vs. C	17.748 (1.427–220.746)	0.025
	B vs. A	10.002 (0.757–132.148)	0.080
	A vs. C	1.774 (0.315–10.010)	0.516
Age	<60 years	4.963 (0.922–26.710)	0.062

OR, odds ratio; 95% CI, 95% confidence interval; HCV, hepatitis C virus; ISDR, interferon-sensitivity-determining-region.
Normal range of ALT: 7–40 IU/L.

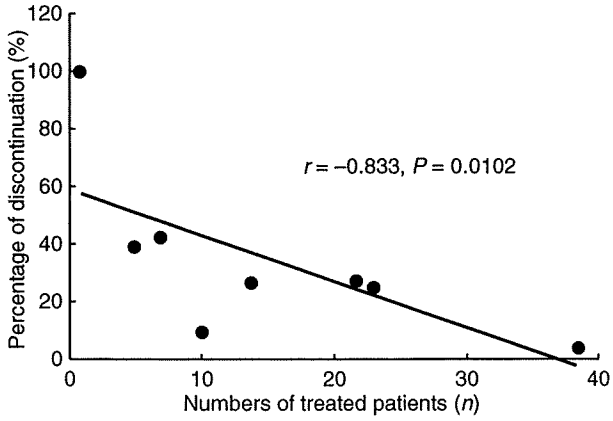

Figure 4. Correlation between the number of treated patients and percentage of discontinuation at each hospital. All 25 patients who stopped treatment within 48 weeks of treatment were analysed. Pearson's correlation coefficient is indicated on the graph.

enrolled per hospital was negatively associated with the numbers of patients with treatment discontinuation. These findings imply that the differences in improved adherence could be the result of physician-driven care and continuity based on the experience of each physician, because almost all of our patients were seen by the same treating physician on a monthly basis throughout the trial. Moreover, low attrition rates in hospitals where a greater numbers of patients were cared could be explained by the patients' knowledge concerning the adverse and beneficial effects of this combination therapy informed by physicians before and during treatment.

Limitation of this study is the small number of patients as compared to the predicted sample size. Thus, the statistical power is weaker than that of the initial design, and the possibility of ß-error remains.

In conclusion, extension of the treatment duration with peginterferon-alfa-2b plus ribavirin up to 96 weeks significantly increased the likelihood of achieving SVR in HCV genotype 1-infected late responders whose serum HCV RNA became undetectable for the first time during 12–48 weeks after treatment. Treatment extension did not increase the rate of dose reduction or treatment discontinuation.

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Review Article

Strategy and mechanism for the prevention of hepatocellular carcinoma: Phosphorylated retinoid X receptor α is a critical target for hepatocellular carcinoma chemoprevention

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Hepatocellular carcinoma (HCC) is a major health care problem worldwide. The prognosis of patients with HCC is poor because even in the early stages when surgical treatment might be expected to be curative, the incidence of recurrence in patients with underlying cirrhosis is very high due to multicentric carcinogenesis. Therefore, strategies to prevent recurrence and second primary HCC are required to improve the prognosis. One of the most practical approaches to prevent the multicentric development of HCC is 'clonal deletion' therapy, which is defined as the removal of latent (i.e. invisible) (pre)malignant clones from the liver in a hypercarcinogenic state. Retinoids, a group of structural and functional analogs of vitamin A, exert their biological function primarily through two distinct nuclear receptors, retinoic acid receptors and retinoid X receptors (RXR), and abnormalities in the expression and function of these receptors are highly associated with the development of various cancers, including HCC. In particular, a malfunction of RXRα due to phosphorylation by the Ras–mitogen-activated protein kinase signaling pathway is profoundly associated with the development of HCC and thus may be a critical target for HCC chemoprevention. Acyclic retinoid, which has been clinically shown to reduce the incidence of a post-therapeutic recurrence of HCC, can inhibit Ras activity and phosphorylation of the extracellular signal-regulated kinase and RXRα proteins. In conclusion, the inhibition of RXRα phosphorylation and the restoration of its physiological function as a master regulator for nuclear receptors may be a potentially effective strategy for HCC chemoprevention and clonal deletion. Acyclic retinoid, which targets phosphorylated RXRα, may thus play a critical role in preventing the development of multicentric HCC. (*Cancer Sci* 2009; 100: 369–374)

Hepatocellular carcinoma is the fifth most common cancer worldwide and the third most common cause of cancer mortality. HCC is unique in that it usually occurs within an established background, chronic liver disease and cirrhosis. The development of HCC is frequently associated with chronic inflammation of the liver induced by a persistent infection with hepatitis B virus or hepatitis C virus. Therefore, this cancer is a major health care problem in Eastern as well as Western countries where hepatitis virus infection is endemic.[1,2] Patients with viral liver cirrhosis are a high-risk group for HCC because the annual rate for this cancer in those patients is approximately 7%. Even in the early stages when surgical treatment might be expected to be curative, the incidence of recurrence in patients with underlying cirrhosis is approximately 20–25% a year. Therefore, the recurrence rate at 5 years after curative treatment may exceed 70%.[3–6] In addition, at least one-third of secondary tumors are primary *de novo* cancers.[7] Based on these clinical characteristics, the prognosis of patients with HCC is poor. Thus, it is a task of pressing urgency to develop more effective strategies for the chemoprevention of HCC and, for this purpose, there is a critical need to elucidate the molecular mechanisms underlying liver carcinogenesis.

Cancer chemoprevention is defined as an approach where a natural or synthetic chemical compound works to arrest or reverse premalignant cells by using physiological pathways.[8] We previously reported that, in a clinical trial, the administration of ACR, a novel synthetic retinoid (Fig. 1), reduced the incidence of post-therapeutic HCC recurrence and improved the survival rate of patients.[9–11] We have also revealed that a malfunction of RXRα, a nuclear retinoid receptor, due to aberrant phosphorylation is associated with carcinogenesis in the liver.[12–14] The aim of the present paper is to review the evidence that ACR exerts its chemopreventive effects on the development of HCC by targeting p-RXRα. In addition, the concept of 'clonal deletion', which is one of the most practical approaches to preventing multicentric HCC development, is reviewed and the possibility of 'combination chemoprevention', which uses ACR as a key drug and might be an effective strategy to prevent this malignancy by pharmacological synergism, is discussed.

Retinoids and their receptors

Retinoids, a group of structural and functional analogs of vitamin A, exert fundamental effects on the regulation of epithelial cell growth, differentiation, and development.[15,16] A small portion of dietary retinoids is converted to RA, which is an active metabolite of retinoids. Retinoids exert their biological functions primarily by regulating gene expression through two distinct nuclear receptors, the RXR and the RAR, which are both composed of three subtypes (α, β, and γ) that are characterized by a modular domain structure. RXR is specific for 9-*cis* RA, whereas RAR binds both 9-*cis* RA and all-*trans*

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Abbreviations: ACR, acyclic retinoid; AFP-L3, lectin-reactive α-fetoprotein isoform 3; Erk, extracellular signal-regulated kinase; HCC, hepatocellular carcinoma; HER2, human epidermal growth factor receptor-2; IFN, interferon; MAPK, mitogen-activated protein kinase; PIVKA-II, protein induced by vitamin K absence or antagonist-II; p-RXR, retinoid X receptor; RA, retinoic acid; RAR, retinoic acid receptor; RTK, receptor tyrosine kinase; RXR, retinoid X receptor; VK_2, vitamin K_2.

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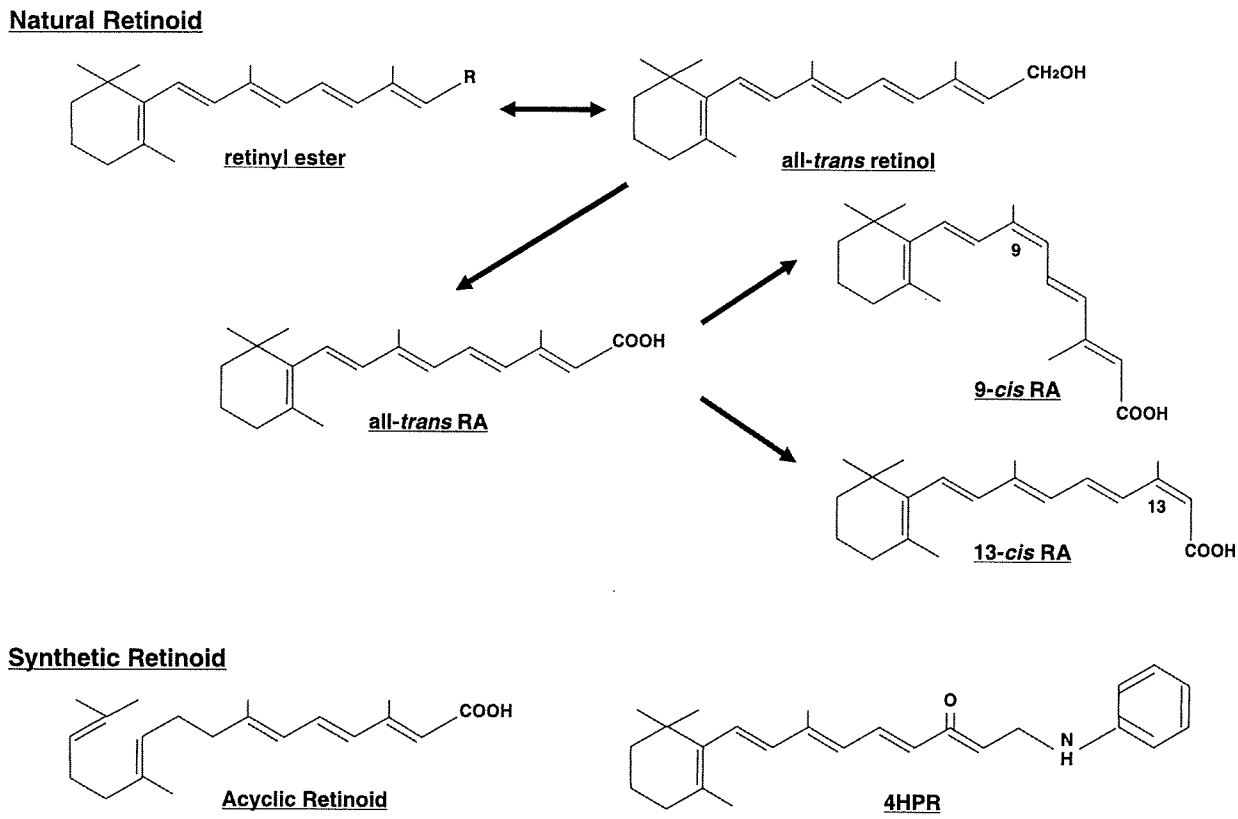

Fig. 1. Chemical structures of natural and representative synthetic retinoids. Retinyl esters (mainly retinyl palmitate; R, fatty acid), stored in the liver stellate cells, are hydrolyzed to retinol, which is then transported to target cells through the circulation after binding to retinol-binding protein. Retinoic acid (RA) is biosynthesized from retinol via the intermediate metabolite retinal by oxidization in the cells of peripheral tissues. Three well-known isomers of RA, all-*trans* RA, 9-*cis* RA, and 13-*cis* RA activate the retinoid receptor retinoic acid receptor (RAR), whereas only 9-*cis* RA activates the other receptor, retinoid X receptor (RXR). A number of synthetic retinoids have been developed to carry out their pharmacological applications including cancer chemoprevention. Acyclic retinoid and *N*-(4-hydroxyphenyl) retinamide (4HPR) successfully prevented the development of hepatocellular carcinoma and breast cancer, respectively, in clinical trials (see review reference[27]).

retinoic acid (Fig. 1). Nuclear retinoid receptors are ligand-dependent transcription factors. After ligand binding, RXR form a homodimer as well as heterodimer with RAR, which interacts with the retinoid X response element or the RAR responsive element located in the promoter region of the target genes, thereby modulating gene expression. RXR also form a heterodimer with other nuclear receptors, such as peroxisome proliferator-activated receptor.[17] Among the retinoid receptors, RXRα is thought to be one of the most important receptors with respect to regulation of fundamental cell activities, including normal cell proliferation and metabolism, and act as the master regulator of nuclear receptors.[15,16]

In addition to the binding of specific ligands, recent studies have also revealed that phosphorylation processes are crucial for regulating RAR- and RXR-mediated transcriptional activity.[18,19] For instance, the phosphorylation of RXRα at its N-terminal domain plays a role in the activation of a subset of RA-responsive genes and in the antiproliferative effect of RA, indicating that RXRα 'positively' regulates the transactivation of target genes through phosphorylation.[20] In contrast, there are some reports that show the phosphorylation of RXRα to 'negatively' modulate the function of its heterodimeric binding partners. Indeed, MAPK-mediated phosphorylation of the omega loop of the RXRα ligand binding domain impairs the transcriptional activity of RXR–RAR[12,21] and RXR–vitamin D_3 receptor[22,23] heterodimers. These 'negative' effects of RXRα via its phosphorylation might be associated with certain types of human diseases, including malignant disorders.[12,24–26]

Hepatocellular carcinoma and RXRα phosphorylation

Because retinoids and their receptors play an essential role in normal cell proliferation and differentiation, abnormalities in the expression and function of these molecules are highly associated with the development of various human malignancies and therefore might be critical targets for cancer chemoprevention and chemotherapy.[27] HCC is no exception in this concern. In the rodent model, we found that retinol was locally deficient in the HCC but not in the adjacent normal liver tissues and this was associated with aberrant metabolism of retinol.[28] The expression of RXRα was also decreased not only in HCC and adenoma, but also in glutathione S-transferase placental form-positive foci, a precancerous lesion of HCC, suggesting that the repression of RXRα occurs even in an early stage of liver carcinogenesis.[29]

In addition, we have previously shown that hepatocarcinogenesis is accompanied by the accumulation of the phosphorylated (i.e. inactivated) form of RXRα.[30] Specifically, RXRα protein is anomalously phosphorylated at serine and threonine residues, and accumulated both in human HCC tissue as well as in HCC cell lines.[12] Phosphorylation at serine 260 of RXRα, a consensus site of MAPK, is closely linked to its retarded degradation, low transcriptional activity, and the promotion of cancer cell growth. In addition, the abrogation of phosphorylation by MAPK-specific inhibitors restored the degradation of RXRα in a ligand-dependent manner.[12,31] Furthermore, in a normal liver and in non-proliferating hepatocyte cultures, RXRα is

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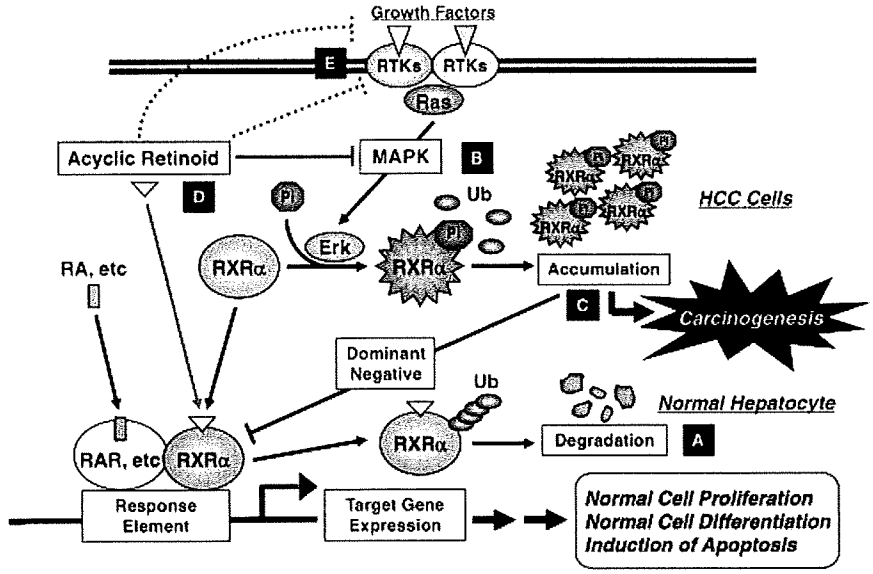

Fig. 2. Retinoid refractoriness due to phosphorylation of retinoid X receptor (RXR) α and its restoration by acyclic retinoid (ACR) in hepatocellular carcinoma (HCC) cells. In normal hepatocytes, when the ligand binds to and activates RXRα, the receptor becomes able to heterodimerize with other nuclear receptors, such as retinoic acid receptor (RAR), and then activates the expression of target genes, which may regulate normal cell proliferation and differentiation, by binding to the specific response elements. Thereafter, RXRα dissociates from the dimer, is ubiquitinated (Ub), and is degraded by the proteasome. The whole process from ligand binding to proteasomal breakdown of RXRα is estimated to take approximately 6 h (A).[14] In HCC cells, the Ras–mitogen-activated protein kinase (MAPK) pathway is highly activated and phosphorylates RXRα at serine residues, thus impairing dimer formation and the subsequent transactivation functions of the receptor. Furthermore, phosphorylated RXRα (pi-RXRα) escapes from ubiquitination and proteasomal degradation (B). Therefore, pi-RXRα accumulates and interferes with the physiological function of the remaining unphosphorylated RXRα, presumably, in a dominant-negative manner, thereby playing a critical role in the development of HCC (C). ACR is not only a ligand for RXRα but also suppresses the Ras–MAPK signaling pathway, inhibiting phosphorylation of RXRα, restoring the function of the receptor, and thus subsequently activating the transcriptional activity of the responsive element (D). ACR also directly or indirectly inhibits the ligand (i.e. specific growth factor)-dependent receptor tyrosine kinase (RTK) activities in cancer cells (E). These effects may contribute to the inhibition of extracellular signal-regulated kinase (Erk) and RXRα phosphorylation, thus causing inhibition of the growth of HCC cells.

unphosphorylated and highly ubiquitinated, thus rendering it sensitive to proteasome-mediated degradation. In contrast, p-RXRα is resistant to ubiquitination and proteasome-mediated degradation in both human HCC tissues and a human HCC cell line.[14] In addition, the phosphorylation of RXRα abolishes its ability to form heterodimers with RARβ and this might be associated with uncontrolled cell growth and resistance to retinoids.[13] These findings suggest that the accumulation of p-RXRα (i.e. non-functional RXRα) may interfere with the function of normal RXRα in a dominant-negative manner, thereby playing a critical role in the development of HCC (Fig. 2). Therefore, the inhibition of RXRα phosphorylation and the restoration of its physiological function as a master regulator of nuclear receptors, such as heterodimeric activity with other nuclear receptors, may be an effective and important strategy for inhibiting the growth of HCC cells.

Chemoprevention of HCC by ACR: Experimental study

Acyclic retinoid (NIK-333; Kowa Pharmaceutical Co., Tokyo, Japan) is a synthetic retinoid and has an agonistic activity for both RXR and RAR.[32,33] In experimental studies, this agent has demonstrated several beneficial effects on inhibition of HCC development. For instance, ACR inhibits chemically induced hepatocarcinogenesis in rats as well as spontaneously occurring hepatoma in mice.[28] ACR also inhibits growth and induces apoptosis in human HCC-derived cells and this might be associated with induction of apoptosis and cell differentiation in these cancer cells.[33–37] In a human HCC cell line, ACR causes an arrest of the cell cycle in G_0–G_1, increases cellular levels of the $p21^{CIP1}$ protein, and decreases levels of the cyclin D1 protein.[38] Moreover, recent studies indicated that ACR is not only the ligand for RXRα. Indeed, in human HCC-derived cells, ACR restores the function of RXRα by inactivating the Ras–Erk signaling system and thereby dephosphorylating RXRα, although 9-*cis* RA fails to suppress phosphorylation of the Erk protein and subsequent RXRα phosphorylation.[31] Both *in vivo*[39,40] and *in vitro*[41,42] studies have demonstrated that ACR reduces the development of HCC and prevents growth of cancer cells by inhibiting the activation of RTK, which play a critical role in stimulation of the Ras–MAPK signaling pathway.[43] Therefore, in addition to direct inhibition of the Ras–Erk signaling system[31] ACR may also cause dephosphorylation of the Erk and RXRα proteins by inactivating RTK, the upstream molecules of Ras, and thus restoring the function of RXRα. These findings suggest that ACR is a promising agent for the chemoprevention of HCC and that p-RXRα is a useful molecular target of ACR (Fig. 2).

Chemoprevention of HCC by ACR: Clinical study

The chemopreventive effects of ACR on recurrent and secondary HCC were confirmed in patients who received anticancer treatment for an initial HCC in a double-blind and placebo-controlled clinical study.[9–11] Oral administration of ACR (600 mg per day) for 12 months significantly reduced the incidence of post-therapeutic HCC recurrence in patients who underwent potentially curative treatments.[9] The survival rate was also significantly improved by the administration of this compound after a median follow up of 62 months.[10] Moreover, the preventive effects of ACR lasted up to 199 weeks after randomization (or 151 weeks after completion of ACR administration).[11] Therefore, administration of ACR for only 12 months confers a long-term effect over several years, without

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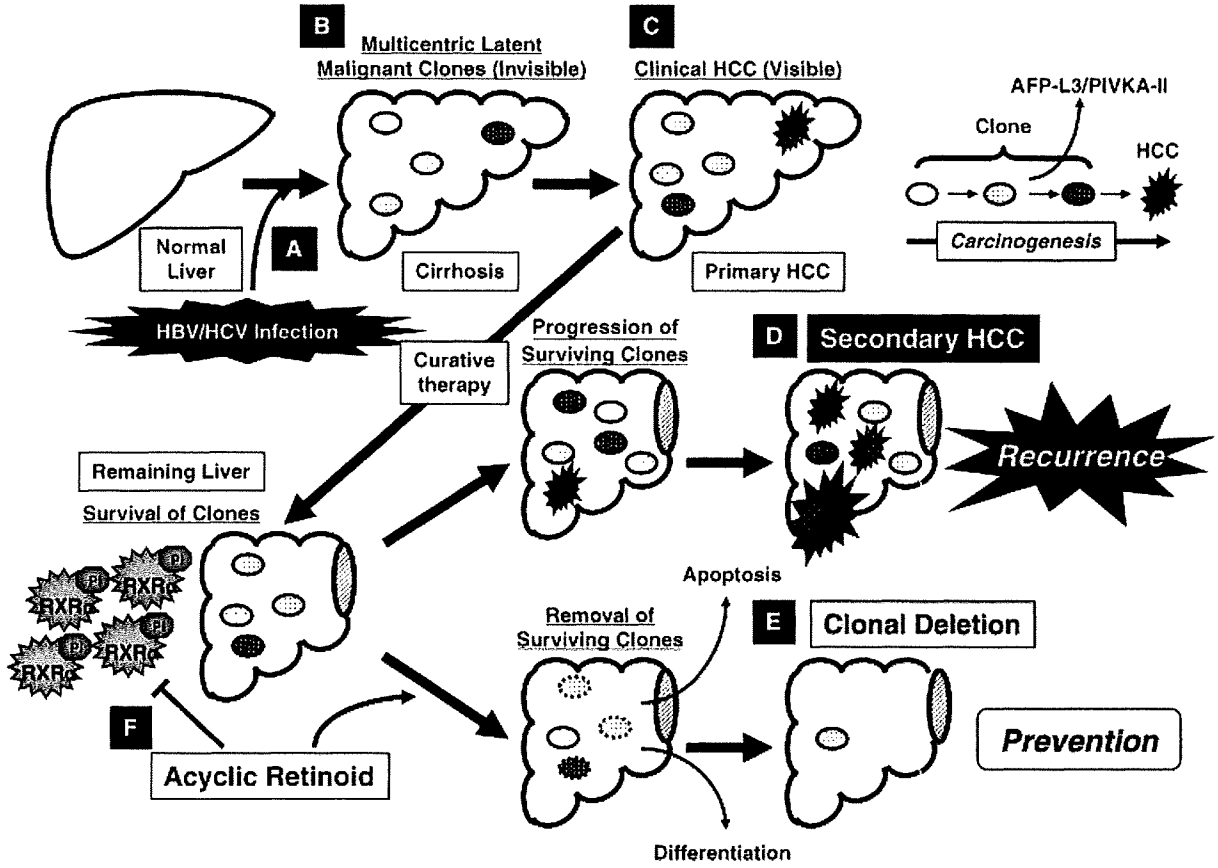

Fig. 3. The concept of 'clonal deletion'. Persistent inflammation caused by hepatitis B virus (HBV) or hepatitis C virus (HCV) infection transforms the liver into a 'precancerous field' (A). Therefore, the high incidence of hepatocellular carcinoma (HCC) as well as its recurrence in cirrhotic patients strongly suggests the presence of latent malignant clones that arise through multicentric carcinogenesis and are undetectable clinically by image analysis (invisible) (B). These multiple clones demonstrate different grades of malignancy (atypia) in the cirrhotic liver and, at some point, turn into clinical HCC (visible) (C). Even when primary HCC could be found in an early stage and surgical treatment might be expected to be curative, other clones still survive in the remaining liver and thus grow into secondary HCC again (D). Therefore, the eradication of such transformed clones, referred to as 'clonal deletion', may be one of the most effective strategies to prevent secondary HCC (E). Clinical experience suggests that acyclic retinoid (ACR), which inhibits phosphorylation (Pi) of retinoid X receptor (RXR) α (F), reduces the recurrence of HCC on the basis of this concept because this agent causes a decrease in the serum levels of lectin-reactive α-fetoprotein isoform 3 (AFP-L3) and protein induced by vitamin K absence or antagonist-II (PIVKA-II), which are produced by latent malignant clones, by eradicating or inhibiting these clones. Once such clones are deleted, the preventive effect on HCC lasts several years without any continuous administration of ACR. In fact, 1-year administration of ACR inhibited secondary HCC for the next 3 years.[11] Therefore, this agent can significantly improve the survival rate of such patients.

causing any severe adverse effects of retinoids, such as dry skin, cheilitis, or conjunctivitis. However, headache or hyperlipidemia was reported in one case ACR.[9] A phase II/III trial of this compound to test its effect in preventing second primary HCC is currently proceeding as a large-scale randomized controlled study. This trial is scheduled to be completed around 2009–10 and it is expected to yield positive results.

Concept of 'clonal deletion'

Pathologically, the high incidence of the development of second primary HCC may be explained by its characteristic mode of carcinogenesis, multicentric carcinogenesis, which is also expressed by the term 'field cancerization'.[44] Once a liver is exposed to continuous carcinogenic insults, such as hepatitis virus infection, the whole liver is regarded as a precancerous field that possesses multiple as well as independent premalignant or latent malignant clones. Therefore, the most effective strategy for HCC chemoprevention is the deletion of latent malignant clones (clonal deletion) as well as inhibition of the evolution of such clones (clonal inhibition) before they expand into a clinically detectable tumor. We therefore propose the benefits of 'clonal deletion' therapy for the prevention of HCC recurrence, which is defined as the removal of latent malignant (or premalignant) clones that are invisible by diagnostic imaging from the liver in a hypercarcinogenic state[11,45–47] (Fig. 3).

This concept has been clinically demonstrated and implemented in a clinical trial using ACR. Indeed, in that trial, ACR significantly reduced the serum levels of AFP-L3, which indicates the presence of latent (i.e. invisible) HCC cells in the remnant liver, after 12 months of administration.[45] The administration of ACR also caused a decrease in the serum levels of protein induced by PIVKA-II, which may also be produced by latent HCC cells.[11] These results strongly suggest that ACR deleted such malignant clones producing AFP-L3 or PIVKA-II before they expanded to clinically detectable tumors, thereby inhibiting second primary HCC. Therefore, once such latent clones are eradicated or inhibited, it may take several years for the next cancer clone to arise clinically.[11] Moreover, ACR also prevented the appearance of AFP-L3 in patients who had been negative at entry, although there was a significant increase in the incidence of AFP-L3-positive patients in the placebo group and these patients had a significantly higher risk of second primary HCC.[45] This is also one of the reasons for the long-term benefit of ACR after only a

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12-month treatment with this agent.[11] Therefore, we suggest that the concept of 'clonal deletion' seems more of a therapy rather than prevention and that ACR is a more affirmative agent to inhibit the development of HCC (Fig. 3).

Possibility of 'combination chemoprevention' with ACR

The combined use of two or more agents is often advantageous as it may permit lower clinical dosages, consequently decreasing the overall toxicity and thus providing the potential for synergistic effects between specific agents, including retinoids.[24,48] Therefore, the beneficial effects, such as synergism, between ACR and other agents to inhibit the growth of HCC cells have been examined. For instance, ACR acts synergistically with IFN in suppressing growth and inducing apoptosis in human HCC cell lines and this synergism was associated with the upregulation of type 1 IFN receptor expression by ACR.[49] The combination of ACR plus OSI-461, a potent derivative of sulindac sulfone, exerts synergistic inhibition of cell growth and induction of apoptosis in HepG2 human HCC cells.[50] In addition, the combination of ACR plus VK_2 also synergistically induced apoptosis and inhibited the growth of HCC cells without affecting the growth of normal human hepatocytes.[51] The findings that both IFN and VK_2 enhance the effects of ACR seem to be of interest because these agents are expected to reduce the development and recurrence rates of HCC.[52,53]

In the above study,[51] VK_2 inhibited phosphorylation of the RXRα protein through the inhibition of Ras activation and Erk phosphorylation, and the inhibition of RXRα phosphorylation by VK_2 was enhanced when the cells were cotreated with ACR. In addition, ACR and trastuzumab, the humanized anti-HER2 monoclonal antibody, cooperatively inhibit the activation of HER2 and its downstream signaling pathways, subsequently inhibiting the phosphorylation of RXRα and the growth of HCC cells.[54] Therefore, ACR may support the effect of the agents that target RTK, thus cooperatively or synergistically inhibiting HCC by targeting RXRα phosphorylation. These findings, together with those of previous reports,[39–41] suggest that the combination of ACR plus a specific agent that targets RTK and the Ras–MAPK signaling pathway may be able to inhibit the phosphorylation of RXRα and it may therefore be a promising strategy to prevent the development of HCC.

Conclusion

The very high incidence of secondary HCC is mainly responsible for the poor prognosis of patients with this malignancy. This fact suggests that, in turn, the establishment of a new effective strategy to prevent the recurrence of HCC will significantly improve the outcome of these patients and thus be an urgent task worldwide. One of the most practical approaches to prevent the development of HCC is 'clonal deletion' and clinical trials using ACR theoretically proved the significance of this therapy in HCC chemoprevention.[11,45] Experimental studies strongly suggest that phosphorylated RXRα is associated with HCC carcinogenesis and thus may be a critical target for HCC chemoprevention. ACR, which targets phosphorylated RXRα, may therefore play a critical role in preventing the development of HCC when it is used alone or combined with other agents.

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