efficacious and generally well tolerated in combination with PegIFN-α/RBV [9]. Clinical interest is increasingly focusing on exploring DAA-only regimens without PegIFN-α/RBV, whose potential benefits might include better tolerability and compliance, and a reduced duration of therapy. One recent PegIFN-α/RBV-sparing study of DCV plus ASV (AI447017) has examined the efficacy and safety of this combination for 24 weeks in a small cohort of ten GT1b null responders, in whom an SVR rate of 90% was observed [10]. The study was then expanded to include an additional cohort of null responders and a group of patients ineligible to receive, or intolerant of, PegIFN-α/RBV [11].

As with other antiviral agents, the efficacy of DCV and ASV can be compromised by the development of drug resistance. *In vitro* data suggest that DCV and ASV should provide additive or synergistic activity that enhances the genetic barrier to resistance [8]. Here we characterize virologic escape observed on DCV plus ASV treatment in the expanded AI447017 study [11]; its associations with baseline characteristics, including *IL28B* genotype and HCV polymorphisms; and an assessment of on- and off-treatment genotypic changes in NS5A and NS3 protease and their phenotypic consequences.

Patients and methods

Study design and patients

This was an open-label, phase 2a study (AI447017; clinicaltrials.gov identifier NCT01051414) evaluating the antiviral activity and safety of DCV plus ASV in 43 patients with HCV GT1 infection. Patients comprised (a) 21 PegIFN-α/RBV null responders (<2 $\log_{10}$ decline in plasma HCV RNA after 12 weeks) and (b) 22 patients who discontinued previous PegIFN-α/RBV within 12 weeks for intolerance or were considered medically poor candidates for PegIFN-α/RBV for reasons such as advanced age, complications of depression, anemia, myelosuppression, diabetes, or cardiovascular or renal dysfunction. Patients enrolled in four cohorts; two each of null responders and ineligible/intolerant patients. The initial sentinel cohort of null responders has been described previously [10]. All enrolled patients were infected with GT1b.

Patients received DCV 60 mg once daily with ASV 200 mg twice daily for 24 weeks, with a further 48 weeks of post-treatment follow-up. ASV dosing in the expanded study was reduced from the 600 mg twice-daily administration used in the sentinel cohort, following reports of hepatic enzyme elevations at this dose, in another clinical study [12].

The full study design, including inclusion/exclusion criteria, and safety/efficacy endpoints, is described elsewhere [11]. Briefly, eligible patients were men and women aged 20–75 years with HCV GT1 infection ⩾6 months and HCV RNA ⩾10^5 IU/ml. Patients were excluded if they had evidence of liver cirrhosis within 24 months of screening; a history of hepatocellular carcinoma, other chronic liver disease, variceal bleeding, hepatic encephalopathy, or ascites requiring diuretics or paracentesis; co-infection with hepatitis B virus or HIV; or other clinically significant medical conditions.

Laboratory assessments

Plasma samples for resistance testing were collected at baseline and study weeks 1, 2, 4, 6, 8, 10, 12, 16, 20, and 24 and post-treatment weeks 4, 8, 12, 24, 36, and 48. HCV RNA was determined at a central laboratory using the Roche COBAS® TaqMan® HCV Auto assay (Roche Diagnostics KK, Tokyo, Japan) with a lower limit of quantitation (LLOQ) of 15 IU/ml. HCV genotype and subtype and *IL28B* genotype (rs12979860 single-nucleotide polymorphism) were determined by polymerase chain reaction (PCR) amplification and sequencing.

Genotypic and phenotypic analysis of clinical samples

Testing was performed on all baseline samples and on samples indicative of slow virologic response at week 1 or virologic failure with HCV RNA levels ⩾1000 IU/ml. Virologic failure, for the purpose of the study, was defined as an HCV RNA level (a) ⩾LLOQ at week 4 (futility rule), (b) >1 $\log_{10}$ IU/ml above nadir or ⩾LLOQ after confirmed undetectable (virologic breakthrough), or (c) ⩾LLOQ at any follow-up visit after being undetectable at the end of treatment (relapse).

Population sequencing of PCR amplicons was performed using methods described elsewhere [13–15]. For clonal analysis, amplicons were cloned into the TOPO vector and transformed into TOP10 *Escherichia coli* using a commercially available kit (TOPO® TA-cloning® kit, Invitrogen, Carlsbad, CA) according to manufacturer's instructions, with ⩾20 individual colonies expanded and sequenced for each analysis.

Phenotypic analyses of resistance-associated substitutions were performed by employing *in vitro* HCV replicon systems according to previously published methodologies [15–17].

Results

Viral response to DCV and ASV

Overall, plasma HCV RNA was undetectable in 77% (33/43) of patients at 24 weeks post-treatment. SVR was higher among the null responders than in the PegIFN-α/RBV ineligible population; all viral breakthroughs (n = 3) and relapses (n = 4) occurred in the ineligible/intolerant subpopulation. Three patients discontinued the study without subsequent SVR or virologic failure (Tables 1 and 2) [11].

Null responders

Virologic response.

Rapid and similar decreases in plasma HCV RNA levels were observed among patients who initiated treatment with ASV 600 mg (Fig. 1A) or ASV 200 mg (Fig. 1B). Mean reduction in HCV RNA at week 1 was comparable for both groups (−4.4 *vs.* −4.3 $\log_{10}$ IU/ml, respectively). Of the patients still receiving treatment (P-6 discontinued at day 16 due to an AE), all but one patient (P-13) had HCV RNA <15 IU/ml at week 4 and 52% had undetectable HCV RNA at this time.

Baseline analysis. Baseline *IL28B* genotype and naturally occurring polymorphisms associated with ASV or DCV resistance (resistance-associated variants [RAVs]) are shown in Table 1. As anticipated for this prior null responder population, the majority (18/21) were non-CC *IL28B*. The NS5A polymorphism Y93H (24-fold DCV resistance [13]) was observed in three patients. Other polymorphisms conferring minimal (two- to three-fold) DCV resistance were detected in two patients (NS5A-L28M-R30Q and NS5A-L31M). Polymorphisms associated with minimal to low-level resistance to select NS3 protease inhibitors (one patient, NS3-T54S-Q80L; one patient, NS3-Q80L-V170I/M; two patients, NS3-Q80L) [4,5,18] were also observed.

Baseline polymorphisms and *IL28B* genotype did not appear to influence either the week 1 response or SVR rate (Fig. 2A). Five patients had RNA levels ⩾1000 IU/ml after 1 week, of whom one (P-21) had significantly slower initial HCV RNA declines when compared with mean reductions (standard deviation [SD]) in HCV RNA for null responders on the study (−3.4 *vs.* −4.35 ± 0.49 $\log_{10}$ IU/ml). This patient had a CC *IL28B* genotype and an NS5A polymorphism (Q54L; no fold-change in DCV resistance). The other four patients had polymorphisms that have been associated with DCV and NS3 protease inhibitor low-level resistance [13,19]—specifically NS5A-Q54H/Q-Q62Q/E-Y93H/Y with NS3-T54S-Q80L (P-1, no fold-change to DCV/ASV), NS3-Q80L-V170I/M (P-2, no fold-change to ASV), NS5A-R30Q

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Table 1. Baseline viral and host characteristics among genotype-1b null responders and their virologic outcome.

Patient	*IL28B* GT	HCV RNA, $\log_{10}$ IU/ml	NS5A polymorphism(s)[a]	NS3 polymorphism(s)[a]	Virologic outcome
P-1	CT	7.2	Q54H/Q-Q62Q/E-Y93H/Y	T54S-Q80L	SVR
P-2	CT	7.0		Q80L-V170I/M	SVR
P-3	CT	7.4	Q54H		SVR
P-4	CT	6.7	R30Q		SVR
P-5	CT	7.0	L31L/M-P58P/S		SVR
P-6	CC	5.3	P58P/T-Q62E		D/C at Wk2 due to SAE[b]
P-7	CC	7.2		S122S/G	SVR
P-8	CT	7.0	Q54H	Q80L	SVR
P-9	CT	7.1	Q54H-Y93H/Y	S122N	SVR
P-10	CT	6.4	L28M-R30Q		SVR
P-11	CT	6.8			D/C at Wk12 due to AE; SVR
P-12	CT	6.4	Q54H-P58S-Q62E		SVR
P-13	CT	7.4	Q54H		D/C at Wk6; PDR not achieved[c]
P-14	CT	6.5			SVR
P-15	CT	6.3	R30Q/R-Q62Q/R		SVR
P-16	CT	6.6	Q54H		SVR
P-17	CT	6.6	Q54H-Q62E		SVR
P-18	CT	6.9	Q54Y	Q80L	SVR
P-19	CT	6.6	Q54H-Y93H	N77A	SVR
P-20	CT	7.0	R30Q	S122G	SVR
P-21	CC	6.6	Q54L		SVR

[a]All NS3 and NS5A amino acids were examined with focus on polymorphisms at positions known to be associated with resistance to NS3 protease inhibitors (36, 43, 54, 55, 77, 78, 79, 80, 122, 123, 138, 155, 156, 158, 168, 170, 175) and NS5A inhibitors (21, 23, 24, 28, 30, 31, 32, 54, 58, 62, 92, 93). When a mixture of substitutions is indicated, the most predominant is identified first.
[b]HCV RNA undetectable at post-treatment week 24.
[c]PegIFN-α/RBV added; HCV RNA undetectable at post-treatment week 24 following 52 weeks of therapy.
AE, adverse event; D/C, discontinued; GT, genotype; HCV, hepatitis C virus; PDR, protocol-defined response; SAE, serious adverse event; SVR, sustained virologic response; Wk, week.

with NS3-S122G (P-20, no fold-change to either DCV/ASV), or NS5A-Q54H (P-13, no fold-change to DCV). P-13 was the only patient with HCV RNA <15 IU/ml (target detectable) at week 6 and was, therefore, considered a treatment failure. Treatment-emergent resistance at week 1 in the five patients could not be determined because of PCR failure. A comparison of initial virologic response *vs.* dose and polymorphisms associated with resistance revealed no differences. Among null responders who received ASV 600 mg, mean HCV RNA declines at week 1 for those with *vs.* without RAVs were −4.6 *vs.* −4.3 $\log_{10}$ IU/ml, which were similar to the week 1 declines among those who received ASV 200 mg (−4.5 $\log_{10}$ IU/ml with RAVs [one patient] *vs.* −4.3 $\log_{10}$).

Baseline HCV RNA levels did not have an impact on response to treatment; patients with high baseline viral load still experienced rapid and robust responses to therapy (Fig. 1; Table 1).

Ineligible/intolerant patients

Virologic response.

Virologic response at week 4 was greater in PegIFN-α/RBV ineligible patients than in null responders. Undetectable HCV RNA at week 4 was observed in 86% of the ineligible group *vs.* 52% of null responders. However, by week 12, undetectable HCV RNA was similar in both groups. Early HCV RNA declines appeared unaffected by *IL28B* genotype, the presence of baseline polymorphisms associated with resistance, or virologic outcome (Fig. 3). Adherence to therapy, assessed through pill counts, was found to be high in six of the seven patients experiencing virologic failure. However, DCV/ASV exposures were high in the one non-compliant patient (P-31) who subsequently experienced relapse.

Baseline analysis.

Baseline *IL28B* genotype, polymorphisms associated with resistance, and virologic outcome are shown in Table 2 and Fig. 2B. Three patients presented with DCV resistance at baseline: one (P-25) with an NS5A-L31M-Y93H combination (7105-fold DCV resistance [13]) and two with an NS5A-Q54Y-Y93H (58-fold resistance). All three subsequently experienced viral breakthrough at week 10 or 16.

Other patients had baseline polymorphisms conferring minimal or low-level resistance to DCV and/or protease inhibitors; NS5A-Y93H (n = 4), NS5A-L28M-R30L (n = 1), NS3-T54S (n = 1), and NS3-Q80L (n = 5). Variable responses were observed among these patients (Fig. 2B); the majority responded, but two patients with baseline NS5A-Y93H experienced post-treatment relapse. One patient (P-24) with baseline NS5A-L28M-R30L-Q54H-A92T and NS3-Q80L-S122G had a slower response to treatment at week 1 when compared with mean HCV RNA reductions (SD) for ineligible/intolerant patients on the study (−3.4 *vs.* −4.74 [0.58] $\log_{10}$ IU/ml), but subsequently achieved SVR with only 16 weeks of treatment. Neither NS3-Q80L-S122G nor NS5A-L28M-R30L-Q54H-A92T conferred resistance to ASV or DCV, respectively.

Baseline viral load did not appear to affect response; mean HCV RNA levels (SD) were 6.4 (0.7) $\log_{10}$ IU/ml among patients

Table 2. Baseline viral and host characteristics among genotype-1b ineligible/intolerant patients and their virologic outcome.

Patient	*IL28B* GT	HCV RNA, $\log_{10}$ IU/ml	NS5A polymorphism(s)[a]	NS3 polymorphism(s)[a]	Virologic outcome
P-22	CC	7.1			SVR
P-23	CC	6.9	A92T	Q80L-S122G/S	SVR
P-24	CC	6.6	L28M-R30L-Q54H-A92T	Q80L-S122S/G	D/C at Wk12 due to AE; SVR
P-25	CT	6.8	L31M/L-Y93H/Y		VBT (Wk16)
P-26	CC	5.3			SVR
P-27	CC	6.9	Q54H-Y93H/Y	T54S	SVR
P-28	CC	6.8	Y93H/Y	Q80L	SVR
P-29	CT	6.7	Q54Y-Y93H/Y	Q80L	VBT (Wk16)
P-30	CT	6.7	Q54H		SVR
P-31	CC	6.6	P58S/P-Y93Y/H	S122G	Relapse (FU Wk12)
P-32	CT	6.7	P58L	S122G	Relapse (FU Wk4)
P-33	CT	5.2	Q54H-Q62P/S		D/C at Wk12 due to patient request; SVR
P-34	CC	6.6		Q80L	SVR
P-35	CC	6.4	Q54H-Q62E/A-A92T		SVR
P-36	CC	7.1		S122S/C	Relapse (FU Wk4)
P-37	CC	6.6	Y93H		Relapse (FU Wk4)
P-38	CC	7.5		S122T	SVR
P-39	CC	5.1	R30Q/R		SVR
P-40	CC	6.8	Q54H-A92A/T	Q80L	D/C at Wk8[b]
P-41	CC	6.0		S122G	SVR
P-42	CC	6.5	A92T		SVR
P-43	CT	7.0	Q54Y-Y93H	S122G	VBT (Wk10)

[a]All NS3 and NS5A amino acids were examined with focus on polymorphisms at positions known to be associated with resistance to NS3 protease inhibitors (36, 43, 54, 55, 77, 78, 79, 80, 122, 123, 138, 155, 156, 158, 168, 170, 175) and NS5A inhibitors (21, 23, 24, 28, 30, 31, 32, 54, 58, 62, 92, 93). When a mixture of substitutions is indicated, the most predominant is identified first.
[b]Treatment discontinued at patient request; subsequently lost to follow-up.
AE, adverse event; D/C, discontinued; FU, follow-up; GT, genotype; HCV, hepatitis C virus; SVR, sustained virologic response; VBT, viral breakthrough; Wk, week.

achieving SVR compared with 6.8 (0.3) $\log_{10}$ IU/ml among patients experiencing virologic failure. However, four of six patients with the *IL28B* CT allele subsequently failed treatment (three breakthroughs, one relapse) *vs.* only three of 16 patients with *IL28B* CC (all relapsed).

Genotypic analysis of patients with viral breakthrough. Treatment-emergent RAVs were assessed through post-treatment week 48 in the three patients with virologic breakthrough (Table 3).

Patient P-25: This patient had an *IL28B* CT genotype with a baseline HCV RNA level of 6.8 $\log_{10}$ IU/ml and a linked baseline NS5A-L31M-Y93H/Y polymorphism. Despite undetectable HCV RNA by week 4 (Fig. 4A), viral breakthrough occurred at week 16, associated with high-level resistance to both DCV (NS5A-L31M-P58A-Y93H; 65,000-fold) and ASV (D168A; ~120-fold in GT1b). Other minor variants detected at baseline by clonal analysis (NS5A-Q62R, -A92T) were not present at breakthrough. NS5A variants present at the end of therapy persisted through follow-up week 48, and, although P58A had largely changed to P58G (73% of 33 clones, Fig. 5A) by week 36, a similar ratio of P58G to A was detected at follow-up week 48. By contrast, NS3-D168A had mostly been replaced by wild type at week 48 (83% of 64 clones).

Patient P-29: This patient had an *IL28B* CT genotype, with a baseline HCV RNA level of 6.7 $\log_{10}$ IU/ml and a pre-existing linked NS5A-Q54Y-Y93H/Y and NS3-Q80L (Fig. 5B). Undetectable HCV RNA by week 3 was followed by viral breakthrough at week 16 (Fig. 4A) associated with NS5A-L31M-Q54Y-Y93H (6467-fold DCV resistance) and NS3-Q80L-D168V (~280-fold ASV resistance). These RAVs remained stable through 48 weeks post-treatment.

Patient P-43: This patient had an *IL28B* CT genotype with a baseline HCV RNA level of 7.0 $\log_{10}$ IU/ml, and a pre-existing NS5A-Q54Y-Y93H variant (Fig. 5C). HCV RNA was undetectable at week 2, and breakthrough occurred at week 10 (Fig. 4A), which was associated with a linked NS5A-L31M-Q54Y-Y93H variant (Fig. 5C; 6467-fold DCV resistance) and an NS3-D168V variant (~270-fold ASV resistance). Again, NS5A variants remained stable through week 48 post-treatment, while NS3-D168V was replaced by wild type (100% of 60 clones).

For the three patients experiencing viral breakthrough, DCV and ASV trough exposures were less than drug levels required to achieve a 90% effective concentration (EC_{90}) value against emergent RAVs (Table 3).

Genotypic analysis of patients experiencing post-treatment relapse. Four ineligible patients, with undetectable HCV RNA at the end of treatment, experienced relapse (Fig. 4B). Resistance polymorphisms through week 48 off-treatment are shown in Table 3. Baseline polymorphisms associated with resistance were not detected in two patients (P-32 and P-36), but both displayed post-relapse resistance by follow-up weeks 8 and 4, respectively. Patient P-32 relapsed with NS5A-L31M-P58L-Y93H (8300-fold DCV resistance) and NS3-D168V (270-fold ASV resistance).

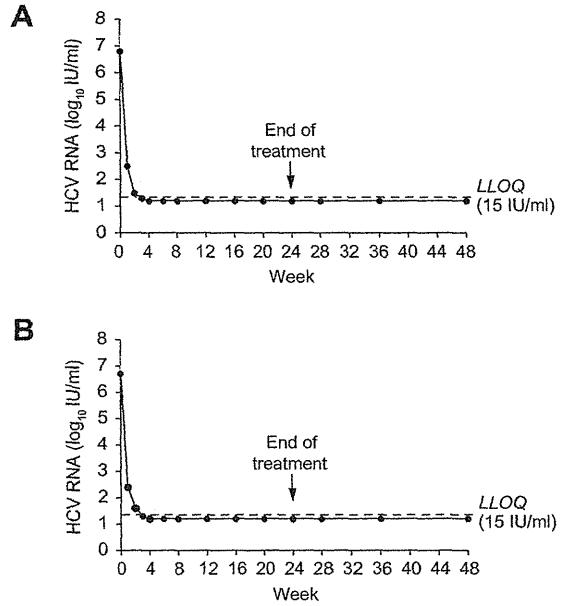

Fig. 1. HCV RNA levels among genotype-1b null responders. Treatment was initiated with (A) asunaprevir 600 mg BID or (B) asunaprevir 200 mg BID, in combination with daclatasvir 60 mg QD. Individual patient HCV RNA levels are shown in grey. Mean HCV RNA levels are shown in black. BID, twice daily; HCV, hepatitis C virus; LLOQ, lower limit of quantitation; QD, once daily.

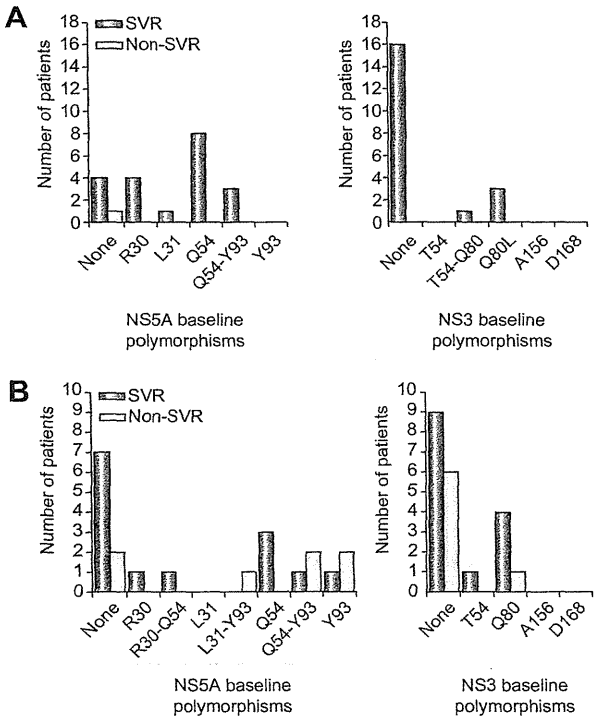

Fig. 2. Impact of baseline polymorphisms associated with resistance on virologic outcome among genotype-1b (A) null responders or (B) ineligible/intolerant patients. The ineligible/intolerant analysis excludes one patient (P-40) who discontinued therapy and was subsequently lost to follow-up. SVR, sustained virologic response.

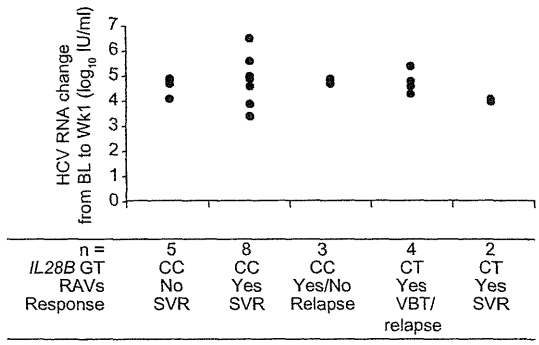

Fig. 3. Early (week 1) declines in HCV RNA were similar among PegIFN-α/RBV ineligible or intolerant patients with and without baseline polymorphisms associated with resistance, virologic failure, and *IL28B* CT genotype. BL, baseline; GT, genotype; HCV, hepatitis C virus; RAV, resistance-associated variant; SVR, sustained virologic response; VBT, viral breakthrough.

Patient P-36 relapsed with an NS5A-L31V/M-Y93H genotype (L31V-Y93H: 14,789-fold DCV resistance *vs.* L31M-Y93H: 7105-fold) [13] and NS3-D168V. The remaining two patients had detectable NS5A-Y93H at baseline (24-fold DCV resistance) and additional substitutions at NS5A-L31 and NS3-D168 were detected after relapse. Patient P-31 displayed NS5A-L31M-Y93H (7105-fold DCV resistance) [13] and NS3-D168A (~120-fold ASV resistance); patient P-37 relapsed with the same NS5A-L31V/M-Y93H and NS3-D168V, as described for patient P-36.

Baseline HCV RNA and *IL28B* genotype did not appear to influence relapse; three of four relapse patients were *IL28B* CC genotype, and baseline HCV RNA was not appreciably higher than for those with SVR (mean HCV RNA [SD]: 6.8 [0.4] *vs.* 6.4 [0.7] log_{10} IU/ml, respectively).

Changes in the DCV resistance pattern present at relapse through follow-up week 48 were seen in three of four relapsers, with Y93H changing to wild type (100% of 68 clones) in patient P-32. Clonal analysis of the baseline sequence revealed the presence of Y93H as a minor species (~2%; 1/61 clones). Genotypic changes resulting in a lower level of phenotypic resistance (L31V-Y93H to L31M-Y93H) were detected in patients P-36 and P-37. NS3 substitutions observed at relapse were not detectable by population sequencing by follow-up week 36. The D168V substitution detected in patient P-37 was replaced by D168E (78-fold ASV resistance [19]) at follow-up weeks 36 and 48. As with the patients who experienced virologic breakthrough, ASV and DCV trough values in the three drug-compliant patients who relapsed were less than the observed EC_{90} values for the respective RAVs.

Discussion

This study assessed resistance and virologic failure in a difficult-to-treat population of null responders and PegIFN-α/RBV ineligible/intolerant patients treated with the dual oral combination of DCV and ASV. Overall, 77% achieved an SVR [11], with all viral breakthroughs and post-treatment relapses occurring in the ineligible/intolerant subpopulation. It is possible that pharmacokinetics may have played a role in these failures, since patients experiencing failure had DCV and/or ASV trough values below median or documented non-compliance [11]. However, since

Table 3. Emergence of resistance-associated variants among genotype-1b ineligible/intolerant patients experiencing viral breakthrough or relapse.

Patient	Time point	DCV/ASV C_{trough} range, nM	NS5A RAVs				DCV EC_{90}, nM	NS3 RAVs			ASV EC_{90}, nM
			L31	Q54	P58	Y93		Q80	S122	D168	
VBT patients											
P-25	BL		M/L	-	-	H/Y	<137	-	-	-	
	Wk16 (VBT)		M	-	A	H	>1000	-	-	A	540
	Wk20		V	-	A	H		-	-	A	
	Wk24	190-261/25-41	M	-	A	H		-	-	A	
	FU Wk4		M	-	A	H		-	-	A	
	FU Wk36		M	-	G	H	>5000	-	-	D/A	
	FU Wk48		M	-	G/A	H		-	-	-	
P-29	BL		-	Y	-	H	0.04	L	-	-	1.6
	Wk16 (VBT)		ND	ND	ND	ND	ND	ND	ND	ND	ND
	Wk20	116-198/18-33	M/V	Y	-	H	750	L	-	V	55
	FU Wk4		M	Y	-	H		L	-	V	
	FU Wk36		M	Y	-	H		L	-	V	
	FU Wk48		M	Y	-	H		L	-	V	
P-43	BL		-	Y	-	H	0.49	-	G	-	2.8
	Wk10 (VBT)		M	Y	-	H	435	-	G	V	279
	FU Wk4	243/69	M	Y	-	H		-	G	V	
	FU Wk36		M	Y	-	H		-	G	-	
	FU Wk48		M	Y	-	H		-	G	-	
Relapse patients											
P-31	BL	573-620/ 153-327	-	-	S/P	Y/H	0.02	-	G	-	
	FU Wk16		ND					-	-	A	
	FU Wk24		M	-	-	H	351	-	G	-	
	FU Wk36		-	-	-	-		-	-	-	
	FU Wk48		-	-	-	-		-	-	-	
P-32	BL	151-306/19-42	-	-	L	-	0.004	-	G	-	
	FU Wk8		M	-	L	H		-	G	V/D	
	FU Wk12		M	-	L	H	543	-	G	-	
	FU Wk36		M	-	L	-	1.5	-	G	-	
	FU Wk48		M	-	L	-		-	G	-	
P-36	BL	138/26	-	-	-	-		-	-	-	
	FU Wk8		V/M	-	-	H		-	-	V	1190
	FU Wk12		V	-	-	H	349	-	-	-	
	FU Wk24		M/V	-	-	H		-	-	V/D	
	FU Wk36		M	-	-	H	137	-	-	-	
	FU Wk48		M	-	-	H		-	-	-	
P-37	BL	75-134/40-93	-	-	-	H	0.49	-	-	-	
	FU Wk8		V	-	-	H		-	-	V	
	FU Wk12		V/I	-	-	H		-	-	V	
	FU Wk24		M	-	-	H		-	-	V	
	FU Wk36		M	-	-	H		-	-	E/D	
	FU Wk48		M	-	-	H		-	-	-	

When a mixture of substitutions is indicated, the most predominant is written first. ASV-resistant variants conferred no cross-resistance to DCV and *vice versa* in a replicon assay. Dashes indicate consensus with control sequence GT1b (Con1).

ASV, asunaprevir; BL, baseline; DCV, daclatasvir; EC_{90}, 90% effective concentration; FU, follow-up; ND, not determined as multiple amplifications failed; RAV, resistance-associated variant; VBT, viral breakthrough; Wk, week.

most patients with troughs below the median achieved SVR, the influence of drug exposure is hard to assess.

NS5A-Y93H was identified as the predominant polymorphism at baseline in all three patients with viral breakthrough and in two of the four patients with relapse. However, three null responders and two ineligible/intolerant patients also had a pre-existing NS5A-Y93H polymorphism and all achieved SVR, making the significance of Y93H alone, for response in the broader patient population, difficult to assess. Furthermore, where Y93H polymorphisms existed at baseline, their effects on

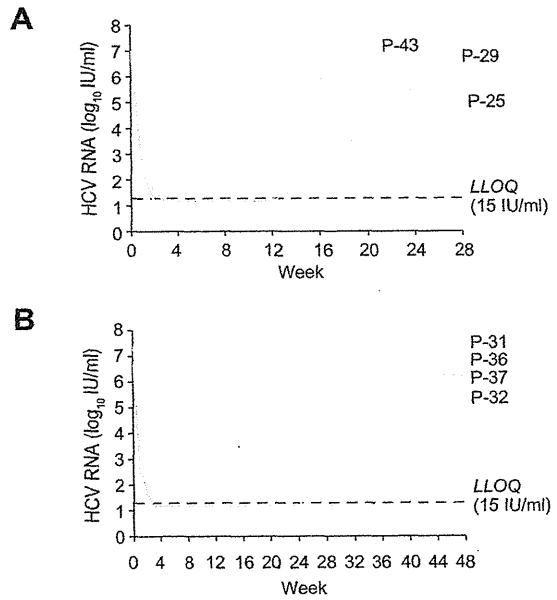

Fig. 4. HCV RNA levels on-treatment and during post-treatment follow-up for genotype-1b ineligible/intolerant patients experiencing (A) viral breakthrough or (B) relapse. Solid lines indicate on-treatment period. Dashed lines indicate post-treatment follow-up. HCV, hepatitis C virus; LLOQ, lower limit of quantitation.

DCV inhibition were minimal (Y93H EC_{50} = 49 pM [6]) compared with C_{trough} values that ranged from 75 to 620 nM. The global prevalence of NS5A-Y93H is approximately 4%, based on data from the Los Alamos database [20] and unpublished data from nine DCV studies, and is approximately 11% in other recent Japanese DCV studies [21], which is considerably lower than the 23% (10/43) prevalence observed in this study. Further analysis of DCV study data indicates that Y93H pre-exists at higher levels in patients infected with GT1b (10%) than GT1a (1%); however, the link with *IL28B* is not so clear given that most failures to date with DCV have been observed in GT1a patients with no baseline Y93H. Other polymorphisms observed at a higher frequency among this GT1b population included NS3-Q80L (~19%, 8/43) *vs.* Q80K, which has been observed more frequently in GT1a populations [18,19].

Baseline HCV RNA did not appear to influence virologic response in either population, and response was too rapid to allow successful genomic sequencing after 1 week of treatment. ASV dose (600 mg or 200 mg twice daily) did not impact the initial decline in HCV RNA in null responders, and the *IL28B* CT allele, present in 86% (18/21) of null responders, did not prevent patients from achieving a very high (90%) SVR. By contrast, although only 27% (6/22) of ineligible/intolerant patients were *IL28B* CT, this genotype was present in all three viral breakthroughs and one of four relapses. While *IL28B* genotype is known to influence response to PegIFN-α/RBV, its apparent impact on virologic suppression in alfa-sparing regimens is unexpected. However, given the small number of patients, any such correlation will require evaluation in a larger dataset.

The emergent RAVs at viral breakthrough or relapse (signature NS5A-L31 and -Y93 substitutions for DCV and NS3-D168 substitutions for ASV) were similar to observations from other

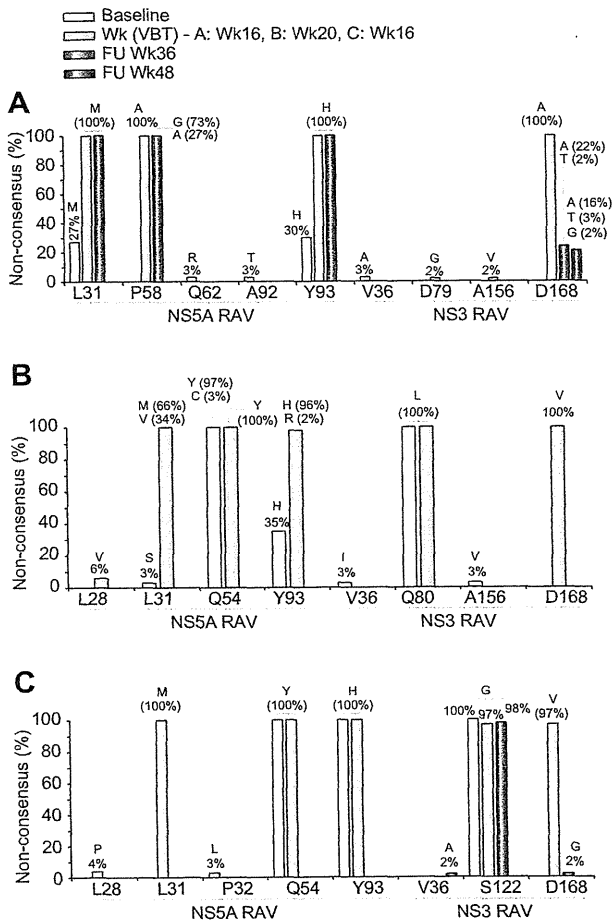

Fig. 5. Clonal analysis of NS3 protease and NS5A resistance-associated variants in patients experiencing virologic breakthrough. (A) Patient P-25. NS5A RAV: baseline 30 clones; Wk16 39 clones; FU Wk36 33 clones; FU Wk48 not performed (no change from FU Wk36 by population sequencing). NS3 RAV: baseline 32 clones; Wk16 41 clones; FU Wk36 56 clones; FU Wk48 63 clones. (B) Patient P-29. NS5A RAV: baseline 37 clones; Wk20 50 clones; FU Wk36/48 analyses not performed (no change from VBT by population sequencing). NS3 RAV: baseline 34 clones; Wk20 47 clones; FU Wk36/48 analyses not performed (no change from VBT by population sequencing). (C) Patient P-43. NS5A RAV: baseline 32 clones; Wk10 47 clones; FU Wk36/48 analyses not performed (no change from VBT by population sequencing). NS3 RAV: baseline 31 clones; Wk10 32 clones; FU Wk36 103 clones; FU Wk48 60 clones. FU, follow-up; VBT, viral breakthrough; RAV, resistance-associated variant.

clinical studies of DCV, and from *in vitro* GT1b replicon resistance studies with ASV [19], although this study represents the first demonstration of emergent clinical ASV resistance. It is possible that signature resistance variants to both DCV and ASV pre-existed as minor species, and subsequently enriched by selective pressure, as predicted by viral kinetic modeling [22]. Although a combination of these NS3 and NS5A variants was not detected by clonal sequencing at baseline, their low-level pre-existence cannot be ruled out. However, assessment of minor NS3 plus NS5A variants from the same RNA sequence is currently not feasible using available deep-sequencing technologies. Nevertheless, additional studies to assess the presence and dynamics of minority baseline variants under drug selection are indicated.

Interestingly, ASV-resistant NS3-D168 substitutions generally decayed during the off-drug follow-up period, implying a lack of replicative fitness relative to wild type, in the absence of selective drug pressure. Indeed, a reduction in replicative fitness has been observed for D168 variants in replicons [19]. Neither of the secondary variants associated with D168V in this study (Q80L or S122G) had an impact on fitness *in vitro* (replication capacity similar or higher than that observed for parental GT1b [Con1] replicon), with both double variants possessing replicative capacities similar to D168V alone [19]. However, clonal analysis indicated that ASV-resistant variants were still detectable in some post-treatment samples as minority species, although not detectable by population sequencing. Deeper sequencing techniques will be required to fully establish the dynamics of decay and whether ASV-resistant strains remain enriched for long periods relative to baseline. Since the re-treatment of patients with prior NS3 protease inhibitor failure has only been assessed in small studies [23], it is not clear whether these NS3 RAVs will form a stable minority capable of rapid overgrowth on re-treatment. By contrast, NS5A variants associated with DCV resistance were observed to be linked and relatively stable through at least 48 weeks post-treatment, although change of DCV-resistance substitutions was noted in four of seven patient samples. As described above, the prevalence of the NS5A variant Y93H, which confers low level resistance to DCV, is approximately 10% in the general HCV GT1b population. Linked NS5A RAVs conferring high level resistance to DCV are less prevalent (<1%). While NS3 RAVs (substitutions at positions V36, T54, R155, or D168) associated with first-generation protease inhibitors have been reported to be present at $\leqslant$2.7% by population sequencing [5,24], emergent NS3 RAVs have been shown to persist for up to 4 years in long-term follow-up studies [25]. Therefore, longer-term studies are indicated to assess what, if any, replicative impairment is conferred by these linked NS5A changes and how long these potentially transmissible drug-resistant strains persist without DCV selection pressure.

In conclusion, high response rates were achieved in this small Japanese study comprising GT1b null responders and PegIFN-α/RBV ineligible/intolerant patients with limited treatment options. Among patients experiencing virologic failure, ASV- and DCV-resistant substitutions emerged together at the time of failure, which were similar to those reported previously. An analysis of persistence demonstrated that DCV-resistant substitutions appeared to have greater fitness over the duration of the study. A loose association with a baseline NS5A polymorphism on virologic outcome was observed; however, further data from larger studies are required. Consequently, a greater understanding of the role and dynamics of pre-existing, emergent, and persistent resistance variants to DCV and ASV will be sought from the planned phase 3 global studies of this combination.

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Conflict of interest

K Chayama has received research grants and consulting fees from Bristol-Myers Squibb, Dainippon Sumitomo Pharma, Mitsubishi Tanabe Pharma, Daiichi Sankyo, Toray Industries, Otsuka Pharmaceutical Company, and GlaxoSmithKline KK. Hiroki Ishikawa, Hideaki Watanabe, Wenhua Hu, Dennis Hernandez, Fei Yu, and Fiona McPhee are employees of Bristol-Myers Squibb. All other authors have no conflicts to report.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Inhibition of hepatocellular carcinoma by PegIFNα-2a in patients with chronic hepatitis C: a nationwide multicenter cooperative study

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Abstract
Background We investigated whether the administration of maintenance doses of interferon prevented hepatocellular carcinoma (HCC) in patients with chronic hepatitis C.
Methods Study 1: A multicenter, retrospective, cooperative study was carried out to determine whether long-term administration of low-dose peginterferon alpha-2a (PegIFNα-2a) prevented HCC development in patients with chronic hepatitis C. In total, 594 chronic hepatitis C patients without a history of HCC were enrolled and treated with 90 μg PegIFNα-2a administered weekly or bi-weekly for at least 1 year. Study 2: HCC developed in 16 of 99 additional patients without PegIFNα-2a treatment during 3.8 years of observation. A propensity-matched control study was then carried out to compare the incidence of

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HCC between the 59 patients who received low-dose PegIFNα-2a (PegIFNα-2a group) and 59 patients who did not receive PegIFNα-2a treatment (control group), matched for sex, age, platelet count, and total bilirubin levels.

Results Study 1: HCC developed in 49 patients. The risk of HCC was lower in patients with undetectable hepatitis C virus RNA, ≤40 IU/L alanine aminotransferase (ALT), or ≤10 ng/L alpha-fetoprotein (AFP) 24 weeks after the start of therapy. Study 2: The incidence of HCC was significantly lower in the PegIFNα-2a group than in the control group.

Conclusions Low-dose and long-term maintenance administration of PegIFNα-2a decreased the incidence of HCC in patients with normalized ALT and AFP levels at 24 weeks compared with patients without normal ALT and AFP levels.

Keywords Chronic hepatitis C · Hepatocellular carcinoma · Peginterferon

Introduction

Hepatocellular carcinoma (HCC), the sixth most common cancer worldwide, often develops because of long-term hepatitis B or C virus infection [1, 2]. In particular, chronic hepatitis C and hepatic cirrhosis increase the risk of HCC; the annual incidence of tumor development in such patients may be as high as 2–4 % [3–5]. The incidence of HCC decreases in patients who achieve a sustained virological response (SVR) to interferon (IFN) treatment, although the incidence remains high in non-SVR patients [6–9]. A detailed analysis of HCC development revealed that chronic hepatitis C patients aged 65 years or more, especially those with advanced fibrosis of the liver, were at an increased risk of developing HCC [10]. For patients 65 years or older with advanced liver fibrosis, the dose of ribavirin is often reduced or the agent is discontinued, resulting in lower SVR rates in those with discontinuation of ribavirin. Establishing an effective treatment strategy for preventing the development of HCC is important for these high-risk patients.

Factors related to the development of HCC have been analyzed in patients who did not achieve an SVR even after IFN treatment; advanced fibrosis of the liver and high levels of serum alanine aminotransferase (ALT), and alpha-fetoprotein (AFP) are risk factors for HCC development [11, 12]. A randomized controlled trial was conducted in Western countries to determine whether combined peginterferon and ribavirin treatment with weekly administration of 90 μg peginterferon alpha-2a (PegIFNα-2a) could prevent HCC in non-responders. A 3.5-year follow up showed that administration of a maintenance dose of PegIFNα-2a did not reduce tumor incidence in these patients [13]. However, after 8.5 years of observation, the incidence of HCC was decreased among those in the PegIFNα-2a group with cirrhosis [14]. Meanwhile, Bruix et al. [15] reported that maintenance therapy with PegIFNα-2b did not prevent HCC in chronic hepatitis C patients with cirrhosis. In Japan, long-term low-dose administration of natural IFN has been reported to decrease the incidence of HCC [16]. In light of these conflicting results, investigations should be carried out in a large number of patients with chronic hepatitis C to resolve the question of whether IFN treatment prevents the development of HCC.

We carried out a multicenter retrospective cooperative study of patients with chronic hepatitis C to determine whether those treated with 90 μg PegIFNα-2a without ribavirin had a reduced incidence of HCC compared with those not treated with IFN.

Patients and methods

Study 1: analysis of risk factors for HCC in patients treated with long-term low-dose-PegIFNα-2a

In total, at 21 hepatitis centers throughout Japan, 743 patients with hepatitis C who had received 90 μg of PegIFNα-2a therapy weekly or bi-weekly for 1 year or more without having received the full dose (180 μg) since December 2003 were examined retrospectively for the development of HCC. The end of enrollment in this study was the end of December 2008 and the end of follow up was the end of December 2010. Patients with a history of HCC before the start of therapy and those with a therapy period of less than 48 weeks were excluded, leaving 594 patients who had undergone long-term administration of PegIFNα-2a for analysis. At the 21 centers involved in this

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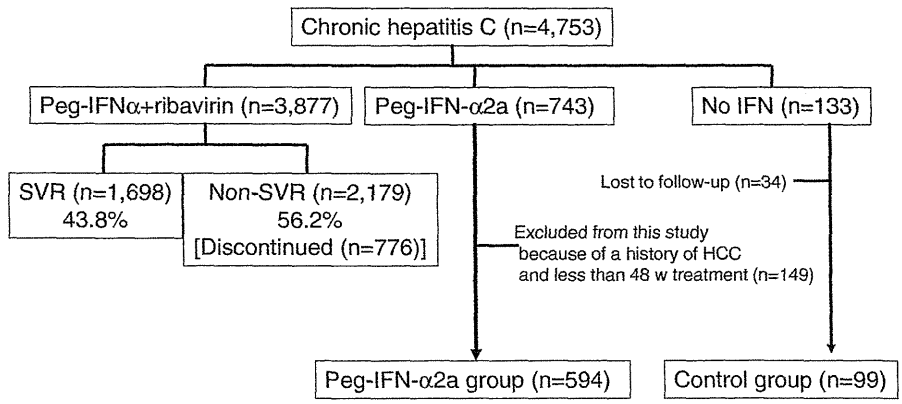

Fig. 1 Flow diagram of the patients' enrollment in the study. *Peg-IFNα* pegylated interferon α, *SVR* sustained viral response, *HCC* hepatocellular carcinoma, *w* week

study, 4,753 patients with chronic hepatitis C had been treated; Peg-IFN and ribavirin combination treatment had been administered to 3,877 patients, 743 patients had received Peg-IFN alone, and 133 patients had not agreed to receive IFN (a flow diagram of the enrollment of patients in this study is shown in Fig. 1). In the patients with Peg-IFN and ribavirin combination treatment, the SVR rate was 43.8 %; SVR was not achieved in 2,179 patients, and in 776 of these patients, the combination therapy was discontinued owing to adverse events or the patient's choice. Patients who failed to achieve an SVR were not included in this study, because the incidence of HCC is known to be reduced even in non-responders to IFN [17].

The backgrounds of the 594 patients studied are shown in Table 1. Findings from the liver biopsies of the patients were classified according to international standards [18]. Long-term PegIFNα-2a treatment is approved by the Japanese Medical Insurance system. Written informed consent was obtained from all patients prior to participation in this study. The study design was approved by the regional ethics committees of the 21 centers involved in this study, including the Musashino Red Cross Hospital, in accordance with the Helsinki Declaration. The 743 patients treated with PegIFNα-2a alone were not indicated for Peg-IFNα and ribavirin combination therapy because of anemia or heart disease. The 133 patients who did not agree to receive IFN served as the control group (see Fig. 1). A large proportion of the 594 study patients had advanced fibrosis of the liver and active inflammation. A dose of 90 μg PegIFNα-2a was administered to 512 and 82 patients weekly and biweekly, respectively, according to the patients' wishes. There were no significant differences between the weekly and biweekly groups in the patients' background data (data not shown).

The median duration of follow up in the PegIFNα-2a group was 1,273 days (range 228–2,768 days) and HCC was observed in 49 of the 594 patients (Table 1). Pretreatment and on-treatment factors associated with the development of HCC were analyzed by Student's *t*-test, the Mann–Whitney *U*-test, and the χ^2 test (Table 2). Independent factors for the development of HCC were assessed by multivariate analysis using logistic regression. The

Table 1 Background data of patients treated with PegIFNα-2a ($n = 594$)

	$n = 594$
Age (years)	61.7 ± 11.7
Sex (male/female)	258/336
BMI	23.2 ± 3.3
Genotype (1/2)	443/151
Diagnosis (ASC/CH/LC)	4/460/130
History of excess alcohol consumption (≥60 g/day; yes/no)	118/376
Fibrosis (F0, 1, 2/F3, 4)	443/151
Inflammatory activity (A0, 1/A2, 3)	469/125
Diabetes mellitus (no/yes)	499/95
LDL cholesterol (mg/dL)	94.2 ± 31.1
Fasting blood sugar (mg/dL)	106.3 ± 28.5
White blood cell count (/mm^3)	4,360 ± 1,470
Red blood cell count (×10^6/μL)	423.8 ± 56.4
Hemoglobin (g/dL)	13.3 ± 1.8
Platelet count (×10^3/μL)	137 ± 56
Albumin (g/dL)	4.0 ± 0.5
Total bilirubin (mg/dL)	0.8 ± 0.6
AST (IU/L)	65.8 ± 47.8
ALT (IU/L)	72.1 ± 68.0
Gamma-GTP (IU/L)	55.2 ± 51.3
Esophageal varices (no/yes)	344/31
Alpha fetoprotein (ng/L)	6.9 (4.2–13.8)
Once weekly or biweekly PegIFNα-2a	512:82
Baseline HCV RNA (KIU/mL)	1,024 (73–2,130)
Development of HCC (no/yes)	545/49

PegIFN pegylated interferon, *BMI* body mass index, *ASC* asymtomatic carrier, *CH* chronic hepatitis, *LC* liver cirrhosis, *LDL* low-density lipoprotein, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *GTP* guanosine triphosphate, *HCV* hepatitis C virus, *HCC* hepatocellular carcinoma

Values are means ± SD, with ranges in parentheses

incidence of HCC was analyzed according to the ALT, AFP, and hepatitis C virus (HCV) RNA levels 24 weeks after the start of PegIFNα-2a administration by using the Kaplan–Meier method. The risk of HCC was analyzed, using the Kaplan–Meier method, only in the non-responders with detectable HCV RNA during PegIFNα-2a administration by dividing them according to the ALT and AFP levels 24 weeks after the start of therapy. The incidence of HCC was compared between the patients with ALT levels of <41 IU/L and those with levels of ≥41 IU/L, and between patients with serum AFP levels of <10 ng/L and those with levels of ≥10 ng/mL at 24 weeks after starting treatment, because at most of the centers participating in the this study, the upper normal range of serum ALT is set at 40 IU/L, and the most significant difference in the incidence of HCC was observed between the PegIFNα-2a and control group with the cut-off serum ALT set at 41 IU/L and cutoff serum AFP set at 10 ng/mL, 24 weeks after starting treatment. The HCV RNA level was measured using the Amplicor Monitor method with a lower detection limit of 50 IU/L (Roche Diagnostics, Tokyo, Japan). A history of excess alcohol consumption was determined as >60 g alcohol per day in order to exclude alcoholic liver disease.

An asymptomatic carrier was defined as a patient with a serum ALT level within the normal range and minimal inflammation or fibrosis in the biopsied tissues of the liver. Chronic hepatitis was defined as mild-to-severe fibrosis of the liver according to liver biopsy [18]. The diagnosis of liver cirrhosis was based on the results of histological examination of the biopsied liver tissues.

Study 2: incidence of HCC in the PegIFNα-2a therapy and non-administration (control) groups in comparison with propensity-matched controls

Ninety-nine of the 133 chronic hepatitis C patients who had not received IFN were examined as controls; patients in this group received liver-protective agents such as glycyrrhizin or were untreated, and the group was observed for more than 1 year. None of the individuals in the control groups had received IFN alone or PegIFNα and ribavirin combination treatment. They were treated for a median of 1,395 days (range 75–6,556 days). Fifty-nine of these patients underwent liver biopsy before the treatment and were considered the control group for the propensity-matched study. For the propensity-matched study, 59 patients were selected from the PegIFNα-2a group according to their age, sex, platelet count, and total bilirubin levels, which had been identified as independent pretreatment risk factors for the development of HCC in Study 1. The rates of HCC were analyzed using the Kaplan–Meier method, and the risk of HCC was analyzed particularly in patients with advanced fibrosis of the liver (F3 and F4).

Table 2 Comparison of HCC and non-HCC patients with long-term PegIFNα-2a administration ($n = 594$)

	Patients with or without development of HCC		p value
	With HCC ($n = 49$)	Without HCC ($n = 545$)	
Pretreatment parameters			
Age (years)	63.8 ± 1.7	61.3 ± 0.5	<0.05
Sex (male/female)	32/17	226/319	<0.01
BMI	24.0 ± 0.5	23.1 ± 0.2	n.s.
Genotype (1/2)	47/6	397/148	n.s.
History of excess alcohol consumption (≥60 g/day; yes/no)	11/38	107/338	n.s.
Fibrosis (F0, 1, 2/F3, 4)	25/24	418/127	<0.001
Inflammatory activity (A0, 1/A2, 3)	7/42	462/83	<0.001
Diabetes mellitus (no/yes)	38/11	461/84	n.s.
LDL cholesterol (mg/dL)	88.2 ± 9.0	94.7 ± 2.6	n.s.
White blood cell count (/mm^3)	4,355 ± 210	4,360 ± 64	n.s.
Red blood cell count (×10^6/μL)	420.8 ± 8.1	424.1 ± 2.6	n.s.
Hemoglobin (g/dL)	13.6 ± 0.3	13.3 ± 0.1	n.s.
Platelet count (×10^3/μL)	106 ± 8	140 ± 2	<0.001
Albumin (g/dL)	3.8 ± 0.1	4.0 ± 0.1	<0.001
Total bilirubin (mg/dL)	1.2 ± 0.1	0.8 ± 0.1	<0.001
AST (IU/L)	78.1 ± 6.8	64.6 ± 2.1	n.s.
ALT (IU/L)	72.8 ± 9.7	72.0 ± 2.9	n.s.
Gamma-GTP (IU/L)	68.7 ± 7.5	53.9 ± 2.3	n.s.
Alpha fetoprotein (ng/L)	17.1 (4.4–36.8)	16.7 (4.1–23.1)	n.s.
Esophageal varices	29.0 % (9/31)	6.4 % (22/344)	<0.01
On-treatment parameters			
ALT (IU/L)	59.4 ± 5.7	44.6 ± 1.8	<0.05
Alpha fetoprotein (ng/L)	9.8 (4.6–17.4)	5.5 (3.7–11.1)	<0.01
HCV RNA level (KIU/mL)	236 (<0.5–2,210)	21 (<0.5–1,780)	<0.05

n.s. not significant

Statistical analysis

Categorical data were compared using the χ^2 test or Fisher's exact test. The distributions of continuous variables were analyzed using Student's t-test and the Mann–Whitney U-test for two groups. Multivariate analysis was

conducted using logistic regression. The cumulative incidence curve was determined using the Kaplan–Meier method and differences between groups were assessed by the log-rank test. For all methods, the level of significance was set at $p < 0.05$. Multivariate analysis of the risk of HCC was carried out using the Cox proportional hazard model. Statistical analyses were performed using the Statistical Package for the Social Sciences software version 11.0 (SPSS, Chicago, IL, USA). In Study 1, age, sex, platelet count, and total bilirubin levels were identified as independent factors for the development of HCC; therefore, these factors were selected for the propensity-matched control study (Study 2) in which 59 patients from the PegIFNα-2a group were included.

Results

Study 1

We analyzed the factors involved in the development of HCC in patients who received 90 μg PegIFNα-2a weekly or biweekly for more than a year. The incidence of HCC did not differ significantly between the groups treated with PegIFNα-2a weekly and biweekly (34 of 512 vs. 15 of 82, respectively). As shown in Table 2, univariate analysis revealed statistically significant differences in the pretreatment parameters including age, sex, fibrosis of the liver, platelet count, albumin level, and total bilirubin, between patients who developed HCC and those who did not. Endoscopy was carried out in 375 patients, and esophageal varices were noted in 31 of them. The incidence of HCC was higher in patients with esophageal varices than in those without varices [29.0 % (9 of 31) vs. 6.4 % (22 of 344)]. Assessment of on-treatment factors by univariate analysis revealed statistically significant differences in serum ALT, AFP, and HCV RNA levels 24 weeks after the start of PegIFNα-2a maintenance treatment (Table 2).

Multivariate analysis including pretreatment parameters revealed that age, sex, fibrosis of the liver, platelet count, and total bilirubin were independent risk factors for HCC development (Table 3). Multivariate analysis including on-treatment parameters identified ALT levels of ≥41 IU/L and AFP levels of ≥10 ng/L 24 weeks after the start of the PegIFNα-2a therapy as independent risk factors for HCC development (Table 3).

The incidence of HCC was significantly lower in patients with ALT levels of ≤40 IU/L than in those with ALT levels of ≥41 IU/L 24 weeks after the start of observation (Fig. 2). The incidence of HCC was also significantly lower in patients with AFP concentrations of <10 ng/mL at 24 weeks after the start of observation than in those with AFP concentrations of ≥10 ng/mL (Fig. 3). The dose of PegIFNα-2a was reduced to 45 μg in 16 patients because of neutropenia and thrombocytopenia. In addition, PegIFNα-2a was discontinued in 18 patients because of adverse events, including depression (7 patients), interstitial pneumonitis (3 patients), thrombocytopenia (3 patients), neutropenia (1 patient), itching (1 patient), and ascites (3 patients). No statistically significant differences were found between the patients with reduced dosage or treatment interruption and those without treatment modifications with respect to overall survival, HCC incidence, ascites formation, variceal bleeding, hepatic encephalopathy, and 2-point increases in the Child-Pugh score. No patients underwent liver transplantation.

Table 3 Independent risk factors for HCC development in patients treated with 90 μg PegIFNα-2a weekly or bi-weekly, evaluated by multivariate analysis (logistic regression analysis)

	Multivariate analysis		
	Odds ratio	95 % Confidence interval (CI)	*p*
Age (years) (every 5 years)	2.24	1.76–9.33	<0.005
Sex (male/female)	3.16	1.56–10.7	<0.005
Fibrosis (F3, 4/F0, 1, 2)	1.69	1.18–5.2	<0.01
Platelet count (<120 × 10^3/μL vs. ≥120 × 10^3/μL)	3.24	1.44–27.6	<0.01
Total bilirubin (mg/dL)	1.59	1.09–2.58	<0.05
ALT (at 24 weeks) (≥41 vs. <40 IU/L)	2.49	1.51–8.28	<0.05
AFP (at 24 weeks) (≥10 vs. <10 ng/L)	3.78	1.92–11.8	<0.01

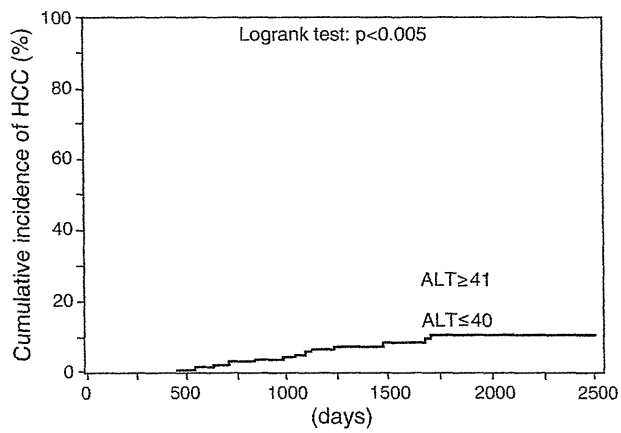

Fig. 2 Comparison of HCC rates in patients administered with PegIFNα-2a ($n = 594$) with respect to alanine aminotransferase (*ALT*) levels 24 weeks after the start of therapy. *Black line* patients with ALT ≥41 IU/L in the first 24 weeks, *gray line* patients with ALT ≤40 IU/L in the first 24 weeks

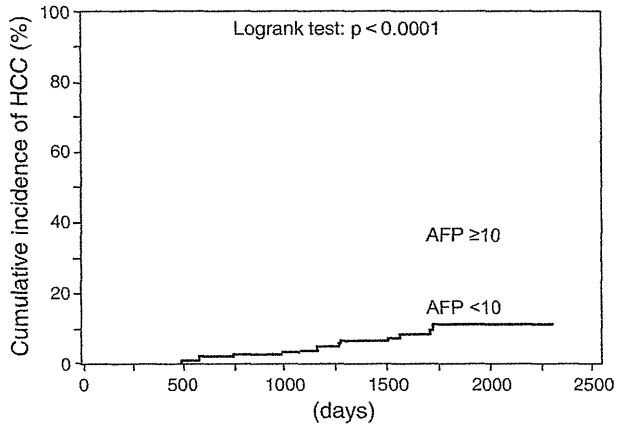

Fig. 3 Comparison of HCC rates in patients administered PegIFNα-2a ($n = 594$) with respect to alpha-fetoprotein (*AFP*) levels in the first 24 weeks after the start of therapy. *Black line* patients with AFP ≥10 ng/mL at 24 weeks, *gray line* patients with AFP <10 ng/mL at 24 weeks

Study 2

We compared the incidence of HCC between 59 patients in the control group and the same number of patients in the PegIFNα-2a group using the matched-pair test. The backgrounds of the patients are shown in Table 4. The PegIFNα-2a group had higher rates of advanced fibrosis (F3 and F4) and active inflammation (A2 and A3). No other differences were found between the two groups, except for the white blood cell count (Table 4).

Development of HCC was observed in 2 patients in the PegIFNα-2a group and 8 in the control group. The incidence of HCC was compared between the two groups, using the Kaplan–Meier method. The incidence of HCC in the PegIFNα-2a group was significantly lower than that in the control group (log-rank test, $p = 0.0187$; Fig. 4). Among the patients with advanced fibrosis of the liver (F3 and F4), those in the PegIFNα-2a group had a lower incidence of HCC than those in the control group. The independent risk factors for the development of HCC were analyzed using the stepwise Cox proportional hazard model. Only PegIFNα-2a administration and age were identified as independent risk factors for the development of HCC (Table 5).

Discussion

The number of HCC cases resulting from HCV infection continues to increase worldwide [19]. To date, IFN therapy is the most effective preventive measure against HCC in patients with chronic hepatitis C; furthermore, the incidence of HCC is reduced in patients who achieve an SVR to IFN [6–9] Therefore, achieving an SVR is the most effective approach for reducing the risk of developing HCC. In Japan, the incidence of HCC is elevated in older patients with hepatitis C. Corroborating this finding, the results of a Japanese study show a higher risk of HCC in patients aged 65 years and more [10]. Therefore, prevention of HCC in aged patients is an important challenge.

In the present multicenter, cooperative, retrospective study conducted in Japan, the incidence of HCC was reduced in patients who received 90 μg PegIFNα-2a weekly or biweekly and had AFP values of <10 ng/mL and ALT values of ≤40 IU/L 24 weeks after the start of the treatment. The results of the matched case–control study of the PegIFNα-2a group and the non-IFN control group show that the incidence of HCC was significantly lower in the PegIFNα-2a group than in the control group, especially in patients with advanced fibrosis of the liver (F3 and F4). However, there could have been a selection bias between

Table 4 Backgrounds of the patients in the propensity-matched control study (PegIFNα-2a group, $n = 59$; control group, $n = 59$)

	PegIFNα-2a group ($n = 59$)	Control group ($n = 59$)	p value
Age (years)	60.5 ± 13.0	63.3 ± 10.5	n.s.
Gender (male/female)	24/35	25/34	n.s.
BMI	22.9 ± 3.6	22.9 ± 3.4	n.s.
Genotype (1/2)	49/10	46/13	n.s.
History of excess alcohol consumption (60 g/day; yes/no)	10/49	4/55	n.s.
Fibrosis (F0, 1, 2/F3, 4)	37/22	43/16	<0.05
Development of HCC (F0–2/F3, 4)	1/1	1/7	n.s.
Inflammatory activity (A0,1/A2, 3)	19/40	30/29	<0.05
Diabetes mellitus (no/yes)	57/2	56/3	n.s.
LDL cholesterol (mg/dL)	95.3 ± 23.8	117.0 ± 4.2	n.s.
White blood cell count (/mm^3)	4,260 ± 1,239	5,193 ± 2,078	<0.05
Red blood cell count (×10^{-4}/μL)	430 ± 57.8	441 ± 44.9	n.s.
Hemoglobin (g/dL)	13.6 ± 1.5	13.6 ± 1.9	n.s.
Platelet count (×10^{-3}/μL)	14.5 ± 5.7	15.8 ± 5.7	n.s.
Albumin (g/dL)	4.1 ± 0.5	4.1 ± 0.4	n.s.
Total bilirubin (mg/dL)	0.7 ± 0.5	0.9 ± 0.7	n.s.
AST (IU/L)	58.3 ± 47.7	49.7 ± 26.6	n.s.
ALT (IU/L)	63.6 ± 68.7	58.0 ± 39.2	n.s.
Gamma-GTP (IU/L)	78.3 ± 81.3	55.3 ± 75.1	n.s.
Baseline alpha-fetoprotein (AFP) (ng/L)	7.2 (4.3–14.2)	7.7 (3.9–13.8)	n.s.
Baseline HCV RNA level (KIU/mL)	1,230 (24–3,870)	1,024 (38–3,110)	n.s.

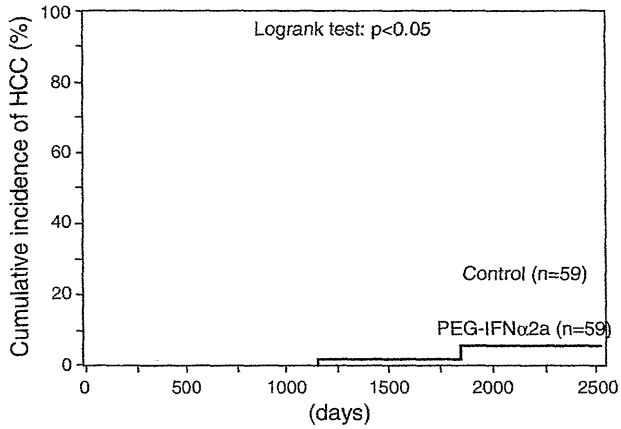

Fig. 4 Comparison of HCC rates between the long-term PegIFNα-2a administration group ($n = 59$) and non-administration group ($n = 59$) in the propensity-matched control study (Kaplan–Meier log-rank test, $p = 0.019$)

Table 5 Risk factors for HCC in the propensity-matched control study (Cox proportional hazard model)

Variables	Risk ratio	95 % CI	*p* value
PegIFN versus control	0.17	0.03–0.75	<0.05
Age (every 1 year)	1.12	1.02–1.25	<0.05
Fibrosis (F3, 4 vs. F0, 1, 2)	1.70	0.75–4.16	n.s.
Platelet count (every 10 × 10^3/μL)	0.89	0.73–1.09	n.s.
Albumin (every 1.0 g/dL)	0.80	0.10–6.68	n.s.
On-treatment AFP (<10 vs. ≥10 ng/L)	4.07	0.59–40.12	n.s.

the PegIFNα-2a group and the control group (patients who did not agree to receive IFN treatment), because this was a retrospective and non-randomized study. However, concordant with the findings of the HALT-C study [14], the present results show that PegIFNα-2a inhibits the development of HCC in patients with advanced fibrosis of the liver.

Recent studies show that polymorphisms in the host *IL28B* gene are important factors in the response to PegIFNα and ribavirin combination therapy [20, 21]. However, the mechanism of IL28B involvement in the response to PegIFNα and ribavirin has not been elucidated completely. A recent report has shown that IL28B is a significant factor in the development of HCC as well as in the response to IFN therapy [22]. Further studies are warranted to analyze the relationship between IL28B and inhibition of the development of HCC by PegIFNα in chronic hepatitis C.

Risk factors for the development of HCC have been discussed previously. Increased intrahepatic fat is involved in the development of HCC in chronic hepatitis C patients [23, 24]. In addition, diabetes-associated fat disorder [25, 26], hepatic iron overload [27], advanced fibrosis, older age, and fatty deposits in the liver are risk factors for HCC development [4]. Therefore, it is important to establish strategies to mitigate these risk factors to prevent the development of HCC and thus improve the outcomes of hepatitis C patients.

IFN therapy after HCC treatment is reported to inhibit the recurrence of tumors [28, 29], and a meta-analysis has revealed a trend toward inhibition of the recurrence of HCC [30, 31]. The prevention of HCC is an important issue that needs to be addressed to improve the survival of chronic hepatitis C patients. The findings of the present study and the HALT-C trial [14] indicate the effectiveness of long-term administration of maintenance IFN for preventing the development of HCC in chronic hepatitis C patients without an SVR. Improvement in ALT levels is also known to be an important predictor for the prevention of HCC [32]. A low AFP value during IFN administration is also recognized as a significant indicator of a lower risk of HCC [33, 34]. Recently, Osaki et al. [35] reported that a decrease of serum AFP during treatment with IFN was associated with a reduced incidence of HCC. Taking these findings and our own together, we conclude that maintenance administration of low-dose PegIFNα-2a weekly or biweekly to non-SVR patients with chronic hepatitis C decreases the incidence of HCC, especially in patients whose serum ALT and AFP levels are within the normal range 24 weeks after the start of treatment. The preventive effects of IFN against the development of HCC without elimination of the virus may be associated with its anticarcinogenic effects [16, 35]; however, the precise mechanism should be investigated.

The limitations of the present study are that it is retrospective and multicentric; therefore, potentially there may have been a selection bias. However, the reduction of the rate of development of HCC by maintenance administration of PegIFNα-2a in the patients in whom serum ALT and AFP levels were within the normal ranges 24 weeks after the start of treatment may be attributable to the anticarcinogenic effects of IFN without elimination of the virus.

Conclusion

The incidence of HCC was lower in non-SVR patients with chronic hepatitis C who were administered with maintenance low-dose PegIFNα-2a; especially in those whose serum ALT and AFP levels were within the normal ranges 24 weeks after the start of treatment.

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Conflict of interest Namiki Izumi received lecture fees from Chugai Co. and MSD Co. in 2011.

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Hepatology Research

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Original Article

Randomized controlled trial of a new procedure of radiofrequency ablation using an expandable needle for hepatocellular carcinoma

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Aim: To evaluate the efficacy of a new ablation procedure for the stepwise hook extension technique using a SuperSlim needle for radiofrequency ablation (RFA) treatment of hepatocellular carcinoma (HCC), a randomized controlled trial was performed.

Methods: Thirty patients with HCC measuring 20 mm or less were randomly treated with a conventional four stepwise expansion technique (group 1) and the new stepwise expansion technique (group 2; the electrode was closed in the shaft after the same three steps of the conventional procedure and then fully extended). All patients underwent the RFA procedure using a 10-hook expandable electrode of 17-G diameter (LeVeen SuperSlim 30 mm). We compared the ablation time, required energy and ablated lesions in the two groups.

Results: The long and short diameters of RFA-induced necrosis were significantly larger in group 2 (37 and 28 mm) than group 1 (30 and 26 mm, $P = 0.001$ and $=0.045$, respectively). Irregular and small needle expansion resulting in the parachute-like or irregularly shaped ablated zone was observed in more cases in group 1 than in group 2. The new technique made all tines expand uniformly and largely, which produced a near-oval ablated zone of which the long axis is perpendicular to the needle shaft.

Conclusion: The two kinds of stepwise procedures allow the selection of a more suitable procedure according to the tumor size and shape in each RFA.

Key words: expandable needle, hepatocellular carcinoma, radiofrequency ablation, randomized controlled trial

INTRODUCTION

PERCUTANEOUS TREATMENT INCLUDING radiofrequency ablation (RFA) and percutaneous ethanol injection (PEI) is often used for small-size hepatocellular carcinoma (HCC) because it is less invasive than surgical therapy. RFA has become the first-choice local treatment because of the excellent outcome; the efficacy of RFA in HCC tumors measuring less than 2 cm in diameter is similar to that of PEI but it requires fewer treatment sessions, and the efficacy in HCC tumors of more than 2 cm in diameter is better than with PEI.[1] In addition, RFA is also more cost-effective than surgical resection of small HCC.[2] With three commercially-available RFA apparatuses – the radiofrequency tumor coagulation system (RTC system; Boston-Scientific, Natick, MA, USA), radiofrequency interstitial thermal ablation system (RITA; AngioDynamics, Latham, NY, USA) and cool-tip RF system (Valleylab, a division of Tyco Healthcare Group, Boulder, CO, USA) – the volume ablated during one RFA session is of a diameter less than 3.0–4.0 cm, except in ablation with the Starburst XL RFA device (RITA).[3] RFA therapy is currently restricted to tumors measuring less than 3 cm. In this regard, previous studies reported that the necrotic area could be enlarged by saline injection prior to RFA,[4,5] combination of RFA with PEI,[6,7] RFA with ethanol lipiodol injection,[8] RFA with transcatheter arterial embolization[9] and RFA with transient arterial obliteration.[10–12]

Among the above three RFA apparatuses, the RTC system and RITA have adopted the use of expandable needles. We reported previously the efficacy of the

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stepwise hook extension technique for RFA therapy of HCC.[13] The technique allows rapid roll-off at lower power and lower energy and reduces any possible increase in intra-tissue pressure that may cause scattering of intrahepatic metastasis.[14–17]

A more slender expandable needle has been developed (17-G, SuperSlim; Boston Scientific, Natick, MA, USA) for easier and safer insertion into the liver. However, insertion of the slim needle into the liver tissue could result in deformation of the needle and hence possible reduction of the size of the ablated area. To overcome this shortfall, we designed a new technique involving full re-expansion after stepwise extension, to ensure full expansion of the needle. We have already reported the experimental study using healthy pig livers *in vivo* to show that this technique can produce a larger necrotic zone than the conventional stepwise procedure.[18]

The aim of this study was to evaluate the efficacy of the new ablation procedure for the stepwise hook extension technique for RFA therapy of HCC of a patient with cirrhosis or without cirrhosis in a randomized controlled trial.

METHODS

Patients and tumors

FROM NOVEMBER 2006 to March 2010, 30 consecutive patients who met the following criteria were enrolled in this study: (i) HCC confirmed either histopathologically or radiologically; and (ii) diameter of the hepatic tumor of no more than 20 mm. They included 20 men and 10 women, with a median age of 57 years (range, 43–73). Seventeen patients were with cirrhosis and the other 13 were without cirrhosis. Table 1 lists the clinical background of patients of both groups. There were no significant differences between the groups.

A typical hypervascular HCC was diagnosed by typical hypervascular stain on digital subtraction angiography. In addition, one of the following three criteria was used to diagnose a tumor as a well-differentiated HCC: (i) histopathological diagnosis as well-differentiated HCC; (ii) hypo-enhanced lesion on computed tomography (CT) during hepatic arteriography (CTHA) and hypoperfusion on CT during arterial portography (CTAP); and (iii) hypo-enhanced lesion on the equilibrium phase of dynamic CT or hypo-perfused lesion on CTAP and hypointense on the hepatocyte-specific phase of multiple resonance imaging (MRI) using gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid (Gd-EOB-DTPA) (Primovist; Bayer Schering Pharma, Osaka, Japan). A total of 30 patients were treated by the RFA protocol.

The study protocol was approved by the Human Ethics Review Committee of Toranomon Hospital and a signed consent form was obtained from each patient.

RFA protocol

We used the RTC system comprising a slim expandable needle (30 mm, 17-G LeVeen needle, SuperSlim), which consists of 10 expandable monopolar array electrodes, and the RF3000 generator, with a maximum power output of 120 W, and four electrode pads placed on the patient's skin. Instead of using the standard method recommended by the manufacturer, we adopted two types of stepwise hook extension techniques.[18] Patients were randomly divided into two groups based on the RFA protocol used. In group 1, after placing the needle electrode shaft into the tumor with the array retracted, using real-time ultrasound guidance, the electrode tines were expanded to a quarter, a half, three-quarters of the length and full-length in the first, second, third and final steps, respectively. The diameter of the array at each step was 10, 15, 25 and 30 mm, respectively. In group 2, the

Table 1 Background of the patients in groups 1 and 2

	Group 1 (conventional method)	Group 2 (new method)	*P*
Male : female	8:8	12:2	0.042
Age†	69 (45–82)	71 (60–84)	0.270
With cirrhosis: without cirrhosis	11:5	6:8	0.160
Tumor diameter, mm†	12 (9–18)	16 (6–19)	0.179
Hypervascular, yes : no	13:3	11:3	0.520

†Data are median (range).
NS, not significant.

first, second and third steps were similar to those of g1. After the third step, the tines were again closed within the shaft and then fully expanded.[18]

Power was first applied at 30 W and then increased at 10-W increments every minute in each step to a maximum of 120 W. The power was fixed once it reached 120 W. The necessary electric power and tissue impedance were recorded every 15 s. The procedure was applied continuously until a rise in impedance (caused by coagulation necrosis) with a corresponding drop in delivered power (a phenomenon called "roll-off"). The energy requirement for ablation was integration of the electric power (W) over the ablation time (s), which could be calculated approximately by summing a product of 15 (s) and the electric power measured every 15 s.

Image analysis

Tumor size, location and vascularity were evaluated before RFA using contrast-enhanced CT or MRI. Dynamic CT scans were performed using nonionic contrast material unless the patient was allergic to the iodine medium, for whom MRI was performed. Dynamic CT consisted of the arterial phase (30-s delay), hepatic portal phase (60-s delay) and hepatic venous phase (120-s delay) with slice thickness of 5 mm after the start of injection, respectively. Contrast-enhanced MRI was performed with i.v. injection of contrast material Gd-EOB-DTPA (EOB-MRI). Dynamic MRI consisted of the arterial phase (30-s delay), hepatic portal phase (60-s delay) and hepatic venous phase (120- and 180-s delay) with a thickness of 5 mm and hepatocyte-specific phase (>20 min delay) with a thickness of 3 mm. The tumor was appraised as "hypervascular" when it was stained denser on the arterial phase image compared to the surrounding liver parenchyma.

One to three days after the treatment, the size and shape of the RF-induced lesion was evaluated by measuring three perpendicular dimensions of portal phase images of the contrast-enhanced CT or MRI, calculating the hypothetical volume of the ablated zone. In cases in which CT/MRI images were taken along the needle trace and those perpendicular to the needle, we measured the length of the ablated area along the needle tract and the diameter of the area perpendicular to it.

Statistical analysis

The duration of ablation, required energy and the size of the ablated lesions were compared between the two groups using the Mann–Whitney *U*-test. All values were expressed as median. A *P*-value less than 0.05 denoted the presence of a statistically significant difference.

RESULTS

Ablation time and required energy

ROLL-OFF WAS achieved at each step of ablation in all 30 RFA procedures. Table 2 shows the time to reach roll-off at each step and total ablation time in the two groups. These results indicate that the durations of the first step, second step and third step were similar for groups 1 and 2 ($P = 0.356$, $= 0.457$ and $= 0.590$, respectively), while that of the fourth step and total session were longer for group 2 than group 1 ($P < 0.001$ and <0.001, respectively). The energy required for one procedure was 18.1 kJ (range, 10.7–31.3) and 59.9 kJ (range, 35.1–119.5) for groups 1 and 2, respectively, indicating more energy requirement for group 2 than group 1 ($P < 0.0001$).

Needle expansion

Figure 1 depicts CT images showing the tines in the tumor in the final step; Figure 1(a,b) shows a cross-

Table 2 Comparison of ablation time (in min/s) and radio frequency-induced areas between groups 1 and 2

	Group 1	Group 2	*P*
Duration of the first step	1′ 53″ (0′ 54″–3′ 43″)	2′ 37″ (1′ 00″–4′ 34″)	0.356
Second step	2′ 14″ (0′ 40″–4′ 57″)	2′ 21″ (0′ 16″–3′ 35″)	0.457
Third step	1′ 26″ (0′ 52″–2′ 46″)	1′ 30″ (0′ 57″–4′ 38″)	0.590
Fourth step	1′ 36″ (1′ 02″–3′ 55″)	9′ 20″ (6′ 39″–17′ 13″)	<0.001
Total ablation time	7′ 36″ (5′ 07″–10′ 13″)	15′ 07″ (11′ 22″–25′ 05″)	<0.001
Required energy for ablation, kJ	18.1 (10.7–31.3)	59.9 (35.1–119.5)	<0.001
Long diameter, mm	30 (21–37)	37 (31–60)	0.001
Short diameter, mm	26 (16–32)	28 (25–39)	0.045
Axial diameter, mm	35 (20–45)	40 (30–50)	0.018

Data are median (range).