

Fig. 5. FGF3 and FGF4 overexpression and drug sensitivity to sorafenib *in vitro* and *in vivo*. (A) Growth inhibitory assay examining sorafenib in various cancer cell lines *in vitro*. The growth inhibitory effect of sorafenib was examined using an MTT assay. The IC₅₀ values of each cell line are shown in the graph. The black bars show that the IC₅₀ values were below 1 μM. Amp, gene amplification. (B) Cancer cell lines stably overexpressing EGFP, FGF3, or FGF4 were established and designated as A549/EGFP, A549/FGF3, and A549/FGF4. Western blot analysis confirmed that exogenously expressed FGF3 and FGF4 were secreted into the culture medium. Sup., supernatant. (C) NIH-3T3 cells were exposed to indicated concentrations of sorafenib for 2 hours and were then stimulated with FGF4-conditioned medium for 20 minutes. (D) Mice inoculated with A549/EGFP, A549/FGF3, or A549/FGF4 (*n* = 20 each) were treated with a low dose of oral sorafenib (*n* = 10, 15 mg/kg/day) or without (*n* = 10, vehicle control). **P* < 0.05.

largely unclear. Relatively small cohort studies have reported that one out of 20 HCCs exhibited *FGF3* amplification as determined via CGH analysis,¹⁷ and 3 out of 45 HCCs examined using Southern blot analysis had a copy number >5;¹⁸ meanwhile, amplification was not detected in 0 out of 42 surgically resected HCCs.¹⁹ In the present study, two of the 82 (2.4%) HCC samples exhibited *FGF3/FGF4* gene amplification in the HCC series. If only 2%-3% of HCC patients harbor the *FGF3/FGF4* amplification, its value as a biomarker seems to be limited in clinics because a frequency of 2%-3% is too low to stratify the patients for specific targeted therapy. However, a combination of biomarkers—including *FGF3/FGF4* amplification, lung metastasis, tumor differentiation, and other unrevealed dysregulation of FGFR signaling—may increase the response prediction. In addition, 2%-3% of *FGF3/FGF4* amplification may be a promising therapeutic target for future FGFR-targeted therapies in the treatment of HCC.

Tumor shrinkage might be due to the mixed effect (sorafenib + 5FU + interferon) of combination therapy in the initially described patient. However, during

this patient's long clinical course, tumor regrowth was observed following withdrawal of sorafenib because of oral hemorrhage, and tumor reshrinkage was observed when sorafenib treatment recommenced. Thus, we considered that tumor shrinkage might be achieved by the effect of sorafenib on its own, rather than 5FU + interferon.

Regarding determinants of drug sensitivity to sorafenib, the mechanism of hypersensitivity in the gastric cancer cell lines HSC-39, HSC-43, and KATO-III is *FGFR2* gene amplification and is thought to be the addiction of these cell lines to this gene,¹⁴ since sorafenib has a relatively weak but significant inhibitory effect on FGFR1 at a concentration of 580 ± 100 nM.³ This result suggests that the blockade of FGFR signaling by sorafenib may lead to a significant treatment response, at least in *FGFR2*-amplified cells. In this study, we found that *FGF4*, but not *FGF3* overexpression, was partially involved in the sensitivity to sorafenib *in vivo*. The limitations of the study are the small number of responder patients and the potential bias in their selection because of the retrospective study design. Further clinical study of responders to

sorafenib is necessary. We are presently undertaking a prospective molecular translational study (2010-2012) in a cohort of Japanese patients with sorafenib-treated HCC.

Multiple lung metastases were frequently observed among responders to sorafenib (38%) but were less common among nonresponders (5%). Based on a Japanese follow-up survey of patients with primary HCC, lung metastasis was observed in 7% (169/2355) of the patients at the time of autopsy.²⁰ Another study demonstrated that 15% of patients were found to have extrahepatic metastases, and lung metastasis was detected in 6% of 995 consecutive HCC patients.²¹ When compared with these data from large-scale studies, the frequency of lung metastasis among responders to sorafenib seems quite high. In addition, a poorly differentiated histological type tended to be more common among responders, although the correlation was not significant.

In conclusion, we found that *FGF3/FGF4* gene amplification, multiple lung metastases, and a poorly differentiated histological type may be involved in the response to sorafenib.

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Genome-Wide Association Study Confirming Association of HLA-DP with Protection against Chronic Hepatitis B and Viral Clearance in Japanese and Korean

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Abstract

Hepatitis B virus (HBV) infection can lead to serious liver diseases, including liver cirrhosis (LC) and hepatocellular carcinoma (HCC); however, about 85–90% of infected individuals become inactive carriers with sustained biochemical remission and very low risk of LC or HCC. To identify host genetic factors contributing to HBV clearance, we conducted genome-wide association studies (GWAS) and replication analysis using samples from HBV carriers and spontaneously HBV-resolved Japanese and Korean individuals. Association analysis in the Japanese and Korean data identified the *HLA-DPA1* and *HLA-DPB1* genes with $P_{meta} = 1.89 \times 10^{-12}$ for rs3077 and $P_{meta} = 9.69 \times 10^{-10}$ for rs9277542. We also found that the *HLA-DPA1* and *HLA-DPB1* genes were significantly associated with protective effects against chronic hepatitis B (CHB) in Japanese, Korean and other Asian populations, including Chinese and Thai individuals ($P_{meta} = 4.40 \times 10^{-19}$ for rs3077 and $P_{meta} = 1.28 \times 10^{-15}$ for rs9277542). These results suggest that the associations between the *HLA-DP* locus and the protective effects against persistent HBV infection and with clearance of HBV were replicated widely in East Asian populations; however, there are no reports of GWAS in Caucasian or African populations. Based on the GWAS in this study, there were no significant SNPs associated with HCC development. To clarify the pathogenesis of CHB and the mechanisms of HBV clearance, further studies are necessary, including functional analyses of the HLA-DP molecule.

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Introduction

Overall, one-third of the world's population (2.2 billion) is infected with hepatitis B virus (HBV), and about 15% of these are chronic carriers. About 75% of the chronic carriers live in the east-south Asia and east pacific area, and there are 1.3–1.5 million chronic carriers living in Japan [1]. Of chronic carriers, 10–15% develop liver cirrhosis (LC), liver failure and hepatocellular carcinoma (HCC), and the remaining individuals eventually achieve a state of nonreplicative infection, resulting in hepatitis B surface antigen (HBsAg) negative and hepatitis B core antibody (anti-HBc) positive, i.e. HBV-resolved individuals [2–3]. In Japan, although the major route of HBV transmission was perinatal transmission and horizontal transmission in early childhood, infant HBV carriers have successfully been reduced since 1986 through a selective vaccination policy by the Japanese government [4–7]. However, the prevalence of HBV genotype A in acute HBV (AHB) infection has increased markedly since 2000, reaching approximately 52% in 2008 due to the lack of a universal HB vaccination, and around 10% of AHB cases could be persistent infection [8–9]. Viral factors, as well as host factors, are thought to be associated with persistent HB infection.

In 2009, significant associations between chronic hepatitis B (CHB) and a region including *HLA-DPA1* and *HLA-DPB1* were identified using 786 Japanese individuals having CHB and 2,201 control individuals through a two-stage genome-wide association study (GWAS) [10]. The same group was also subjected to a second GWAS using a total of 2,667 Japanese persistent HBV infection cases and 6,496 controls, which confirmed significant associations between the *HLA-DP* locus and CHB, in addition to associations with another two SNPs located in the genetic region including the *HLA-DQ* gene [11]. The associations between *HLA-DP* variants with HBV infection were replicated in other Asian populations, including Thai and Han Chinese individuals [10,12–13]. With regard to HBV clearance, the association between the human leukocyte antigen (HLA) class II allele and clearance of HBV was confirmed by the candidate gene approach in African, Caucasian and Asian populations [14–18]. However, in a previous GWAS using samples of Japanese CHB and control individuals, the clinical data on HBV exposure in the control individuals were unknown, and this may have led to bias. Moreover, there have been no reports of GWAS using samples from HBV carriers and HBV-resolved individuals to identify host genetic factors associated with HBV clearance other than HLA class II molecules.

Here, we performed a GWAS using samples from Japanese HBV carriers, healthy controls and spontaneously HBV-resolved individuals in order to confirm or identify the host genetic factors related to CHB and viral clearance. In the subsequent replication analysis, we validated the associated SNPs in the GWAS using two independent sets of Japanese and Korean individuals. In our study, healthy controls were randomly selected with clinically no evidence of HBV exposure, therefore, HBV-resolved individuals were prepared to clearly identify the host genetic factors related with CHB or HBV clearance.

Results

Protective Effects Against Chronic Hepatitis B in Japanese and Korean Individuals

In this study, we conducted a GWAS using samples from 181 Japanese HBV carriers (including asymptomatic carriers (ASC), CHB cases, LC cases and HCC cases, based on the criteria described in Materials and Methods) and 184 healthy controls in

order to identify the host genetic factors related to progression of CHB. All samples were genotyped using a genome-wide SNP typing array (Affymetrix Genome-Wide Human SNP Array 6.0 for 900 K SNPs). Figure 1a shows a genome-wide view of the single point association data based on allele frequencies using the SNPs that met the following filtering criteria: (i) SNP call rate $\geq 95\%$; (ii) minor allele frequency (MAF) $\geq 1\%$ for HBV carriers and healthy controls; and (iii) no deviation from Hardy-Weinberg equilibrium (HWE) $P \geq 0.001$ in healthy controls. We identified significant associations of protective effects against CHB with two SNPs (rs3077 and rs9277542) using the allele frequency model, both of which are located in the 3' UTR of *HLA-DPA1* and in the sixth exon of *HLA-DPB1*, respectively (rs3077, $P = 1.14 \times 10^{-7}$, and rs9277542, $P = 5.32 \times 10^{-8}$, respectively). The association for rs9277542 reached a genome-wide level of significance in the GWAS panel (Bonferroni criterion $P < 8.36 \times 10^{-8}$ (0.05/597,789)).

In order to validate the results of GWAS, a total of 32 SNPs, including the associated two SNPs (rs3077 and rs9277542), were selected for replication in two independent sets of HBV carriers and healthy controls (replication-1:256 Japanese HBV carriers and 236 Japanese healthy controls; and replication-2:344 Korean HBV carriers and 151 Korean healthy controls; Table 1). The associations for the original significant SNP (rs9277542) and marginal SNP (rs3077) on GWAS were replicated in both replication sets [replication-1 (Japanese); rs3077, $P = 2.70 \times 10^{-8}$, OR = 0.48 and rs9277542, $P = 3.33 \times 10^{-6}$, OR = 0.54; replication-2 (Korean); rs3077, $P = 2.08 \times 10^{-6}$, OR = 0.47 and rs9277542, $P = 8.29 \times 10^{-5}$, OR = 0.54, Table 2]. We conducted meta-analysis to combine these studies using the DerSimonian Laird method (random effects model) to incorporate variation among studies. As shown in Table 2, the odds ratios were quite similar across the three studies (GWAS and two replication studies) and no heterogeneity was observed ($P_{het} = 0.80$ for rs3077 and 0.40 for rs9277542). P_{meta} values were 4.40×10^{-19} for rs3077 (OR = 0.46, 95% confidence interval (CI) = 0.39–0.54), and 1.28×10^{-15} for rs9277542 (OR = 0.50, 95% CI = 0.43–0.60). Among the remaining 30 SNPs in the replication study, 27 SNPs were successfully genotyped by the DigiTag2 assay with SNP call rate $\geq 95\%$ and HWE p -value ≥ 0.01 . Two SNPs (rs9276431 and rs7768538), located in the genetic region including the *HLA-DQ* gene, were marginally replicated in the two sets of HBV carriers and healthy controls with Mantel-Haenszel P values of 2.80×10^{-7} (OR = 0.56, 95% CI = 0.45–0.70) and 1.09×10^{-7} (OR = 0.53, 95% CI = 0.42–0.67), respectively, when using additive, two-tailed Cochran Mantel-Haenszel (CMH) fixed-effects model with no evidence of heterogeneity ($P_{het} = 0.67$ for rs9276431 and 0.70 for rs7768538) (Table S1).

Meta-analysis using the random effects model across 6 independent studies, including 5 additional published data, showed $P_{meta} = 3.94 \times 10^{-45}$, OR = 0.55 for rs3077, $P_{meta} = 1.74 \times 10^{-21}$, OR = 0.61 for rs9277535 and $P_{meta} = 1.69 \times 10^{-15}$, OR = 0.51 for rs9277542, with the SNP rs9277535 being located about 4-kb upstream from rs9277542 and showing strong linkage disequilibrium of $r^2 = 0.955$ on the HapMap JPT (Table S2). As shown in Table S2, the odds ratio was very similar among the 6 studies, and heterogeneity was negligible with $P_{het} > 0.01$.

Moreover, based on GWAS using samples from 94 chronic HBV carriers with LC or HCC and 87 chronic HBV carriers without LC and HCC, we found no significant SNPs associated with CHB progression (Figure S1).

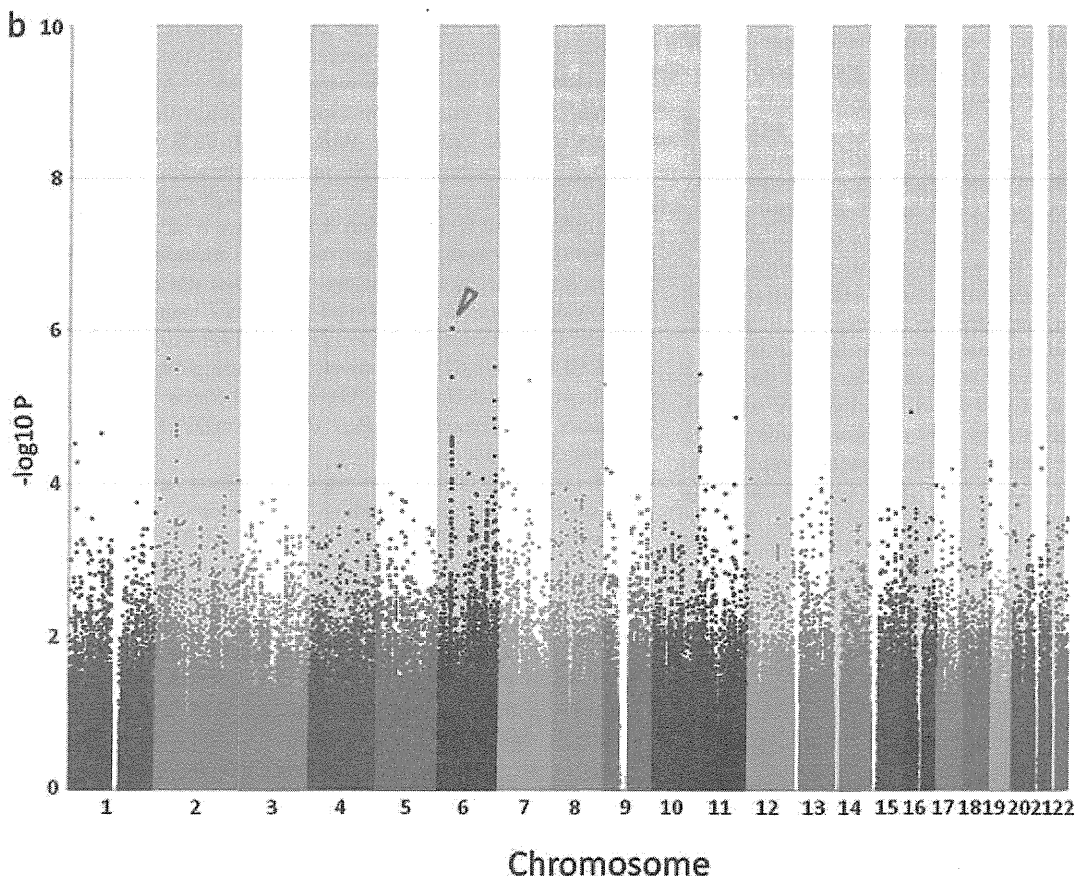
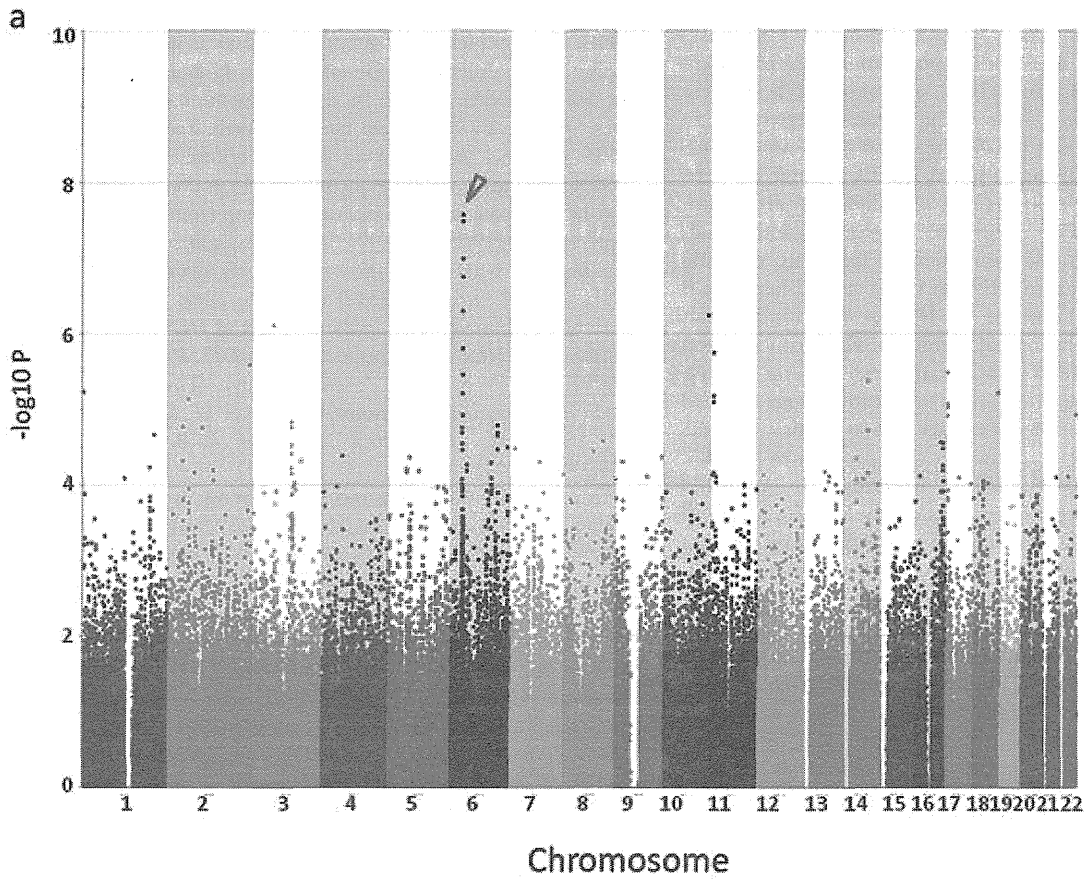


Figure 1. Results of genome-wide association studies. a) HBV carriers and healthy controls, and b) HBV carriers and HBV-resolved individuals were compared. *P* values were calculated by chi-squared test for allele frequencies. Dots with arrows on chromosome 6 show strong associations with protective effects against persistent HB infection and with HBV clearance. doi:10.1371/journal.pone.0039175.g001

Clearance of Hepatitis B virus in Japanese and Korean Individuals

We also conducted a GWAS to identify the host genetic factors related to clearance of HBV in the above 181 Japanese HBV carriers and 185 Japanese HBV-resolved individuals using a genome-wide SNP typing array (Affymetrix Genome-Wide Human SNP Array 6.0 for 900 K SNPs). The same two SNPs (rs3077 and rs9277542) showed strong associations in the allele frequency model ($P = 9.24 \times 10^{-7}$ and $P = 3.15 \times 10^{-5}$) with clearance of HBV (Figure 1b).

The above 32 SNPs, including the two associated SNPs (rs3077 and rs9277542), were selected for a replication study in two independent sets of HBV carriers and HBV resolved individuals (replication-1: 256 Japanese HBV carriers and 150 Japanese HBV resolved individuals; and replication-2: 344 Korean HBV carriers and 106 Korean HBV resolved individuals; Table 1). All 32 SNPs were genotyped using the DigiTag2 assay and 29 of 32 SNPs were successfully genotyped (Table S3). The associations of the original SNPs were replicated in both replication sets [replication-1 (Japanese): rs3077, $P = 3.32 \times 10^{-2}$, OR = 0.72 and rs9277542, $P = 1.25 \times 10^{-2}$, OR = 0.68; replication-2 (Korean): rs3077, $P = 2.35 \times 10^{-7}$, OR = 0.41 and rs9277542, $P = 4.97 \times 10^{-6}$, OR = 0.46; Table 3]. Meta-analysis using random effects model showed $P_{meta} = 1.56 \times 10^{-4}$ for rs3077 (OR = 0.51, 95% CI = 0.36–0.72), and 5.91×10^{-7} for rs9277542 (OR = 0.55, 95% CI = 0.43–0.69). While there was evidence of heterogeneity between these studies for rs3077 ($P_{het} = 0.03$) and no evidence for rs9277542 ($P_{het} = 0.19$), significant associations with HBV clearance were observed with Mantel-Haenszel $P_{meta} = 3.28 \times 10^{-12}$ for rs3077 and 1.42×10^{-10} for rs9277542, when using CMH fixed-effects model. Among the remaining 27 SNPs in the replication study, two SNPs (rs9276431 and rs7768538), located in a genetic region including *HLA-DQ* gene, were marginally replicated in the two sets of HBV carriers and HBV resolved individuals with Mantel-Haenszel *P* values of 2.10×10^{-5} (OR = 0.59) and 1.10×10^{-5} (OR = 0.56), respectively (Table S3), when using CMH fixed-effect model. Due to the existing heterogeneity among three groups (GWAS, Replication-1 and Replication-2) ($P_{het} = 0.03$ for rs9276431 and 0.04 for rs7768538), weak associations were

observed with $P_{meta} = 0.03$ for rs9276431 and 0.02 for rs7768538 by the random effects model meta-analysis.

Meta-analysis across 6 independent studies, including 5 additional published data, showed $P_{meta} = 1.48 \times 10^{-9}$, OR = 0.60 for rs3077, $P_{meta} = 1.08 \times 10^{-17}$, OR = 0.66 for rs9277535 and $P_{meta} = 5.14 \times 10^{-5}$, OR = 0.55 for rs9277542 (Table S4). As shown in Table S4, the OR for the rs9277535 and rs9277542 were similar among the 6 independent studies, and heterogeneity was negligible ($P_{het} = 0.03$ for rs9277535 and 0.14 for rs9277542). However, significant level of heterogeneity for rs3077 was observed with $P_{het} = 9.57 \times 10^{-6}$ across 5 independent studies, including our study.

URLs

The results of the present GWAS are registered at a public database: https://gwas.lifesciencedb.jp/cgi-bin/gwasdb/gwas_top.cgi.

Discussion

The recent genome-wide association study showed that the SNPs located in a genetic region including *HLA-DPA1* and *HLA-DPB1* genes were associated with chronic HBV infection in the Japanese and Thai population [10,11]. In this study, we confirmed a significant association between SNPs (rs3077 and rs9277542) located in the same genetic region as *HLA-DPA1* and *HLA-DPB1* and protective effects against CHB in Korean and Japanese individuals. Meta-analysis using the random effects model across 6 independent studies including our study suggested that, widely in East Asian populations, variants in antigen binding sites of *HLA-DP* contribute to protective effects against persistent HBV infection (Table S2).

On GWAS and replication analysis with Japanese and Korean individuals, we identified associations between the same SNPs (rs3077 and rs9277542) in the *HLA-DPA1* and *HLA-DPB1* genes and HBV clearance; however, no new candidate SNPs from the GWAS were detected on replication analysis (Table S3). When the data of reference#18 was excluded from the meta-analysis across 6 independent studies, heterogeneity among 4 studies was estimated to be $P_{het} = 0.15$ and significant association of rs3077 with HBV clearance was observed with $P_{meta} = 5.88 \times 10^{-24}$, OR = 0.56 (Table S4). In our study, a negligible level of heterogeneity for rs3077 was also observed ($P_{het} = 0.03$) on meta-analysis by adding replication-1 (Table 3). Despite the heterogeneity in replication-1, a marginal association was observed for rs3077 with the same downward trend in the odds ratio ($P = 3.32 \times 10^{-2}$, OR = 0.72). Moreover, meta-analysis using GWAS and replication-2 showed significant association of $P_{meta} = 1.89 \times 10^{-12}$, OR = 0.43 for rs3077 with no evidence of heterogeneity ($P_{het} = 0.75$). Although the reason why heterogeneity was observed in replication-1 is unclear, one possible reason is the clinical heterogeneity due to different kits being used for antibody testing. The associations of *HLA-DPA1*/*-DPB1* with CHB and HBV clearance showed the same level of significance in the comparison of HBV patients with HBV resolved individuals (OR = 0.43 for rs3077 and 0.49 for rs9277542) as the one with healthy controls (OR = 0.46 for rs3077 and 0.50 for rs9277542), when the replication-1 was excluded in the analysis (Table 2 and Table 3). The results of meta-analysis across 6 independent studies including our study also showed the same or slightly weaker associations in the

Table 1. Number of study samples.

		GWAS	Replication-1	Replication-2
population		Japanese	Japanese	Korean
HBV carriers	Total	181	256	344
	IC	20	94	–
	CH	67	101	177
	LC	3	10	–
	HCC	91	51	167
Healthy controls	184	236	151	
Resolved individuals	185	150	106	

Abbreviation: IC, Inactive Carrier; CH, Chronic Hepatitis; LC, Liver Cirrhosis; HCC, Hepatocellular Carcinoma.

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Table 2. Results of replication study for protective effects against CHB.

dbSNP rsID	Position		MAF ^a (allele)	Allele (1/2)	Stage (population)	HBV carriers			Healthy controls			OR ^b			
	Chr	Buld 36.3 Nearest Gene				11	12	22	11	12	22	HWEp	95% CI	P-value ^c	P _{het} ^d
rs3077	6	33141000 HLA-DPA1	0.44	T/C	GWAS	13	51	117	28	88	67	0.919	0.42	1.14 × 10 ⁻⁷	
					(T)	(7.2)	(28.2)	(64.6)	(15.3)	(48.1)	(36.6)		(0.30–0.58)		
					Replication-1	26	95	134	46	125	65	0.309	0.48	2.70 × 10 ⁻⁸	
					(Japanese)	(10.2)	(37.3)	(52.5)	(19.5)	(53.0)	(27.5)		(0.37–0.62)		
					Replication-2	23	81	111	31	74	40	0.767	0.47	2.08 × 10 ⁻⁶	
(Korean)	(10.7)	(37.7)	(51.6)	(21.4)	(51.0)	(27.6)		(0.35–0.65)							
					Meta-analysis ^e						0.46	4.40 × 10 ⁻¹⁹	0.80		
												(0.39–0.54)			
rs9277542	6	33163225 HLA-DPB1	0.45	T/C	GWAS	18	53	110	29	102	52	0.073	0.42	5.32 × 10 ⁻⁸	
					(T)	(9.9)	(29.3)	(60.8)	(15.8)	(55.7)	(28.4)		(0.31–0.58)		
					Replication-1	30	106	118	54	114	67	0.681	0.54	3.33 × 10 ⁻⁶	
					(Japanese)	(11.8)	(41.7)	(46.5)	(23.0)	(48.5)	(28.5)		(0.42–0.70)		
					Replication-2	30	87	94	35	72	36	0.933	0.54	8.29 × 10 ⁻⁵	
(Korean)	(14.2)	(41.2)	(44.5)	(24.5)	(50.3)	(25.2)		(0.40–0.74)							
					Meta-analysis ^e						0.50	1.28 × 10 ⁻¹⁵	0.40		
												(0.43–0.60)			

^aMinor allele frequency and minor allele in 198 healthy Japanese (ref#19).

^bOdds ratio of minor allele from two-by-two allele frequency table.

^cP value of Pearson's chi-square test for allelic model.

^dHeterogeneity was tested using general variance-based method.

^eMeta-analysis was tested using the random effects model.

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comparison of HBV patients with HBV resolved individuals (OR = 0.56 for rs3077, 0.66 for rs9277535 and 0.55 for rs9277542) than in the one with healthy controls (OR = 0.55 for rs3077, 0.61 for rs9277535 and 0.51 for rs9277542), which was the opposite result as we expected (Table S2 and Table S4). These results may suggest that other unknown immune system(s) exist to eliminate the HBV in the HBV resolved individuals.

Among the HLA class II loci (*HLA-DPA1*, *HLA-DPB1* and *HLA-DQB2*), which were associated with CHB and HBV clearance, a weak linkage disequilibrium ($r^2 < 0.1$) was observed between *HLA-DQB2* locus and *HLA-DPA1/-DPB1* loci in Japanese and Korean populations (Figure S2). We also found that similar linkage disequilibrium blocks (r^2) were observed among three subgroups (HBV carriers, HBV resolved individuals and Healthy controls). Moreover, logistic regression analysis of *HLA-DP* (rs3077 and rs9277542) with use of *HLA-DQ* (rs9276431 and rs768538) as covariates showed that the same level of significant associations of *HLA-DP* with CHB and HBV clearance as shown in the single-point association analysis, while no associations of *HLA-DQ* with $P_{log} > 0.05$ were detected both in Japanese and in Korean (Table S5). These results show that *HLA-DP* is the main genetic factor for susceptibility to CHB and HBV clearance, and the associations of *HLA-DQB2* would result from linkage disequilibrium of *HLA-DPA1/-DPB1*.

In this study, we confirmed the significant associations between *HLA-DPA1* and *HLA-DPB1*, and protective effects against CHB and HBV clearance in Japanese and Korean individuals. These results suggest that the associations between the *HLA-DP* locus, CHB and HBV clearance are widely replicated in East Asian populations, including Chinese, Thai, Japanese and Korean individuals; however, there have been no similar GWAS performed in Caucasian and African populations. Moreover,

there were no significant SNPs associated with HCC development in this study, thus suggesting that it is necessary to increase the sample size. To clarify the pathogenesis of CHB or the mechanisms of HBV clearance, further studies are necessary, including a functional study of the *HLA-DP* molecule, identification of novel host genetic factors other than *HLA-DP*, and variation analysis of HBV.

Materials and Methods

Ethics Statement

All study protocols conform to the relevant ethical guidelines, as reflected in the *a priori* approval by the ethics committees of all participating universities and hospitals. The written informed consent was obtained from each patient who participated in this study and all samples were anonymized.

Genomic DNA Samples and Clinical Data

All of the 1,793 Japanese and Korean samples, including individuals with CHB, healthy controls and HBV-resolved individuals (HBsAg-negative and anti-HBc-positive), were collected at 20 multi-center hospitals (liver units with hepatologists) throughout Japan and Korea. The 19 hospitals in Japan were grouped into the following 8 areas: Hokkaido area (Hokkaido University Hospital, Teine Keijinkai Hospital), Tohoku area (Iwate Medical University Hospital), Kanto area (Musashino Red Cross Hospital, Saitama Medical University, Kitasato University Hospital, University of Tokyo), Koshin area (Shinshu University Hospital, Kanazawa University Hospital), Tokai area (Nagoya City University Hospital, Nagoya Daini Red Cross Hospital), Kinki area (Kyoto Prefectural University of Medicine Hospital, National Hospital Organization Osaka National Hospital, Osaka

Table 3. Results of replication study for clearance of hepatitis B virus.

dbSNP rsID	Position			MAF ^a (allele)	Allele (1/2)	Stage (population)	HBV carriers			Resolved individuals			OR ^b 95% CI	P-value ^c	P _{het} ^d
	Chr	Buld	36.3 Nearest Gene				11	12	22	11	12	22			
rs3077	6	33141000	HLA-DPA1	0.44	T/C	GWAS	13	51	117	29	82	74	0.44	9.24×10 ⁻⁷	
							(7.2)	(28.2)	(64.6)	(15.7)	(44.3)	(40.0)	(0.32–0.61)		
						Replication-1	26	95	134	20	64	60	0.72	3.32×10 ⁻²	
							(10.2)	(37.3)	(52.5)	(13.9)	(44.4)	(41.7)	(0.53–0.97)		
						Replication-2	23	81	111	29	48	28	0.41	2.35×10 ⁻⁷	
							(10.7)	(37.7)	(51.6)	(27.6)	(45.7)	(26.7)	(0.29–0.58)		
			Meta-analysis ^e								0.51	1.56×10 ⁻⁴	0.03		
												(0.36–0.72)			
												0.43	1.89×10 ⁻¹²	0.75	
													(0.34–0.54)		
rs9277542	6	33163225	HLA-DPB1	0.45	T/C	GWAS	18	53	110	28	88	69	0.51	3.15×10 ⁻⁵	
							(9.9)	(29.3)	(60.8)	(15.1)	(47.6)	(37.3)	(0.37–0.70)		
						Replication-1	30	106	118	28	62	52	0.68	1.25×10 ⁻²	
							(11.8)	(41.7)	(46.5)	(19.7)	(43.7)	(36.6)	(0.51–0.92)		
						Replication-2	30	87	94	30	53	22	0.46	4.97×10 ⁻⁶	
							(14.2)	(41.2)	(44.5)	(28.6)	(50.5)	(21.0)	(0.33–0.64)		
			Meta-analysis ^e									0.55	5.91×10 ⁻⁷	0.19	
													(0.43–0.69)		
													0.49	9.69×10 ⁻¹⁰	0.65
														(0.39–0.61)	

^aMinor allele frequency and minor allele in 198 healthy Japanese (ref#19).

^bOdds ratio of minor allele from two-by-two allele frequency table.

^cP value of Pearson's chi-square test for allelic model.

^dHeterogeneity was tested using general variance-based method.

^eMeta-analysis was tested using the random effects model.

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City University), Chugoku/Shikoku area (Tottori University Hospital, Ehime University Hospital, Yamaguchi University Hospital, Kawasaki Medical College Hospital) and Kyushu area (Kurume University Hospital). Korean samples were collected at Yonsei University College of Medicine.

HBV status was measured based on serological results for HBsAg and anti-HBc with a fully automated chemiluminescent enzyme immunoassay system (Abbott ARCHITECT; Abbott Japan, Tokyo, Japan, or LUMIPULSE f or G1200; Fujirebio, Inc., Tokyo, Japan). For clinical staging, inactive carrier (IC) state was defined by the presence of HBsAg with normal ALT levels over 1 year (examined at least four times at 3-month intervals) and without evidence of portal hypertension. Chronic hepatitis (CH) was defined by elevated ALT levels (>1.5 times the upper limit of normal [35 IU/L]) persisting over 6 months (at least by 3 bimonthly tests). Liver cirrhosis (LC) was diagnosed principally by ultrasonography (coarse liver architecture, nodular liver surface, blunt liver edges and hypersplenism), platelet counts <100,000/cm³, or a combination thereof. Histological confirmation by fine-needle biopsy of the liver was performed as required. Hepatocellular carcinoma (HCC) was diagnosed by ultrasonography, computerized tomography, magnetic resonance imaging, angiography, tumor biopsy or a combination thereof.

The Japanese control samples from HBV-resolved subjects (HBsAg-negative and anti-HBc-positive) at Nagoya City University-affiliated healthcare center were used by comprehensive agree-

ment (anonymization in an unlinkable manner) in this study. Some of the unrelated Japanese healthy controls were obtained from the Japan Health Science Research Resources Bank (Osaka, Japan). One microgram of purified genomic DNA was dissolved in 100 µl of TE buffer (pH 8.0) (Wako, Osaka, Japan), followed by storage at -20°C until use.

SNP Genotyping and Data Cleaning

For GWAS, we genotyped a total of 550 individuals, including 181 Japanese HBV carriers, 184 Japanese healthy controls and 185 spontaneously HBV-resolved Japanese individuals (HBsAg-negative and anti-HBc-positive), using the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Inc., Santa Clara, CA), in accordance with the manufacturer's instructions. The average QC call rate for 550 samples reached 98.47% (95.00–99.92%), which had an average sample call rate of 98.91% (93.55–99.74%) by determining the genotype calls of over 900 K SNPs using the Genotyping Console v4.1 software (with Birdseed v1 algorithm) provided by the manufacturer [19]. We then applied the following thresholds for SNP quality control in data cleaning: SNP call rate ≥95% and MAF ≥1% for three groups (HBV carriers, healthy controls and HBV-resolved individuals), and HWE P-value ≥0.001 for healthy controls [20]. Here, SNP call rate is defined for each SNP as the number of successfully genotyped samples divided by the number of total samples genotyped. A total of 597,789 SNPs and 590,278 SNPs on autosomal chromosomes

passed the quality control filters in the genome-wide association analysis using HBV carriers and healthy controls, and using HBV carriers and HBV-resolved individuals, respectively (Figure 1). All cluster plots for the SNPs showing $P < 0.0001$ on association analyses in the allele frequency model were confirmed by visual inspection, and SNPs with ambiguous cluster plots were excluded.

In the following replication stage, we selected a set of 32 SNPs with $P < 0.0001$ in the GWAS using HBV carriers and HBV-resolved individuals. SNP genotyping in two independent sets of 256 Japanese HBV carriers, 236 Japanese healthy controls and 150 Japanese HBV-resolved individuals (Table 1, replication-1), and 344 Korean HBV carriers, 151 Korean healthy controls and 106 Korean HBV-resolved individuals (Table 1, replication-2) was completed for the selected 32 SNPs using the DigiTag2 assay [21,22] and custom TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) on the LightCycler 480 Real-Time PCR System (Roche, Mannheim, Germany).

Statistical Analysis

The observed associations between SNPs and the protective effects on chronic hepatitis B or clearance of hepatitis virus B were assessed by chi-squared test with a two-by-two contingency table in allele frequency model. SNPs on chromosome X were removed because gender was not matched among HBV carriers, healthy controls and HBV-resolved individuals. A total of 597,789 SNPs and 590,278 SNPs passed the quality control filters in the GWAS stage; therefore, significance levels after Bonferroni correction for multiple testing were $P = 8.36 \times 10^{-8}$ ($0.05/597,789$) and $P = 8.47 \times 10^{-8}$ ($0.05/590,278$), respectively. For the replication study, 29 of 32 SNPs were successfully genotyped; therefore, we applied $P = 0.0017$ ($0.05/29$) as a significance level, and none of the 29 markers genotyped in the replication stage showed deviations from the Hardy-Weinberg equilibrium in healthy controls ($P > 0.01$).

The genetic inflation factor λ was estimated by applying the Cochran-Armitage test on all SNPs and was found to be 1.056 and 1.030 in the GWAS using HBV carriers and healthy controls, and using HBV carriers and HBV-resolved individuals, respectively (Figure S3). These results suggest that the population substructure should not have any substantial effect on statistical analysis. In addition, the principal component analysis in a total of 550 individuals in the GWAS stage together with the HapMap samples also revealed that the effect of population stratification was negligible (Figure S4).

Based on the genotype data of a total of 1,793 samples including 1,192 Japanese samples and 601 Korean samples in both GWAS and replication stages, haplotype blocks were estimated using the Gabriel's algorithm using the Haploview software (v4.2) (Figure S2). In the logistic regression analysis, two SNPs (rs9276431 and rs7768538) within the HLA-DQ locus were individually involved as a covariate (Table S5). Statistical analyses were performed using the SNP & Variation Suite 7 software (Golden Helix, MT, USA).

Supporting Information

Figure S1 GWAS using samples from HBV carriers with LC or HCC, and HBV carriers without LC and HCC. P values were calculated using chi-squared test for allele frequencies. (PPTX)

Figure S2 Estimation of linkage disequilibrium blocks in HBV patients, HBV resolved individuals and healthy controls in Japanese and Korean. The LD blocks (r^2) were analyzed using the Gabriel's algorithm. (PPTX)

Figure S3 Quantile-quantile plot for test statistics (allele-based chi-squared tests) for GWAS results. Dots represent P values of each SNP that passed the quality control filters. Inflation factor λ was estimated to be: a) 1.056 in the analysis with HBV carriers and healthy controls; and b) 1.030 with HBV carriers and HBV-resolved individuals. (PPTX)

Figure S4 Principal component analysis on a total of 550 individuals in GWAS, together with HapMap samples (CEU, YRI and JPT). (PPTX)

Table S1 Results for 29 SNPs selected in replication study using samples of HBV carriers and healthy controls. ^a P values by chi-squared test for allelic model. ^bOdds ratio of minor allele from two-by-two allele frequency table. ^cMeta-analysis was tested using additive, two-tailed CMH fixed-effects model. (XLSX)

Table S2 Results of meta-analysis for protective effects against persistent HB infection across 6 independent studies, including this study. ^aMinor allele frequency and minor allele in 198 healthy Japanese (ref#19). ^bOdds ratio of minor allele from two-by-two allele frequency table. ^c P value of Pearson's chi-squared test for allele model. ^dHeterogeneity was tested using general variance-based method. ^eMeta-analysis was tested using the random effects model. (XLSX)

Table S3 Results for 29 SNPs selected in replication study using samples from HBV carriers and HBV-resolved individuals. ^a P values by chi-squared test for allelic model. ^bOdds ratio of minor allele from two-by-two allele frequency table. ^cMeta-analysis was tested using additive, two-tailed CMH fixed-effects model. (XLSX)

Table S4 Results of meta-analysis for clearance of HBV across 6 independent studies, including this study. ^aMinor allele frequency and minor allele in 198 healthy Japanese (ref#19). ^bOdds ratio of minor allele from two-by-two allele frequency table. ^c P value of Pearson's chi-squared test for allele model. ^dHeterogeneity was tested using general variance-based method. ^eMeta-analysis was tested using the random effects model. (XLSX)

Table S5 Logistic regression analysis of HLA-DP (rs3077 and rs9277542) and HLA-DQ (rs9276431 and rs7768538) with susceptibility to CHB and HBV clearance using the HLA-DQ genotypes individually as a covariate. (XLSX)

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Author Contributions

Conceived and designed the experiments: NN HS YT. Performed the experiments: HS Y. Mawatari M. Sageshima YO. Analyzed the data: NN MK AK. Contributed reagents/materials/analysis tools: KM M. Sugiyama SHA JYP SH JHK KS M. Kurosaki YA SM MW ET MH SK EO YI EM AT Y. Murawaki YH IS M. Korenaga KH TI NI KHH YT MM. Wrote the paper: NN M. Kawashima YT KT MM.

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Clinical effectiveness of bipolar radiofrequency ablation for small liver cancers

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Abstract

Background Radiofrequency ablation (RFA) is minimally invasive and can achieve a high rate of cure of liver cancer. This study was conducted to evaluate the efficacy and safety of a bipolar RFA device (CelonPOWER System) in the treatment of Japanese liver cancer patients.

Methods The study was a multicenter, single-group, open-label trial. The indications for RFA were based on the Japanese guidelines for the management of liver cancer. The subjects had a Child-Pugh classification of A or B, and the target tumors were defined as nodular, numbering up to 3 lesions, each of which was 3 cm or less in diameter, or solitary lesions up to 4 cm in diameter. To test for the non-inferiority of the CelonPOWER System, this system was compared with the Cool-tip RF System, which has already been approved in Japan, in terms of the complete necrosis rate (CNR).

Results The CNR obtained with the CelonPOWER System was 97.8 % (88/90 patients). The CNR obtained with the Cool-tip RF System was 86.2 % (50/58 patients), confirming the non-inferiority of the CelonPOWER System ($p < 0.001$, Fisher's exact test based on binomial distribution). Throughout the treatment and follow-up periods, there were no adverse events regarding safety that were uniquely related to the CelonPOWER System and there were no cases of device failure.

Conclusions The CelonPOWER System was confirmed to be an effective and safe RFA device. It could become extensively used as a safe next-generation RFA device, reducing the physical burden on patients.

Keywords Small hepatocellular carcinoma · Radiofrequency ablation (RFA) · Bipolar RFA · Conformance European (CE) mark · Non-inferiority to monopolar RFA

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Introduction

According to a report of the Japanese Ministry of Health, Labor and Welfare in 2010, the number of deaths due to malignancies, including hepatocellular carcinoma (HCC), which is the most common type of primary liver cancer, has tended to increase annually [1]. In the 2007 report of the Japanese Ministry of Health, Labor and Welfare, the mortality of liver cancer was the 3rd highest among malignant diseases, following gastric cancer and lung cancer [2]. HCC appears in cirrhotic liver, and cirrhotic liver often results from alcohol abuse or chronic hepatitis B virus (HBV) or HCV infection. The presence of liver cirrhosis limits HCC treatment options, because surgery and systemic chemotherapy impair residual liver function and can induce fatal liver failure. In addition,

even if the primary tumor is completely resected, there is a very high recurrence rate in the residual liver [3, 4].

Radiofrequency ablation (RFA) is a minimally invasive method that can yield radical localized therapeutic results, and it has become a standard treatment for small liver cancers 3 cm or less in diameter [5].

Three different RFA systems have been introduced in Japan, all consisting of monopolar devices. One of the main problems with monopolar RFA devices is that the electrical current flows between the electrodes and the grounding pad that is used in these devices. The current flows in a wide area of the body, which may cause systemic symptoms, such as heat retention and perspiration. In addition, because the applicator is distant from the grounding pad, its low energy efficiency requires a long ablation time. Moreover, energy concentration can occur owing to an unanticipated current pathway between the applicator and grounding pad, posing a risk of burns at the grounding pad patch site and at non-treatment sites [3, 6–9].

A bipolar system, in contrast to the monopolar systems, features as its principal characteristic an electrical current flowing between two electrodes on a single probe. With a bipolar system, the current pathway is limited to only within the treatment area, thus eliminating the need for a grounding pad. A bipolar RFA system also overcomes such disadvantages of a monopolar system as the occurrence of heat retention and other side effects, low energy efficacy, and thermal injuries at electrode pad sites caused by an electrical current flowing in the body. The simultaneous use of multiple applicators with a bipolar system makes it possible to achieve a sufficiently large thermocoagulation volume with a single ablation procedure. That is, one ablation is usually sufficient for a wide area and this enables a short ablation time. In addition, ablation can be achieved even if the electrodes are not inserted directly into the tumor. The use of the bipolar system with multiple applicators with a wide ablation area maximizes the effectiveness of the bipolar system.

The purpose of this study was to evaluate the safety and efficacy of a bipolar RFA device, the CelonPOWER System, in order to obtain the clinical data necessary for an application for its regulatory approval in Japan. The study and protocol were designed in compliance with Japanese good clinical practice (GCP) based on the advice from the Pharmaceuticals and Medical Devices Agency (PMDA) of the Japanese regulatory authority. In designing this study, we were requested by the PMDA to compare this device with an existing RFA device (that had been already approved in Japan) and we selected the data from the 2002 to 2003 clinical study of the Cool-tip RF System as valid control data. The study of the Cool-tip RF System was also conducted to obtain marketing approval in Japan [10]. This study was sponsored by Olympus Medical Systems Corp.

Patients, materials, and methods

Device

Celon AG Medical Instruments (Teltow, Germany) developed a bipolar RFA device (CelonPOWER System) in order to overcome the disadvantages of monopolar RFA devices. Unlike a monopolar RFA system, the prime characteristic of this new device is its bipolar feature, i.e., two electrodes are located on the same needle (Fig. 1a, b), allowing electricity flow only between the electrodes at the treatment target site, eliminating both the need for a grounding pad and the danger of burns (Fig. 2a, b).

The bipolar characteristics of the CelonPOWER system ensure the return of power to the device, and the simultaneous use of multiple applicators yields an extensive ablated area in a single treatment, which can reduce treatment time and the burden on the patient. This eliminates the need for repeated reinsertion of single monopolar needles to perform overlapping ablation. Another advantage of the bipolar device is that electric current is immediately retrieved, preventing it from flowing to unintended sites. The CelonPOWER System was awarded the Conformance Européenne (CE) mark in 2003, and since then its use has spread mainly in Europe [11–17].

The CelonPOWER System consists of a high-frequency power generator, a water pump, and computerized applicators for regulation of the current frequency. The basic

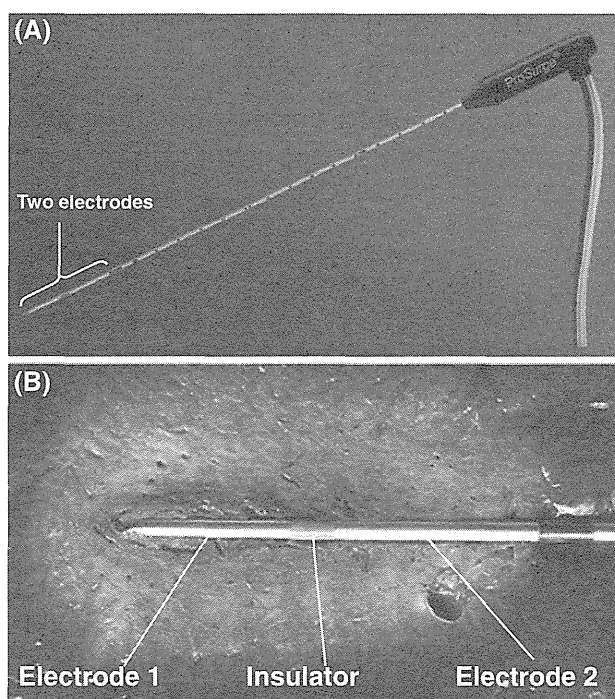


Fig. 1 In the CelonPOWER System, each applicator is needle-shaped and has two electrodes near its tip

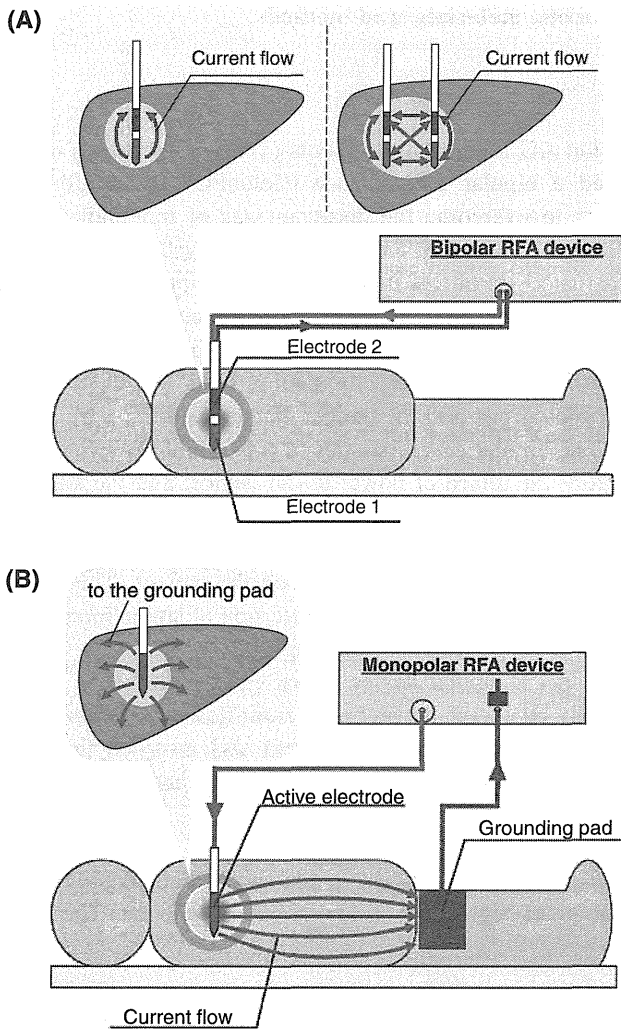


Fig. 2 Differences in the electrical flow routes of **a** the monopolar and **b** the bipolar (CelonPOWER System) radiofrequency ablation (RFA) systems. With the bipolar system (CelonPOWER System), the electrical current flows between the two electrodes, and for this reason the current pathway is limited to the treatment area, allowing lower power to be concentrated in a specific area and yet yielding effects equivalent to those obtained by higher energy monopolar devices, the power of which is dispersed throughout the body to the dispersion grounding pads placed under the patient

frequency of the power generator is 470 kHz, with a maximum output of 250 W. All the needles for RFA are 1.8 mm in width (15 G) but there are 3 different lengths: 20, 30, and 40 mm. The Cool-tip RF System needles are 1.5 mm in width (17 G).

Bipolar applicators

Each applicator is needle-shaped and has two electrodes near its tip. The electrical current flows between the two electrodes on the single probe, limiting the current pathway to within the treatment area. A grounding pad is

unnecessary (Fig. 2a). The applicators are cooled by the internal circulation of chilled water.

Multipolar application

When simultaneously using multiple applicators (up to 3 can be employed simultaneously), it is possible to treat relatively large cancers that could not be sufficiently ablated by means of one insertion of a single applicator. The high-frequency electrical current flows sequentially between the electrodes of the applicators (6 electrode pair combinations when there are 2 applicators, 15 electrode combinations when there are 3 applicators) (Fig. 3a).

Resistance controlled automatic power (RCAP)

RCAP is a function that monitors the change of electric resistance between the electrodes, and automatically

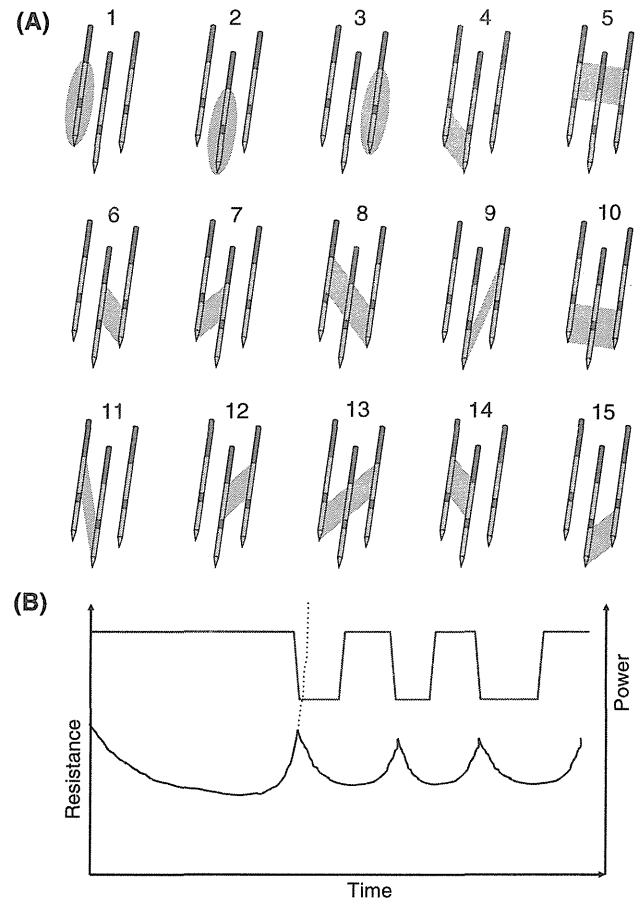


Fig. 3 When 3 applicators are employed, the high-frequency electrical current flows sequentially between 15 combinations of electrode pairs (a), and an image is generated of the automated control of the output by the resistance controlled automatic power (RCAP) function (b). RCAP is a function by which the degree of change in the electrical resistance among the electrodes (increase/decrease in slope) is monitored, and the high-frequency power output is automatically controlled

controls the high-frequency power (Fig. 3b). This function makes it possible to prevent unexpected rapid increases in electrical resistance resulting from tissue necrotization.

Patients

This clinical study was carried out based on the HCC treatment algorithm in the Scientific Data-based Clinical Practice Guidelines for Liver Cancer-2005 Version [18]. We enrolled adult male and female patients aged 20 years or older with primary or metastatic small liver cancers who had provided written informed consent. Target tumors were defined as nodular, numbering up to 3 lesions, each of which was 3 cm or less in diameter, or solitary lesions up to 4 cm in diameter. Exclusion criteria included a Child-Pugh grade of C, or platelet count below 50000/ μ l. Informed consent was obtained from 104 patients, of whom 96 were initially enrolled, but 5 withdrew consent before the trial started. The trial was therefore carried out in a total of 91 patients (112 treated lesions) with intention-to-treat (ITT) analysis, and 90 patients were eligible for the analysis of efficacy.

Patient details

Table 1 summarizes the data on the background characteristics of the 91 patients and 112 treated lesions treated in the study (73 patients had 1 lesion, 15 had 2, and 3 patients had 3 lesions; Table 1). The cohort consisted of 61 men and 30 women, and the mean age (\pm SD) was 69 ± 10 years; 84 patients had primary liver cancer, while 7 had metastatic liver cancer.

Study design

This prospective multicenter, collaborative, single-group, open-label study was conducted at 5 institutions between December 2008 and December 2009. The study protocol was approved by each center's institutional review board. The trial treatment period lasted from the acquisition of written informed consent through completion of the final treatment (maximum 3 treatments), in addition to a follow-up period from the day after the final examinations of the treatment period until the completion of examinations performed 24 weeks later. The non-inferiority of the CelonPOWER System was evaluated relative to the results obtained with a Cool-tip RF System in 2002–2003 [10].

Study methodology

Figure 4 shows the study procedures. During the treatment period, the following procedures were performed, in the order listed: registration of eligible patients, RFA treatment

and examinations including computed tomography (CT) imaging, laboratory tests, and blood pressure measurement. The efficacy was evaluated from the extent of the necrotic area (tumor necrosis; TN) induced by ablation as measured on conventional and dynamic CT imaging. Additional ablation, up to a maximum of 3 sessions, was performed as necessary. The laboratory tests consisted of RBC count, WBC count, hemoglobin level, hematocrit, platelet count, prothrombin time (PT) activity, total bilirubin, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), and creatinine.

In the follow-up phase, at 10 ± 2 weeks (70 ± 14 days) and 24 ± 2 weeks (168 ± 14 days) following the day of the final RFA session, we performed CT imaging, laboratory tests, blood pressure measurement, measurement of alpha-fetoprotein (AFP), and measurement of protein induced by vitamin K absence or antagonist II (PIVKA-II). The CT images and tumor marker data were employed to assess the continuity of the therapeutic effect (TE) of the RFA treatment.

RFA procedure

The procedure with the CelonPOWER System device was similar to the procedure with the existing monopolar RFA devices. In all cases, the procedure was performed percutaneously under ultrasound guidance and local anesthesia.

Assessment of efficacy

TN was assessed using 5 grades, in accordance with the Criteria for Direct Effects of Liver Cancer Treatment (1994) [19]. Class V tumor necrosis (100 % TN) of liver cancer following the final RFA session was defined as "complete necrosis," and the percentage of patients achieving Class V TN was defined as the "complete necrosis rate" (CNR), the primary endpoint. The TN classification was used for short-term (during treatment) evaluation, and this was the only evaluation reported for the Cool-tip RF System in the marketing authorization holder's application for Japanese government approval. However, now the government demands not only short-term evaluation, but also long-term evaluation, for which such parameters as TE, overall response, and complete response (CR) are used.

The secondary endpoints of our study were the number of RFA sessions, the TE, and the overall assessment of the TE. The assessment of the immediate TE and the overall assessment of TE were performed in accordance with the General Rules for the Clinical and Pathological Study of Primary Liver Cancer (2008) [20]. The TE was classified as either CR (total necrosis and normalization of all tumor

Table 1 Patient background factors and lesion characteristics

Patients (<i>n</i> = 91)		Lesions (<i>n</i> = 112)	
Background factors	<i>N</i> (%)	Characteristics	<i>N</i>
Sex		Maximum dimension (cm)	
M	61 (67.0)	<1.0	22
F	30 (33.0)	1.1–2.0	69
Age (years)		2.1–3.0	17
31–40	1 (1.1)	3.1–4.0	4
41–50	4 (4.4)	Mean ± SD	
51–60	9 (9.9)	1.6 ± 0.7	
61–70	32 (35.2)	Subsegment	
71–80	34 (37.4)	S1	0
81–90	11 (12.1)	S2	6
Cancer		S3	9
Primary	84 (92.3)	S4	8
Metastatic	7 (7.7)	S5	18
Underlying disease		S6	20
Cirrhosis	63 (69.2)	S7	18
Chronic hepatitis	22 (24.2)	S8	33
None	6 (6.6)		
Child-Pugh classification			
Grade A	83 (91.2)		
Grade B	8 (8.8)		
Number of treated lesions			
1	73 (80.2)		
2	15 (16.5)		
3	3 (3.3)		
Previous treatment of primary disease			
Yes	40 (44.0)		
No	51 (56.0)		

markers), or others. In addition, ITT analysis was performed in regard to the cumulative local recurrence rate and the overall assessment of the TE.

Assessment of safety

The following safety endpoints were assessed in all 91 patients in whom the study was conducted: overall safety assessment, adverse events, device-related adverse events, device failure, laboratory test values, and blood pressure.

Statistical analysis

Statistical analysis was performed using a one-sided significance level of 2.5 % for the primary endpoint. In principle, a two-sided significance level of 5 % was used for the other endpoints to avoid data dispersion. The CNR (the primary endpoint) was calculated as the percentage of the total number of patients who achieved Class V TN, and its exact one-sided 97.5 % confidence

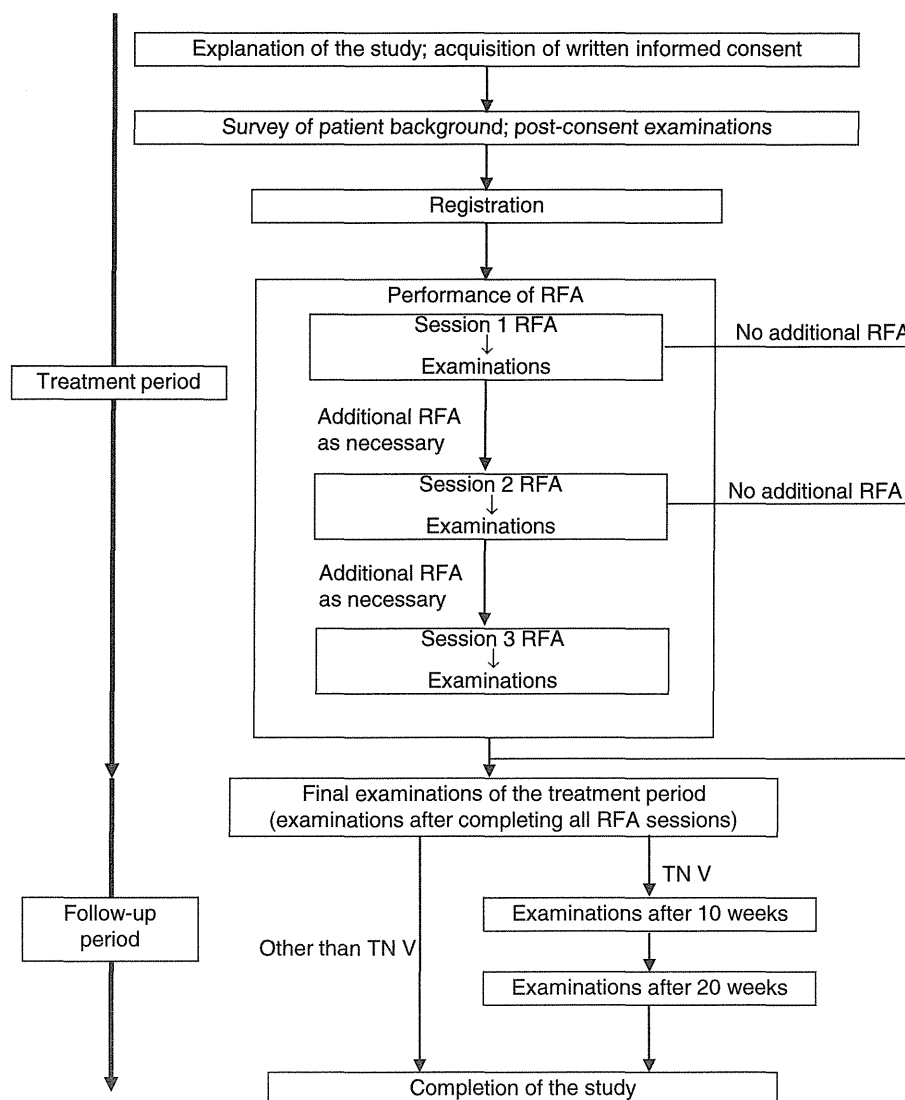
interval was calculated. For the secondary endpoints, the variables and their ratios were compiled, and the basic statistics for the mean and standard deviation were calculated.

Results

Patients

Written informed consent was obtained from 104 patients, including the 96 patients in the study. The study was conducted in 91 of these patients, and treatment was completed in 90 patients. Eighty-eight of the 90 patients (excluding 2 TN4 patients) were followed up. Five patients discontinued the study during the follow-up period, leaving 83 patients who completed the follow-up period. Three patients were excluded because of unacceptable enrollment dates, so the final number of patients eligible for the efficacy analysis was 80.

Fig. 4 Clinical study procedure. *TN* Tumor necrosis



Efficacy

Of the 90 patients who completed this clinical treatment study, 88 showed Class V TN (97.8%). The 2 patients (2.2%) who did not show 100% TN both had primary liver cancers and were categorized as Class IV TN. The CNR was 100% in patients with metastatic liver cancer (7/7 patients) and 97.6% in patients with primary liver cancer (81/83 patients). The Japanese package insert for the Cool-tip RF System [21] states that the CNR obtained by that system was 86.2% (50/58 patients). Assuming a 5% non-inferiority margin, the lower limit of the confidence interval (one-sided 97.5%) was 92.2%, and the p value was <0.001 for the exact test based on binomial distribution.

The initial success rate (Class V TN after 1 session) was 77.8% (70 of 90 patients), while Class V TN was seen in 16 (17.8%) patients following a second session. The remaining 4 (4.4%) patients underwent a third RFA

session, and 2 were rated as Class V TN following that session.

We used 1 applicator in 20 patients, 2 simultaneously in 54 patients, and 3 simultaneously in 16 patients. We used 30-mm electrodes in all the patients, except in 3 of the 16 patients in whom 3 electrodes were used simultaneously; in these 3 patients we used 3 40-mm electrodes. A representative case in which 3 applicators were used is shown in Fig. 5.

Of the 88 patients who proceeded to the follow-up phase, excluding the single out-of-hospital fatality, examination at 24 weeks showed that CR was obtained in 94.3% (82/87). The cumulative local recurrence rate at the end of 24 weeks in the follow-up period was 5.7% (5/87 patients; ITT analysis) (Table 2).

Figure 6a, b shows a comparison of the treatment results of the Cool-tip RF System clinical trial [21] and the number of patients analyzed for the CNR and the efficacy

Fig. 5 Images in a female patient who had hepatocellular carcinoma (HCC) in segment VI. Before treatment, scans obtained on computed tomography during hepatic arteriography (CTHA) (a) and computed tomography during arterial portography (CTAP) (b) showed a nodular HCC (arrow) measuring 2.5 cm. Three applicators were placed in parallel in the HCC in liver segment VI, and then the tumor was ablated in one procedure (total ablation time 13 min 42 s, total applied energy 35.3 kJ). After the procedure, computed tomography (CT) images showed a necrotic area of 46 mm in diameter including the nodular HCC (c [arrows show applicator for insertion paths], d [arrow shows applicator for insertion paths])

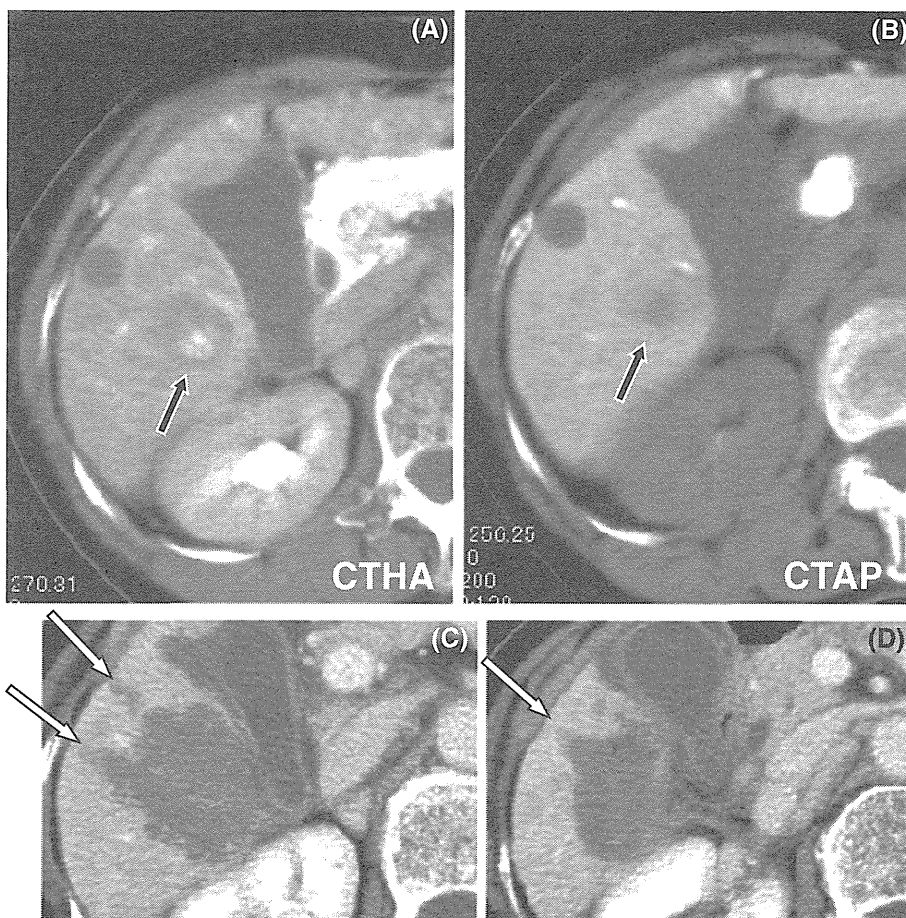


Table 2 Maintenance of the therapeutic effect (TE) (overall assessment of the TE; intention-to-treat (ITT) analysis)

	This clinical study		Patients who underwent local therapy [24]	
	10 weeks	24 weeks ^a	3 months	6 months
Complete response (CR) (no. of patients)	85/88 (96.6 %)	82/87 (94.3 %)	4468/5394 (82.8 %)	4318/5378 (80.3 %)
Other (no. of patients)	3/88 (3.4 %) ^b	5/87 (5.7 %) ^c	926/5394 (17.2 %)	1060/5378 (19.7 %)

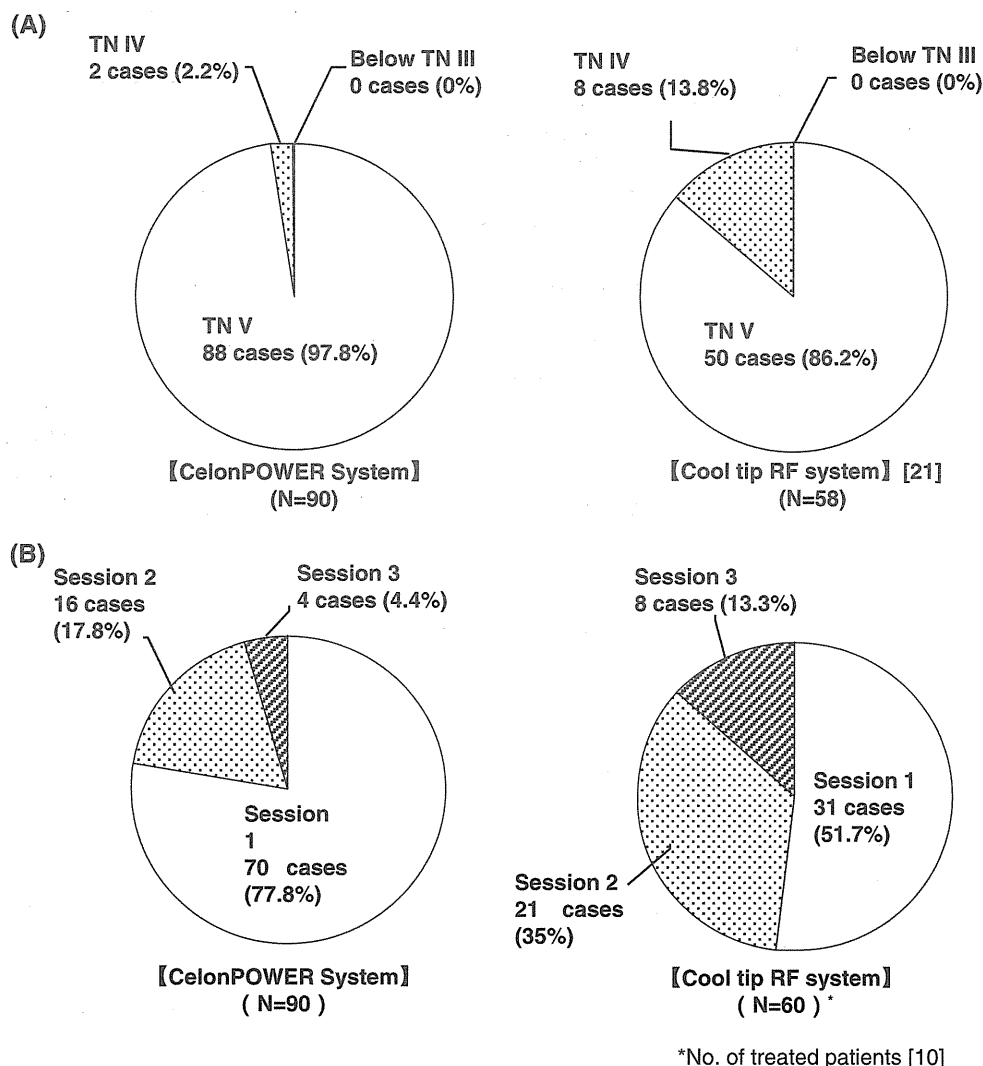
^a One patient who died was omitted from the 24-week assessment
^b Includes 3 patients who developed local recurrence within 10 weeks
^c Includes 5 patients who developed local recurrence within 24 weeks

of each RFA session in the present clinical study. As shown in Fig. 6a, the complete necrosis (Class V TN) rate with the CelonPOWER System was 97.8 % (88/90 patients), which was higher than the rate of 86.2 % (50/58 patients) with the Cool-tip RF System. These results thus confirm the non-inferiority of the CelonPOWER System ($p < 0.001$; Fisher’s exact test based on binomial distribution). As shown in Fig. 6b, the percentage of patients in whom treatment was completed in a single session was 77.8 % (70/90 patients) in the present study with the CelonPOWER System, compared with 51.7 % (31/60 patients) in the Cool-tip RF System study [10].

Safety

The overall safety assessment was performed for the entire clinical study period, i.e., inclusive of the treatment period and the follow-up period. Of the 91 patients included in the safety analysis, no procedure was rated as unsafe, although 2 procedures (2.2 %) were rated as somewhat unsafe, one with an abdominal wall burn and one with biliary peritonitis owing to bile leakage; 78 procedures (85.7 %) were rated as safe overall and 11 procedures (12.1 %) were rated as safe. There was no device failure. In the patient with biliary peritonitis, three 30-mm electrodes had been

Fig. 6 Comparison of the present results obtained with the CelonPOWER System and the clinical study results reported for the Cool-tip RF System. The percentage of Class V tumor necrosis (TN) (TN 100 %) cases (a) and the number of patients in whom each RFA session was completed (b)



simultaneously inserted into an S8 tumor, and treatment was finished in a single ablation.

During the course of the entire clinical study period, serious adverse events (i.e., events for which a causal relationship with the CelonPOWER System could not be ruled out) were seen in 3 patients, consisting of abdominal wall burn, pleural effusion, and biliary peritonitis. Each of those events was judged to be serious because they required prolongation of hospitalization, and each required treatment. In addition, it was judged that each of these serious adverse events was a known adverse event that had been observed with similar, already-approved RFA devices [21–23]. Also, the single fatality, which occurred at home, had occurred in a patient who had been hospitalized for treatment on the suspicion of peritonitis based on the examinations performed after 10 weeks in the follow-up period. The patient’s condition had improved and the patient had been discharged, and it was later confirmed that death had occurred at home. Autopsy revealed the cause of death to have been due to the progression of cirrhosis, and

it was thus thought that the death was not related to the treatment with the CelonPOWER System. Table 3 shows the most common adverse effects (those observed in 5 % of patients or more) and all of these (pleural effusion, nausea, vomiting, postprocedural pain, and fever) have been known to occur with previously approved local therapeutic devices. Moreover, all the adverse events were easily controllable.

Discussion

We set out to prospectively determine whether a bipolar RFA device (CelonPOWER System) was safe and effective in the treatment of liver cancer and whether it could be demonstrated to be non-inferior to a monopolar RFA system currently approved and employed clinically in Japan (Cool-tip RF System).

Treatment was completed in a fewer number of sessions when using the CelonPOWER System than with the Cool-

Table 3 Frequently observed adverse effects (5 % or more) (adverse reactions at an incidence of >5 % in the overall study period)

Adverse event	No. of patients	%	No. of patients treated (%)	Treatments
Aspartate aminotransferase (AST) increase	72	79.1	0 (0)	–
Alanine aminotransferase (ALT) increase	69	75.8	0 (0)	–
Lactate dehydrogenase (LDH) increase	22	24.2	0 (0)	–
Total bilirubin increase	20	22.0	0 (0)	–
Pleural effusion	12	13.2	2 (2.20)	Human serum albumin, cefmetazole sodium, tazobactam piperacillin hydrate
Vomiting	12	13.2	7 (7.69)	Metoclopramide
Nausea	10	11.0	9 (9.89)	Metoclopramide, domperidone, diazepam
Postoperative pain	9	9.9	3 (3.30)	Pentazocine, loxoprofen sodium hydrate, acetaminophen, diclofenac sodium
White blood cell count increase	8	8.8	1 (1.10)	Sulbactam sodium–cefoperazone sodium
Platelet count decrease	6	6.6	0 (0)	–
Alkaline phosphatase (ALP) increase	5	5.5	0 (0)	–
Fever	5	5.5	5 (5.49)	Loxoprofen sodium hydrate, acetaminophen, cefmetazole sodium

tip RF System, suggesting that this new system yields efficacy that is at least equivalent to that achieved with the Cool-tip RF System, while causing less of a treatment burden on the patient.

We assessed the TE level, and its maintenance in ITT cases after 10 weeks and 24 weeks (6 months) in the follow-up period of this clinical study and found that the overall TE assessment was not inferior to that of the National Follow-up Survey Report on Primary Hepatic Carcinoma (2004–2005) [24] issued by the Liver Cancer Study Group of Japan (Table 2). Considering that the method for overall TE assessment in that report was the same as that employed in the present study, it is reasonable to conclude that the TE maintenance with the CelonPOWER System is not inferior to that of other local therapy.

Nishikawa et al. reported on local recurrence when using monopolar systems clinically. They found that, in 269 patients with solitary hypervascular HCCs who had undergone RFA, the 1- and 2-year cumulative local recurrence rates were 12.8 and 23.6 %, respectively [25]. We believe that our present results for the cumulative local recurrence rate (5.7 % for 6 months) with the CelonPOWER System are comparable to those reported results.

The introduction of a new device inevitably raises the question of its safety. In our series, there were 3 adverse events—one event of abdominal wall burn and one of pleural effusion during the treatment period, and one event of biliary peritonitis during the follow-up period. These

adverse events were previously known to be possible adverse events that had been observed with the Cool-tip, RITA, and Boston monopolar RFA systems that have already been approved for clinical use in Japan [21–23]. Therefore, similar caution concerning internal adverse events is necessary when using the CelonPOWER System, although the problem of external burns does not exist with this system.

The high-incidence (≥ 5 %) device-related adverse event rate during the course of our clinical study was similar to the rates with the Cool-tip, RITA, and Boston monopolar RFA systems [21–23].

Therefore, these events are not unique to the CelonPOWER System, and the safety of the CelonPOWER System is not inferior to that of the existing approved RFA devices.

This study has several limitations. First of all, although it was a prospective study, it was not a randomized controlled clinical study. However, all consecutive patients who satisfied the enrollment criteria were offered the opportunity to participate and the study was performed in all those who provided informed consent and decided to receive the treatment. After providing informed consent, 5 patients decided not to participate and 1 ceased treatment after 1 session, due to the difficulty posed by the proximity of the lesion to the heart and lungs.

Although we were able to compare our own results immediately after treatment with those of the Cool-tip RF

System and other systems, we were not able to compare the results 6 months after treatment because of the lack of such data for the Cool-tip RF System, because of the different GCP guidelines in force at the time of the Cool-tip RF System study. However, the 6-month follow-up data of our study were very satisfactory. Furthermore, because there were no such data available in the reports on the Cool-tip RF System, we could not compare the levels of experience of the operators in the two studies.

In conclusion, the present clinical study confirmed that the CelonPOWER System is a very safe and highly effective RFA system for liver cancer in Japanese patients. In addition, because this system is a bipolar device, it operates with high energy efficiency, and because multiple multipolar applicators can be employed simultaneously, coagulation necrosis of an extensive tumor tissue volume can be achieved in a short treatment time. Moreover, throughout the course of this clinical study, most of the patients did not experience hot flushes or perspiration. It is therefore anticipated that the CelonPOWER System will become used as a next-generation RFA system that is not only safer than existing systems, but is highly effective and places less physical burden on the patient.

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