Table 3 Demography and laboratory data of 69 patients in training group

	F1 ($n = 27$)	F2 ($n = 20$)	F3 ($n = 20$)	F4 ($n = 2$)
Demographics				
Men : women	18:9	15:5	13:7	2:0
Age (median, range)	36 (13–64)	45 (14–64)	36.5 (24–59)	32 (25–39)
Laboratory data (median, range)				
WBC (×1000/mm^3)	5.0 (2.8–8.7)	5.8 (2.8–11.6)	5.3 (3.2–8.1)	3.85 (2.7–5.0)
Hemoglobin (g/dL)	14.8 (12.4–17.4)	15.0 (12.4–16.9)	14.4 (11.1–16.4)	14.4 (12.5–16.3)
Platelet (×1000/mm^3)	204 (86–322)	180 (90–275)	147 (90–276)	130 (67–183)
Albumin (g/dL)	4.4 (2.8–5.2)	4.2 (3.5–5.1)	4.3 (3.4–4.9)	4.45 (4.0–4.9)
Bilirubin (mg/dL)	0.9 (0.4–6.4)	0.8 (0.2–1.6)	0.75 (0.4–1.7)	1.15 (1.1–1.2)
AST (IU/L)	52 (17–575)	50.5 (21–272)	65 (22–284)	248.5 (51–446)
ALT (IU/L)	84 (16–1101)	101.5 (19–554)	86.5 (16–1113)	453.5 (74–833)
γ-GTP (IU/L)	42 (14–332)	54 (16–205)	52.5 (13–191)	193 (57–329)
γ-Globulin (g/dL)	1.30 (1.04–1.59)	1.35 (1.18–2.53)	1.62 (1.16–1.97)	1.545 (1.51–1.58)
γ-Globulin (%)	17.9 (14.3–22.1)	19.6 (15.5–30.8)	22.0 (16.5–24.6)	20.15 (19.3–21.0)
α-2-Macroglobulin (mg/dL)	287 (160–687)	270 (89–452)	272.5 (211–463)	389 (313–465)
Haptoglobin (mg/dL)	58 (<5–229)	74 (<5–154)	56.5 (<5–198)	<5 (<5–<5)
Apolipoprotein A-I (mg/dL)	146 (95–216)	137 (87–162)	120 (88–170)	100.5 (74–127)
Hyaluronic acid (μg/L)	27 (<5–113)	36 (10–1050)	59 (14–439)	331 (225–437)
TIMP-1 (ng/mL)	168.5 (83–302)	176 (127–408)	182 (104–303)	390.5 (283–498)
TIMP-2 (ng/mL)	76 (25–143)	86.5 (28–154)	77.5 (32–141)	100.5 (91–110)
Procollagen III peptide (U/mL)	0.71 (0.27–2.20)	0.88 (0.63–2.80)	0.995 (0.60–2.10)	1.75 (1.50–2.00)
Type IV collagen 7S (ng/ml)	3.6 (2.7–17.0)	5.25 (3.3–13.0)	5.7 (3.0–16.0)	15.5 (15.0–16.0)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyl transpeptidase; TIMP, tissue inhibitor of matrix metalloproteinase; WBC, white blood cells.

Recently, non-invasive estimation of severity of liver fibrosis has been reported in patients with HBV-related chronic hepatitis.[2,6–13] However, these studies were principally aimed at differentiation of advanced fibrotic stages of F3 or F4 from mild fibrotic stages of F1 or F2. Those discrimination functions were insufficient to recognize the stepwise progression of viral hepatitis from F1–F4. This dichotomy (mild or severe) of chronic hepatitis B seemed less valuable in the study of disease progression, disease control abilities of antiviral drugs and estimation of histological improvement after anti-inflammatory drugs. A histology-oriented, practical and reliable formula is therefore required for the diagnosis and investigation of chronic hepatitis B.

This study aimed to establish non-invasive evaluation and calculation of liver fibrosis for patients with chronic hepatitis B virus infection. Although it was retrospectively performed as a multicenter study of eight institutions, judgment of histological diagnosis was independently performed by four pathologists in another hospital, who were informed only of the patient's age, sex and positive HBV infection. Objective judgment of the histological staging and grading in sufficient biopsy specimens could be obtained.

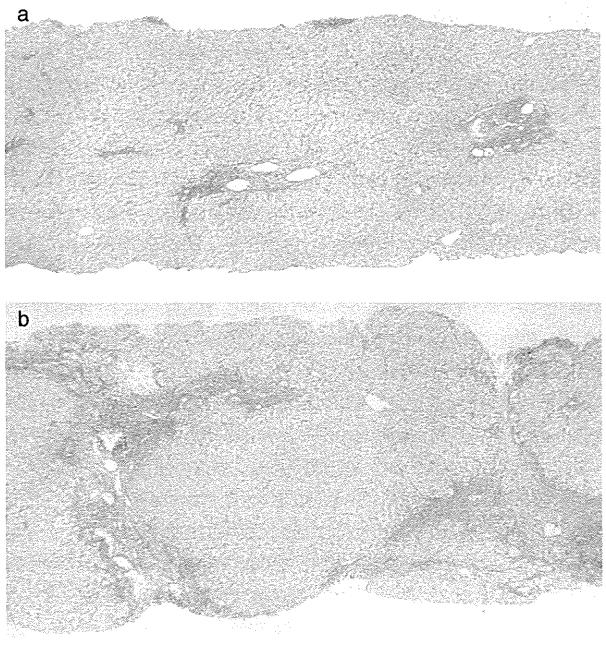

Figure 2 Case presentations of the training set. (a) A 28-year-old man with F1 fibrosis. Final regression function provided his fibrosis score as 0.99. (b) A 45-year-old man with F3 fibrosis. His regression coefficient was calculated as 3.10. Silver stain, ×40.

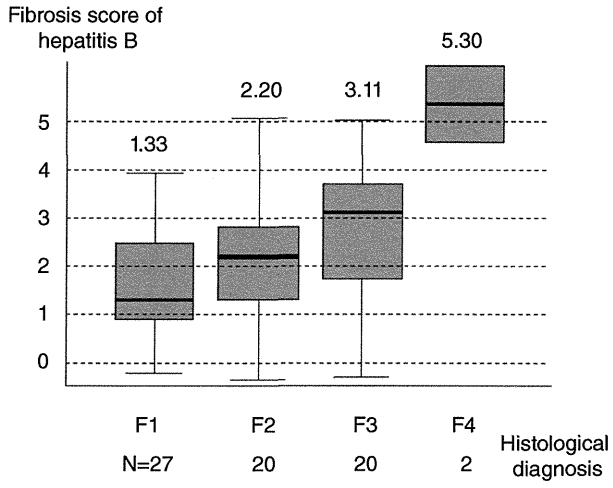

Figure 3 Box and whisker plots of fibrotic score of each group of histological fibrosis in the validation dataset. The fibrosis score of hepatitis B was generated by the function, $z = 1.40 \times ln$ (type IV collagen 7S) (ng/mL) − $0.017 \times$ (platelet count) ($\times 1000^3/mm^3$) + $1.24 \times ln$ (tissue inhibitor of matrix metalloproteinase-2) (ng/mL) + $1.19 \times ln$ (α-2-macroglobulin) (mg/dL) − 9.15.

As many as 227 patients with chronic hepatitis B were analyzed in this study, who had been diagnosed as having chronic hepatitis or cirrhosis by liver biopsy performed in experienced liver units in Japan. To obtain the most suitable equation approximating histological fibrotic stage, multivariate analysis was performed using two demographic parameters (age and sex) and 21 hematological and biochemical markers with or without logarithmic transformation. They included many kinds of fibrosis markers: α-2-macroglobulin, haptoglobin concentration, haptoglobin typing, apolipoprotein A1, hyaluronic acid, TIMP-1, TIMP-2, procollagen III peptide and type IV collagen 7S Multiple regression analysis finally generated a first-degree polynomial function consisting of four variables: type IV collagen 7S, platelet count, TIMP-2 and α2-macroglobulin. A constant numeral (−9.15) was finally adjusted in the regression equation in order to obtain fitted figures for a fibrotic stage of F1–F4. From the magnitude of the standardized partial regression coefficient of individual variable in the function, platelet count demonstrated the most potent contribution toward the prediction of liver fibrosis. Type IV collagen 7S and *ln* (TIMP-2) proved to be the second and third distinctive power in the model, respectively.

The FSB was sufficiently fitted to actual fibrotic stages with certain overlapping as is usually found in histological ambiguity judged by pathologists. Because judgment of fibrosis in chronic hepatitis often shows a transitional histological staging, pathological examination cannot always make a clear-cut diagnosis discriminating F1–F4. Considering the limitation of the pathological difficulty in differentiating the four continuous disease entities, the obtained regression function showed satisfactory high accuracy rates in the prediction of liver disease severity. The FSB can provide one or two decimal places (e.g. 3.2 or 3.24) and the utility of the score is possibly higher than the mere histological stage of F1–F4. The reproducibility was confirmed by the remaining 67 patients' data obtained from the other six hospitals. Although the validation data were collected from a different geographic area and different chronological situation, the FSB showed similar results in prediction of histological staging.

The FSB seemed a very useful quantitative marker in evaluating fibrotic severity of hepatitis B patients without invasive procedures and without any specialized ultrasonography or magnetic resonance imaging. The FSB also has an advantage of measurement, in which old blood samples are available for retrospective assessment of varied clinical settings: for example, old sera from 20 years prior to the time of initial liver biopsy, or paired sera before and after long-term antiviral therapy. These kinds of retrospective assessments of fibrotic staging will be valuable in estimating a long-term progression of liver disease, in evaluating efficacy of long-term medication or other medical intervention, or in making a political judgment from the viewpoints of socioeconomic efficacy.

The score can be calculated for any patients with chronic HBV infection. Although this multiple regression model dealt with appropriate logarithmic transformation for non-normal distribution parameters, the regression analysis was based on a linear regression model. Very slight fibrosis can be calculated as less than 1.00, which is commonly found to a slight degree in chronic hepatitis with tiny fibrotic change as F0. Very severe fibrosis might be calculated as more than 4.00, which is an imaginary and nonsense number in the scoring system of fibrosis. The FSB is, however, very useful and valuable in a real clinical setting: estimation of severity of liver fibrosis in an outpatient clinic, evaluation of the natural progression of a patient's fibrosis over 10 years and assessment of a long-term administration of interferon in patients with chronic hepatitis B from the viewpoint of fibrotic change. Recent development of new nucleoside/nucleotide analogs requires evaluation for long-term histological advantage, for aggravation of hepatitis stage during viral and biochemical breakthrough caused by HBV mutation, and even for

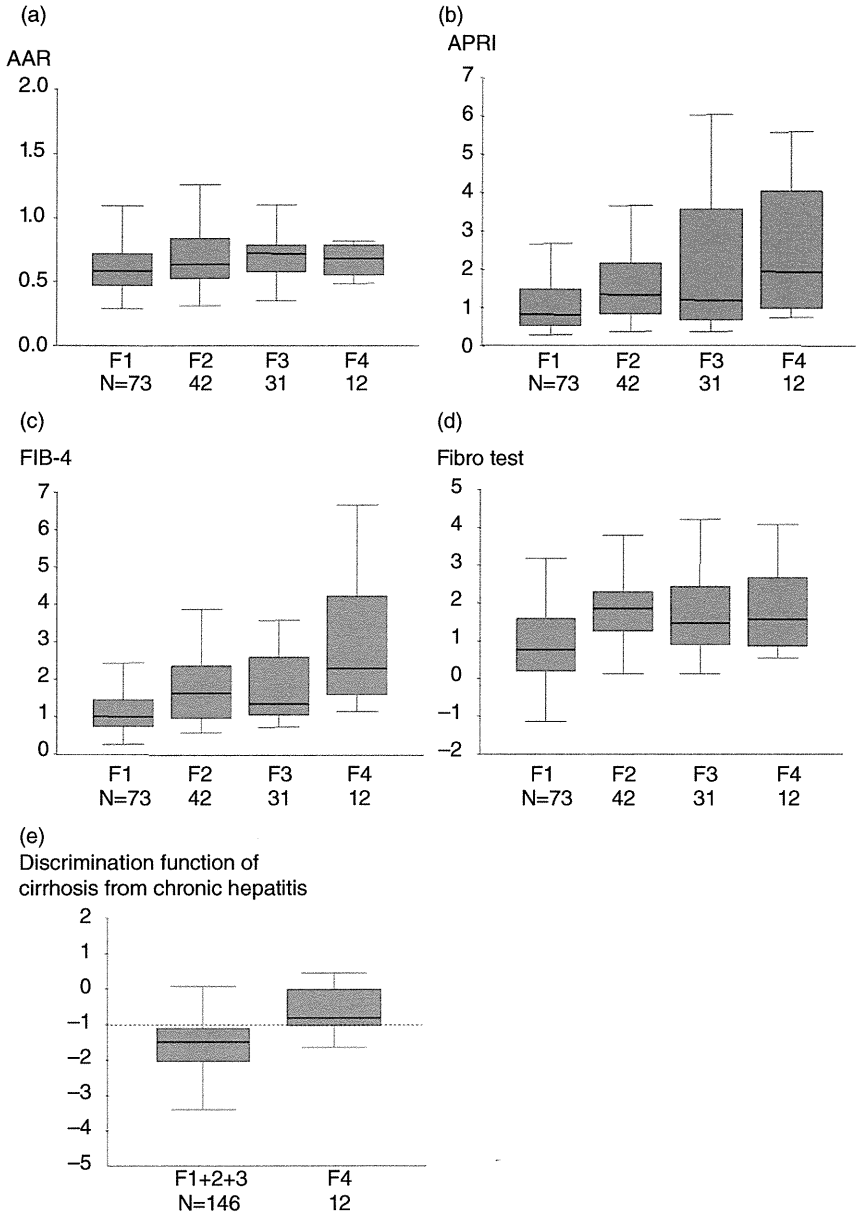

Figure 4 Previously published fibrosis scores. (a) Aspartate aminotransferase/alanine aminotransferase ratio (AAR),[19] (b) aspartate aminotransferase-to-platelet ratio index (APRI),[20] (c) FIB-4,[21] (d) FibroTest[22] and (e) discrimination function of cirrhosis from hepatitis in Japanese patients.[23]

the best management of patients with chronic hepatitis B. The FSB seems one of the ideal methods of approximating the fibrotic stage of chronic hepatitis B. Repeated measurement is quite suitable for patients with an unestablished treatment or trial, every 1 or 2 years, for example. Because the current regression function was generated from the data of HBV-related chronic liver disease, this equation would not be suitable for the recognition of hepatitis C virus-related chronic liver disease, alcoholic liver disease, and other congenital or autoimmune liver diseases. To recognize the latter diseases, other studies of individual diseases must be performed.

We compared the usefulness of the FSB with that of other fibrosis scores.[19–23] The more simple and less expensive AAR or APRI could not estimate fibrotic stages with poor correlation coefficients of 0.199 and 0.265, which are much lower than the coefficient of the FSB of 0.625. FibroTest, which contained three costly fibrosis markers (α-2-macroglobulin, haptoglobin and apolipo-

protein A1), also showed a low correlation coefficient of 0.330, suggesting that its usefulness was limited in HBV positive oriental patients. Although FIB-4 demonstrated the best coefficient of 0.412 among the fibrosis scores, significant overlaps were found between neighboring stages and obtained scores were not coordinated for real histological classification.

In conclusion, the FSB was a useful and reliable biomarker for prediction of liver fibrosis in patients with chronic HBV infection. The FSB is expected to be introduced and utilized in varied kinds of studies and trials. Its accuracy and reproducibility require further validation using higher numbers of patients in several countries other than Japan.

ACKNOWLEDGMENTS

THIS STUDY WAS proposed and initiated by Dr Shiro Iino and the project was performed with a grant from the Viral Hepatitis Research Foundation of Japan.

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J Gastroenterol
DOI 10.1007/s00535-012-0685-x

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Clinical effectiveness of bipolar radiofrequency ablation for small liver cancers

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Received: 2 May 2012 / Accepted: 7 September 2012

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 · Conformite Europeenne (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,

Published online: 10 October 2012

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 Conformite Europeenne (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

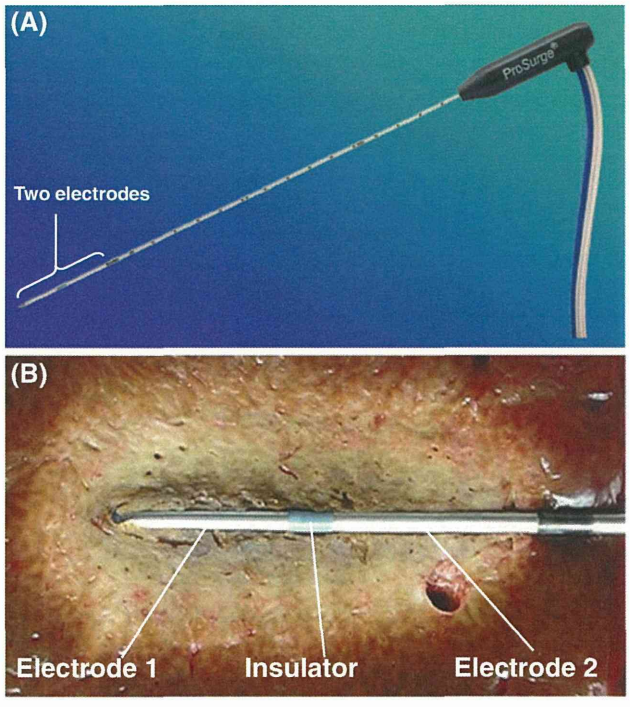

Fig. 1 In the CelonPOWER System, each applicator is needle-shaped and has two electrodes near its tip

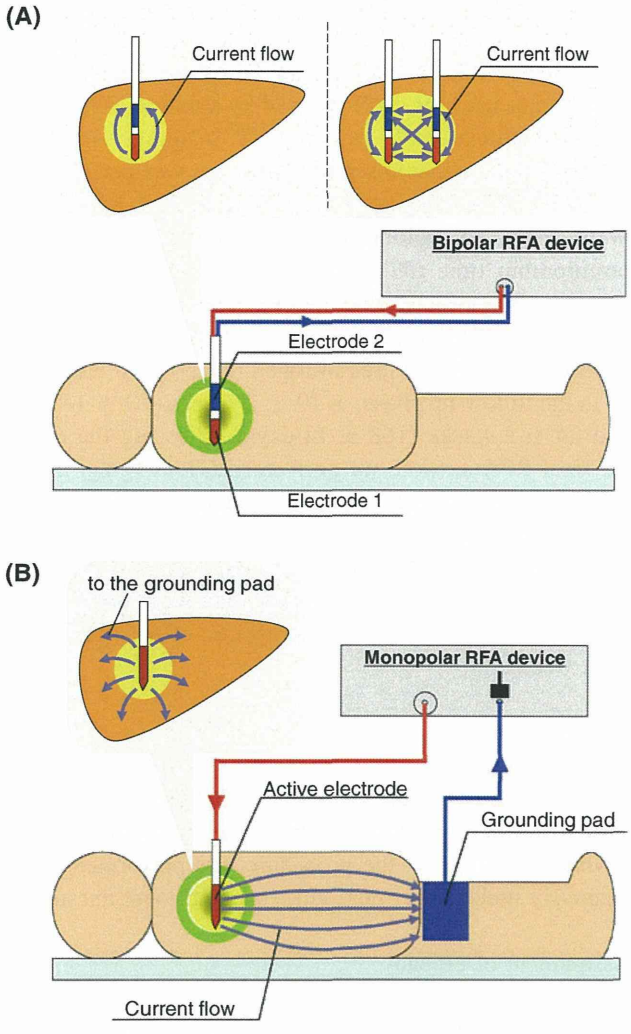

Fig. 2 Differences in the electrical flow routes of **a** the monopolar and **b** the bipolar (CelonPOWER System) radiofrequency ablation (*RFA*) systems. With the bipolar system (CelonPOWER System), the electrical current flows between the two electrodes, and for this reason the current pathway is limited 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

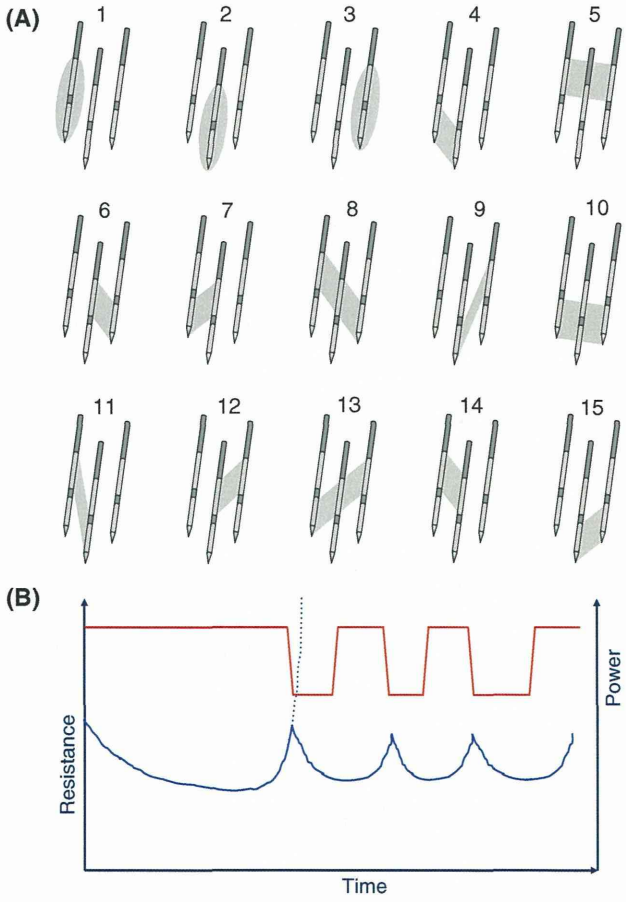

Fig. 3 When 3 applicators are employed, the high-frequency electrical current flows sequentially between 15 combinations of electrode pairs (**a**), and an image is generated of the automated control of the output by the resistance controlled automatic power (RCAP) function (**b**). RCAP is a function 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/μl. 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 (±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 ($n = 91$)		Lesions ($n = 112$)	
Background factors	*N* (%)	Characteristics	*N*
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.

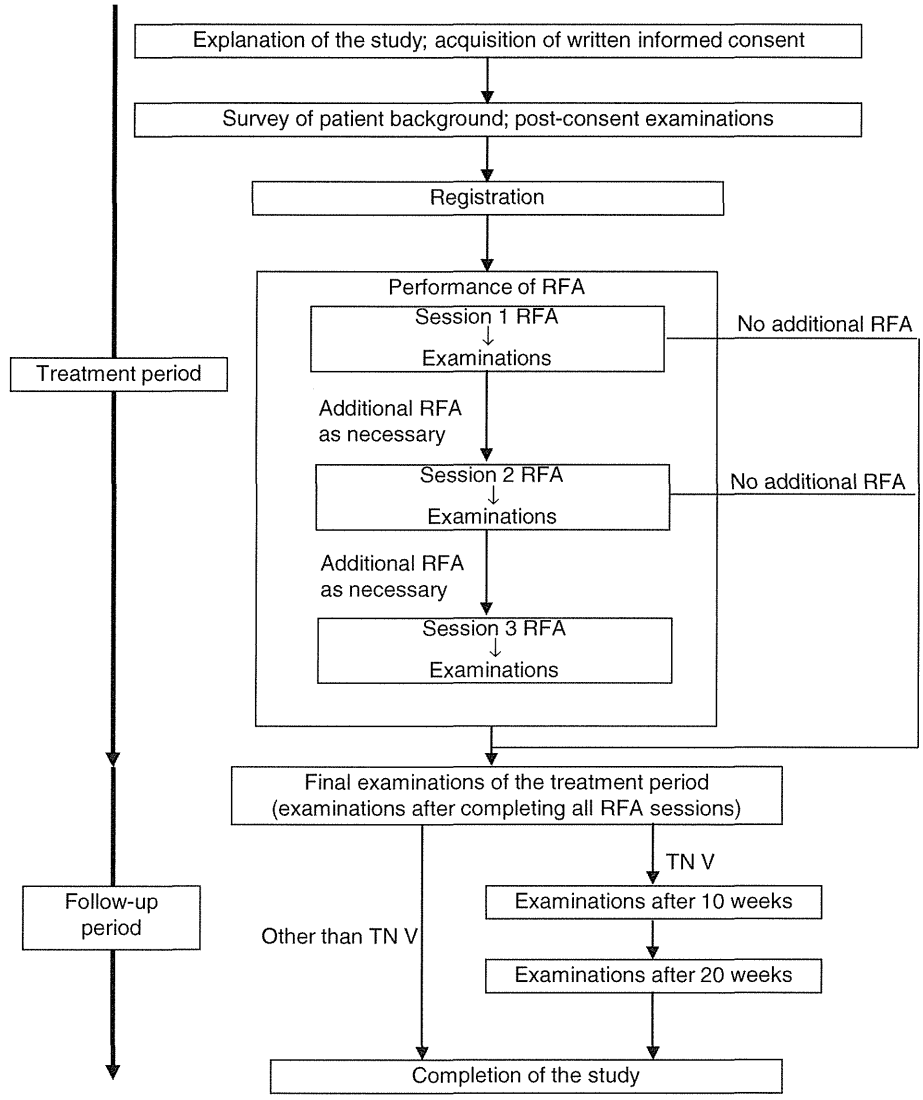

Fig. 4 Clinical study procedure. *TN* Tumor necrosis

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

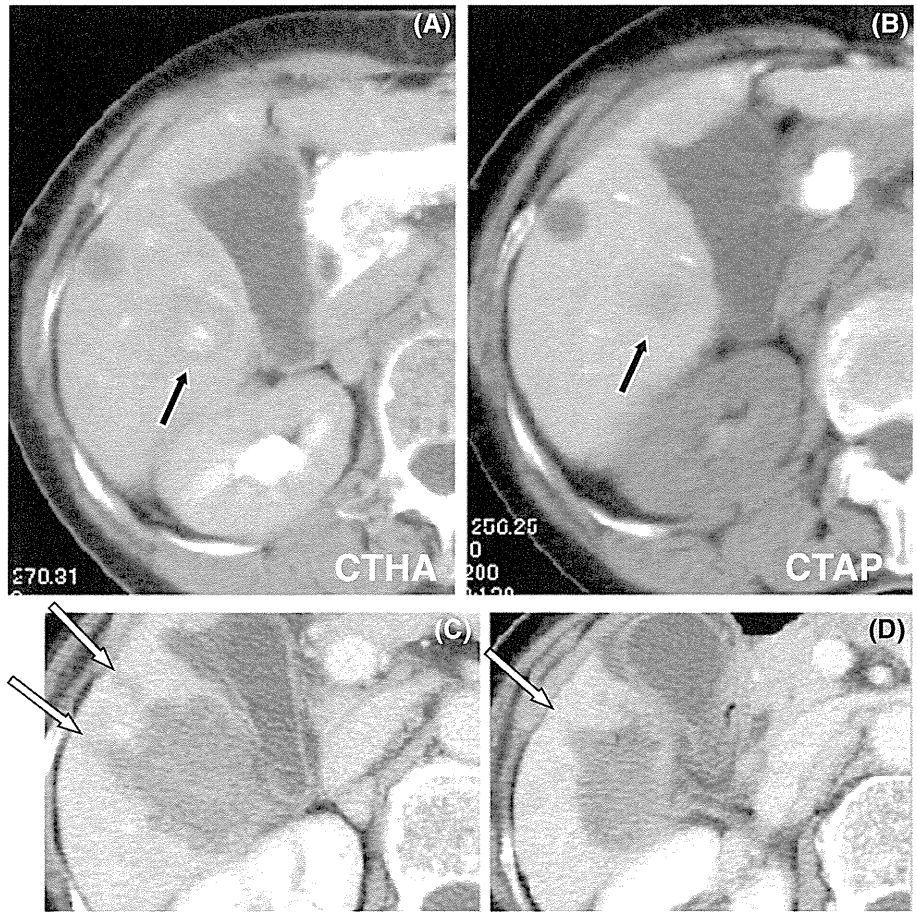

Fig. 5 Images in a female patient who had hepatocellular carcinoma (HCC) in segment VI. Before treatment, scans obtained on computed tomography during hepatic arteriography (CTHA) (**a**) and computed tomography during arterial portography (CTAP) (**b**) showed a nodular HCC (*arrow*) measuring 2.5 cm. 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

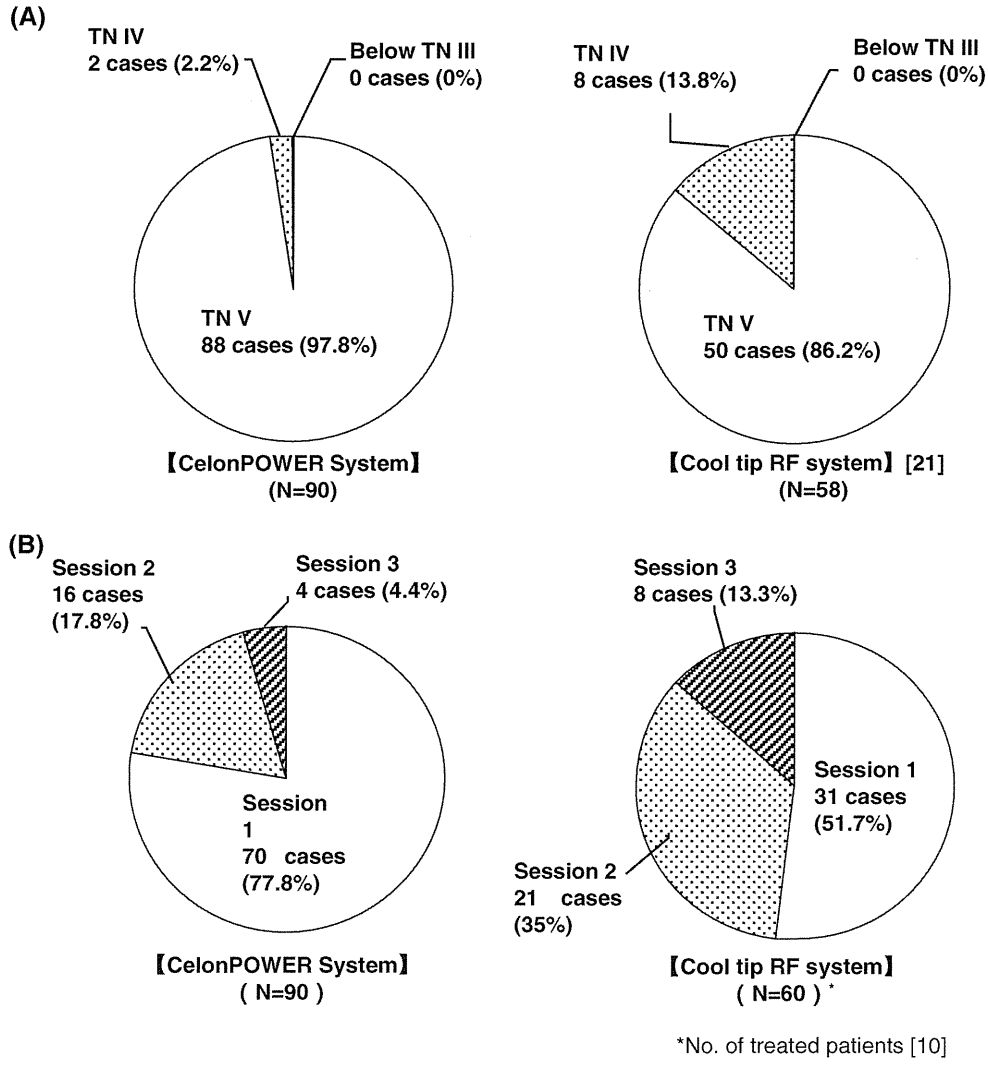

Fig. 6 Comparison of the present results obtained with the CelonPOWER System and the clinical study results reported for the Cool-tip RF System. The percentage of Class V tumor necrosis (*TN*) (TN 100 %) cases (**a**) and the number of patients in whom each RFA session was completed (**b**)

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.

Acknowledgments The authors wish to thank all persons involved in this clinical study for their contributions. The authors are also grateful to Professor J. Patrick Barron, Chairman of the Department of International Medical Communications, Tokyo Medical University, who is also a non-remunerated Editor and Consultant of the *Journal of Gastroenterology*, for retranslating and reediting this manuscript. The authors are also grateful to Mr. Takayuki Ikadai, Group Leader of the Biostatistics Group of JGC Pharma Services Co., for his statistical work in this paper.

Conflict of interest Shinji Hatta received a salary from Olympus Medical Systems Corp., which supported this study.

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HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

FGF3/FGF4 Amplification and Multiple Lung Metastases in Responders to Sorafenib in Hepatocellular Carcinoma

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The response rate to sorafenib in hepatocellular carcinoma (HCC) is relatively low (0.7%-3%), however, rapid and drastic tumor regression is occasionally observed. The molecular backgrounds and clinico-pathological features of these responders remain largely unclear. We analyzed the clinical and molecular backgrounds of 13 responders to sorafenib with significant tumor shrinkage in a retrospective study. A comparative genomic hybridization analysis using one frozen HCC sample from a responder demonstrated that the 11q13 region, a rare amplicon in HCC including the loci for *FGF3* and *FGF4*, was highly amplified. A real-time polymerase chain reaction–based copy number assay revealed that *FGF3/FGF4* amplification was observed in three of the 10 HCC samples from responders in which DNA was evaluable, whereas amplification was not observed in 38 patients with stable or progressive disease ($P = 0.006$). Fluorescence *in situ* hybridization analysis confirmed *FGF3* amplification. In addition, the clinico-pathological features showed that multiple lung metastases (5/13, $P = 0.006$) and a poorly differentiated histological type (5/13, $P = 0.13$) were frequently observed in responders. A growth inhibitory assay showed that only one *FGF3/FGF4*-amplified and three *FGFR2*-amplified cancer cell lines exhibited hypersensitivity to sorafenib *in vitro*. Finally, an *in vivo* study revealed that treatment with a low dose of sorafenib was partially effective for stably and exogenously expressed *FGF4* tumors, while being less effective in tumors expressing *EGFP* or *FGF3*. *Conclusion: FGF3/FGF4* amplification was observed in around 2% of HCCs. Although the sample size was relatively small, *FGF3/FGF4* amplification, a poorly differentiated histological type, and multiple lung metastases were frequently observed in responders to sorafenib. Our findings may provide a novel insight into the molecular background of HCC and sorafenib responders, warranting further prospective biomarker studies. (HEPATOLOGY 2012;00:000-000)

Abbreviations: 5FU, 5-fluorouracil; CGH, comparative genomic hybridization; DMEM, Dulbecco's modified Eagle's medium; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; FFPE, formalin-fixed, paraffin-embedded; FISH, fluorescence in situ *hybridization; HCC, hepatocellular carcinoma; IC_{50}, 50% inhibitory concentration; mRNA, messenger RNA; PCR, polymerase chain reaction; PIVKA-II, protein induced by vitamin K absence or antagonist-II; RPMI-1640, Roswell Park Memorial Institute 1640; RT-PCR, reverse-transcription PCR.*

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Received October 7, 2011; accepted June 25, 2012.

Supported by the Third-Term Comprehensive 10-Year Strategy for Cancer Control (K. N.), a National Cancer Center Research and Development Fund (H22-015, to M. K.), and a Grant-in-Aid for Scientific Research (23650627, to K. N.).

**These authors contributed equally to this work.*

Hepatocellular carcinoma (HCC) is the sixth most common cancer-related cause of death in the world annually, and the development of new primary tumors, recurrences, and metastasis are the most common causes of mortality among patients with HCC.[1,2] Sorafenib (Nexavar; Bayer Healthcare Pharmaceuticals Inc.) is a small molecule kinase inhibitor that is classified as an anti-angiogenic inhibitor.[3] Sorafenib inhibits the kinase activities of Raf-1 and B-Raf in addition to vascular endothelial growth factor receptors, platelet-derived growth factor receptor β, Flt-3, and c-KIT. Two large randomized controlled trials reported a significant clinical benefit of single-agent sorafenib in extending overall survival in both Western and Asian patients with advanced unresectable HCC.[4,5] Consequently, sorafenib is now used as a standard therapy for HCC. The mechanisms of action that lead to these remarkably prolonged overall survival periods are thought to result from the anti-angiogenic effects of sorafenib and its characteristic inhibitory effect on Raf-1 and B-Raf signaling. In these trials, a partial response was observed in 0.7% (2/299) and 3.3% (5/150) of the patients treated with sorafenib.[4-5]

Recently, emerging evidence has demonstrated that some responders exhibit rapid tumor regression as a result of sorafenib treatment for HCC. Complete responses were observed in two patients with advanced HCC and multiple lung metastases, with rapid tumor regression observed even after short-term treatment with sorafenib.[6,7] The drastic tumor response to sorafenib seems to be similar to the tumor response obtained using other tyrosine kinase inhibitors to target a deregulated signal in cancer cells. For example, constitutively active mutations of epidermal growth factor receptor (EGFR) tyrosine kinase in non–small cell lung cancer are associated with a striking treatment response to gefitinib, a selective EGFR tyrosine kinase inhibitor.[8,9] We hypothesized that these HCC cells may harbor a genetic background conducive to a drastic response to sorafenib, rather than the typical anti-angiogenic effect. In this study, we retrospectively searched for genetic changes using mainly formalin-fixed, paraffin-embedded (FFPE) samples from patients with HCC who had undergone sorafenib treatment.

Patients and Methods

Reagent and Cell Culture. Sorafenib was provided by Bayer Healthcare Pharmaceuticals Inc. (Montville, NJ). All cell lines used in this study were maintained in Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Sigma, St. Louis, MO) except for IM95, OUMS23, Colo320, WiDr, HLF, HLE, Huh7, and HepG2 (Dulbecco's modified Eagle's medium [DMEM]; Nissui Pharmaceutical, Tokyo, Japan); LoVo (F12; Nissui Pharmaceutical, Tokyo, Japan); KYSE180, KYSE220, and KYSE270 (RPMI-1640:F12, 1:1); KYSE150 (F12); and KYSE70 (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY) or 2% FBS for the KYSE series plus penicillin and streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. These cell lines were obtained from the American Type Culture Collection (Manassas, VA) and the Japanese Collection of Research Bioresources Collection (Sennan-shi, Osaka, Japan).

Patients and Samples. The inclusion criteria for the study were as follows: patients with histologically confirmed HCC who had been treated with sorafenib, from whom pretreatment tumor samples were available. Finally, the clinical characteristics of a total of 55 cases of HCC from 12 medical centers were evaluated retrospectively. In the gene copy number analysis, four samples were excluded because of an insufficient quantity of DNA, two samples were excluded because of the poor quality of the DNA and two samples were response not evaluable. One not evaluable sample was poor DNA quality. Thus, the copy number assay was performed using the remaining 48 samples. Meanwhile, a series of 82 HCC samples were obtained from frozen specimens of surgical specimens at the Kinki University Faculty of Medicine. The tumor response was evaluated using computerized tomography according to the Response Evaluation Criteria in Solid Tumors; the response was then classified as a complete response, a partial response, stable disease, progressive disease, or not evaluable. The clinico-pathological features evaluated included age, sex, viral infection, alpha-fetoprotein level, protein induced by vitamin K absence or antagonist-II (PIVKA-II), clinical stage, primary tumor size, metastatic lesion, histological type,

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View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep.25956
Potential conflict of interest: Nothing to report.

treatment response, and duration of sorafenib treatment. The present study was approved by the institutional review boards of all the centers involved in the study, and informed consent was obtained from the patients.

Isolation of Genomic DNA. Genomic DNA samples were extracted from deparaffinized tissue sections preserved as FFPE tissue using a QIAamp DNA Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA samples were extracted from surgical frozen sections using a QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions. The DNA concentration was determined using the NanoDrop2000 (Thermo Scientific, Waltham, MA).

Comparative Genomic Hybridization Analysis. The Genome-wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA) was used to perform array comparative genomic hybridization (CGH) on genomic DNA from HCC and paired liver samples according to the manufacturer's instructions. A total of 250 ng of genomic DNA was digested with both Nsp I and Sty I in independent parallel reactions, subjected to restriction enzymes, ligated to the adaptor, and amplified using polymerase chain reaction (PCR) with a universal primer and TITANIUM Taq DNA Polymerase (Clontech, Palo Alt, CA). The PCR products were quantified, fragmented, end-labeled, and hybridized onto a Genome-wide Human SNP6.0 Array. After washing and staining in Fluidics Station 450 (Affymetrix), the arrays were scanned to generate CEL files using the GeneChip Scanner 3000 and GeneChip Operating Software version 1.4. In the array CGH analysis, sample-specific copy number changes were analyzed using Partek Genomic Suite 6.4 software (Partek Inc., St. Louis, MO).

Copy Number Assay. The copy numbers for *FGF3* and *FGF4* were determined using commercially available and predesigned TaqMan Copy Number Assays according to the manufacturer's instructions (Applied Biosystems, Foster City, CA) as described.[10] The primer IDs used for the *FGFs* were as follows: *FGF3*, Hs06336027_cn; *FGF4*, HS01235235_cn. The *TERT* locus was used for the internal reference copy number. Human Genomic DNA (Clontech) and DNA from noncancerous FFPE tissue were used as a normal control.

Real-Time Reverse-Transcription PCR. Real-time reverse-transcription PCR (RT-PCR) was performed as described.[11] In brief, complementary DNA was prepared from the total RNA obtained from each surgical frozen section using a GeneAmp RNA-PCR kit (Applied Biosystems). Real-time RT-PCR amplification was performed using a Thermal Cycler Dice (TaKaRa, Otsu, Japan) in accordance with the manufacturer's instructions under the following conditions: 95°C for 5 minutes, followed by 50 cycles of 95°C for 10 seconds and 60°C for 30 seconds. The primers used for the real-time RT-PCR were as follows: *FGF3*, 5′-TTT GGA GAT AAC GGC AGT GGA-3′ (forward) and 5′-CGT ATT ATA GCC CAG CTC GTG GA-3′ (reverse); *FGF4*, 5′-GAG CAG CAA GGG CAA GCT CTA-3′ (forward) and 5′-ACC TTC ATG GTG GGC GAC A-3′ (reverse); *GAPD*, 5′-GCA CCG TCA AGG CTG AGA AC-3′ (forward) and 5′-ATG GTG GTG AAG ACG CCA GT-3′ (reverse). *GAPD* was used to normalize expression levels in the subsequent quantitative analyses.

Fluorescence* In Situ *Hybridization Analysis. Fluorescence *in situ* hybridization (FISH) was performed as described.[10] Probes designed to detect the *FGF3* gene and *CEN11p* on chromosome 11 were labeled with fluorescein isothiocyanate or Texas red and were designed to hybridize to the adjacent genomic sequence spanning approximately 0.32 Mb and 0.63 Mb, respectively. The probes were generated from appropriate clones from a library of human genomic clones (GSP Laboratory, Kawasaki, Japan).

Immunoblotting. Western blot analysis was performed as described.[11] The following antibodies were used: monoclonal FGF3 (R&D Systems, Minneapolis, MN), FGF4 and FGFR2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and phosphorylated FGFR and horseradish peroxidase–conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA). NIH-3T3 cells were exposed to the indicated concentrations of sorafenib for 2 hours and were then stimulated with FGF4-conditioned medium for 20 minutes.

Cell Growth Inhibitory Assay. To evaluate growth inhibition in the presence of various concentrations of sorafenib, we used an MTT assay as described.[12]

Plasmid Construction, Viral Production, and Stable Transfectants. The methods used in this section have been described.[12] The complementary DNA fragment encoding human full-length *FGF3* or *FGF4* was isolated using PCR and Prime STAR HS DNA polymerase (TaKaRa, Otsu, Japan) with following primers: *FGF3*, 5′-GG GAA TTC GCC GCC ATG GGC CTA ATC TGG CTG CTA-3′ (forward) and 5′-CC CTC GAG GCC CAG CTA GTG CGC ACT GGC CTC-3′ (reverse); *FGF4*, 5′-GG GAA TTC GCC GCC ATG TCG GGG CCC GGG ACG GCC GCG GTA GCG C-3′ (forward) and 5′-CC CTC GAG

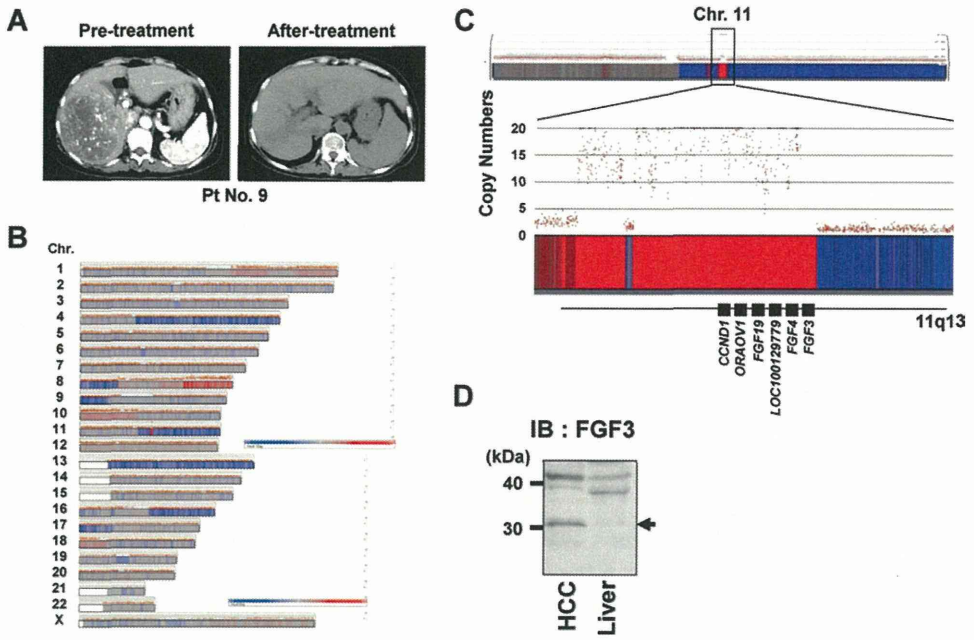

Fig. 1. HCC exhibiting a marked response to sorafenib treatment harbors *FGF3/FGF4* gene amplification. (A) Abdominal CT images obtained pretreatment (left panel) and 2 months after treatment (right panel). (B) CGH analysis of the tumor. Paired background liver tissue was used as a reference sample. A gain (>4 copies, red) and a loss (<0.5 copies, blue) of genomic copy number are shown. (C) Whole copy numbers of chromosome 11 are shown. A highly amplified region is described in the lower panel. (D) Western blot analysis of FGF3 (arrow) in HCC and paired background liver samples. IB, immunoblotting.

GGA GGG TCA CAG CCT GGG GAG GAA GTG GGT GAC CTT C-3′ (reverse). The stable transfectants expressing *EGFP* or *FGF3* or *FGF4* for each cell line were designated as A549/EGFP, A549/FGF3, and A549/FGF4.

Xenograft Studies. Nude mice (BALB/c nu/nu, 6-week-old females; CLEA Japan Inc., Tokyo) were used for *in vivo* studies and were cared for in accordance with the recommendations for the handling of laboratory animals for biomedical research compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiments, Kinki University. Mice were subcutaneously inoculated with a total of 5×10^6 A549/EGFP, A549/FGF3, or A549/FGF4 cells. Two weeks after inoculation, the mice were randomized according to tumor size into two groups to equalize the mean pretreatment tumor size among the three groups (n = 20 mice per group). The mice were then treated with a low dose of oral sorafenib (n = 10, 15 mg/kg/day) or vehicle control (n = 10, Cremophor EL/ethanol/water) for 9 days. Tumor volume was calculated as length × width^2 × 0.5 and was assessed every 2 to 3 days.

Statistical Analysis. The statistical analyses were performed to test for differences between groups using the Student *t* test or Fisher's exact test. $P < 0.05$ was considered statistically significant. All analyses were performed using PAWS Statistics 18 (SPSS Japan Inc., Tokyo, Japan).

Results

Responder to Sorafenib Who Harbored* FGF3/FGF4 *Gene Amplification. A 58-year-old woman was diagnosed as having histologically confirmed advanced HCC (Fig. 1A, left panel) with multiple lung metastases. She received combination treatment with sorafenib, 5-fluorouracil (5FU), and interferon, and a subsequent treatment assessment revealed a partial response. Because the disease was well controlled with sorafenib treatment for 14 months (Fig. 1A, right panel), surgery was performed. To characterize this tumor molecularly, we performed array CGH analysis using frozen surgical specimens of the HCC region and paired background liver tissue as a reference control. The array CGH analysis revealed a low-level gain in the genomic DNA copy number for 1q, 8q, 10p, and 18p and a high level gain at 11q13 (Fig. 1B). Interestingly, the 11q13 region, a rare amplicons in HCC that contains several genes, including *FGF3*, *FGF4*, *CCND1*, and *FGF19*, was highly amplified over 20 copies (Fig. 1C). Western blot analysis revealed that FGF3 was overexpressed in the HCC specimen compared with the paired background liver specimen (Fig. 1D).

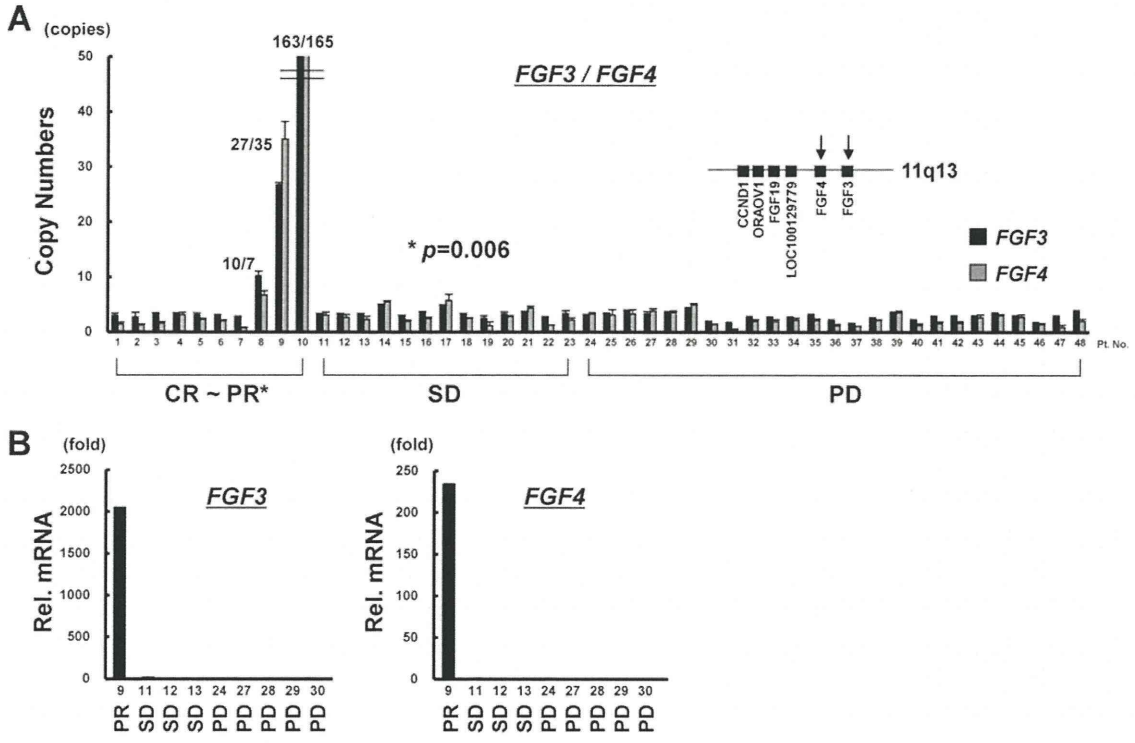

Fig. 2. *FGF3/FGF4* gene amplification is frequently observed in responders to sorafenib in HCC. (A) *FGF3/FGF4* gene amplification was determined using the TaqMan copy number assay in DNA samples obtained from 48 HCC samples that had been treated with sorafenib. *FGF3* amplification of >5 copies was observed in three of the sorafenib responders. *Complete response + partial response versus stable disease + progressive disease. (B) *FGF3/FGF4* gene amplification mediates the overexpression of *FGF3/FGF4* mRNA. The mRNA expression levels of *FGF3* and *FGF4* were examined in nine HCC samples that were available as frozen samples among 48 HCC samples that were treated with sorafenib. Rel. mRNA, *target gene/GAPD* $\times$ 10^6.

The 11q13 locus is known to be a frequently amplified region in several human cancers except HCC.[13] Thus, we hypothesized that the amplification of 11q13 may be involved in a marked response to sorafenib.

FGF3/FGF4 ***Gene Amplification Is Frequently Observed in Responders to Sorafenib.*** To address the question of whether *FGF3/FGF4* gene amplification is also found in the HCC of other responders to sorafenib, we examined HCC specimens collected from 11 other medical centers in Japan. Because most of the HCC samples were collected as FFPE samples, we used a TaqMan Copy number assay.[10] A copy number assay revealed that *FGF3/FGF4* amplification was observed in three of the 10 (30%) HCC samples that responded to sorafenib, whereas no amplification was observed in the 38 specimens from patients with stable or progressive disease ($P = 0.006$, Fig. 2A). The copy numbers for *FGF3/FGF4* were 10.2 $\pm$ 0.8/6.7 $\pm$ 0.8, 26.7 $\pm$ 0.4/35.1 $\pm$ 3.1, and 162.5 $\pm$ 9.0/165.0 $\pm$ 12.5 copies in the amplified samples, whereas the copy numbers of *FGF3* for all the other samples were below 5 copies. The correlation between the *FGF3* locus and the *FGF4* locus copy numbers was very high (R = 0.998), indicating that the DNA copy number assay for *FGF3/FGF4* was a sensitive and reproducible method.

FGF3/FGF4 ***Gene Amplification Mediates the Overexpression of*** **FGF3/FGF4** ***Messenger RNA.*** We examined the messenger RNA (mRNA) expression levels of *FGF3/FGF4* in nine HCC samples that were available as frozen samples among the 48 sorafenib-treated samples, as shown in Fig. 2A. One amplified sample expressed extremely high mRNA levels of *FGF3/FGF4* compared with nonamplified samples (Fig. 2B). The results demonstrated that *FGF3/FGF4* gene amplification mediates the overexpression of *FGF3/FGF4* mRNAs and proteins (Figs. 2B and 1D).

FISH Analysis Confirmed **FGF3/FGF4** ***Gene Amplification.*** We used FISH analysis to examine *FGF3/FGF4* amplification and to verify the results of the above-described PCR-based DNA copy number assay. All *FGF3/FGF4*-amplified clinical samples were confirmed as exhibiting high-level *FGF3* amplification using FISH analysis (Fig. 3). One patient showed multiple scattered signals, whereas two patients showed large clustered signals. Nonamplified HCC yielded a negative result for gene amplification. These results clearly demonstrate the presence of *FGF3/FGF4*-

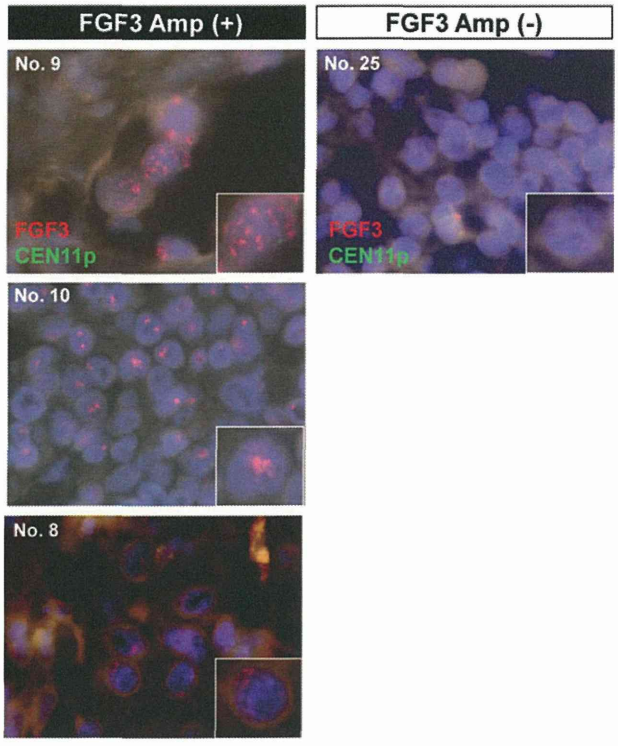

Fig. 3. FISH analysis of *FGF3*-amplified HCC. Patient numbers were indicated. Green staining indicates *CEN11P* loci; red staining indicates *FGF3* loci. High-power images are presented in each inset for a single cancer cell. Amp, gene amplification.

amplified HCC among the clinical samples, and the FISH analysis results were consistent with those for the copy number assay.

Frequency of* FGF3/FGF4 *Gene Amplification in HCC. To determine the frequency of *FGF3/FGF4* gene amplification in HCC, we performed a copy number assay for HCC samples without sorafenib treatment in a series of surgical specimens. Two of the 82 (2.4%) HCC samples exhibited *FGF3/FGF4* gene amplification, with copy numbers of 10.7/15.3 and 133.3/112.7 copies, respectively (Fig. 4). One amplified HCC was a poorly differentiated tumor, whereas the other was a moderately differentiated tumor.

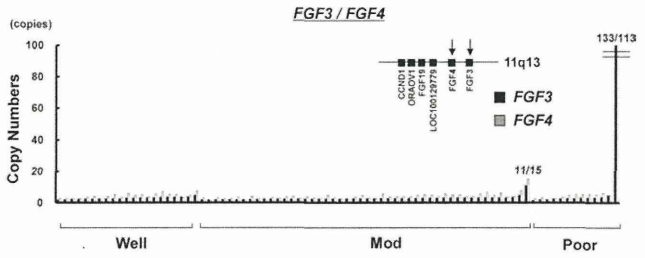

Fig. 4. *FGF3/FGF4* gene amplification in a series of HCC samples without sorafenib treatment. TaqMan copy number assay for *FGF3* and *FGF4* was used to examine DNA samples obtained from 82 surgical specimens. Human normal genomic DNA was used as a normal control. Well, well-differentiated HCC; Mod, moderately differentiated HCC; Poor, poorly differentiated HCC.

Clinicopathological Features of Responders to Sorafenib. The clinico-pathological features of the sorafenib responders are shown in Table 1. A comparison of clinical factors (age, sex, viral status, alpha-fetoprotein level, PIVKA-II, clinical stage, primary tumor size, metastatic status, histological type, and tumor response between responders and nonresponders) is given in Table 2. Notably, multiple lung metastases over five nodules was significantly higher among responders to sorafenib (responders, 5/13 [38%]; nonresponders, 2/42 [5%]; $P = 0.006$). Although the difference was not significant, poorly differentiated HCC tended to be

Table 1. Clinico-pathological Characteristics in Sorafenib Responders

Patient No.	Age, Years	Sex	Viral Status	AFP, ng/mL	PIVKA-II, mAU/mL	Clinical Stage	HCC in the Liver	Lung Metastasis	Other Metastases	Histological Type	Combination Treatment	Treatment Response	*FGF3/FGF4* Amplification
1	52	M	B	198	140	IV	2 cm, ×3	multi	Adrenal gland	Mod	(−)	PR	(−)
2	63	M	B	24	1,983	III	6 cm	(−)	(−)	Mod	(−)	CR	(−)
3	58	M	C	16	14	III	9 cm, multiple	(−)	(−)	Well	(−)	PR	(−)
4	62	M	B	8	130	IV	(−)	×3	(−)	Mod-Poor	(−)	PR	(−)
5	47	F	C	1,872	728	IV	2 cm, multiple	Multiple	(−)	Poor	+TAI	CR	(−)
6	66	M	C	290	18,507*	IV	5 cm	(−)	(−)	Mod	(−)	CR	(−)
7	71	M	C	404,100	1,328	IV	5 cm, multiple	Multiple	(−)	Poor	(−)	CR	(−)
8	66	M	Non	49	7,173	IV	(−)	×2	Pleural, LN	Mod	(−)	PR	Amplification
9	58	F	B	715	101	IV	11 cm	Multiple	(−)	Combination†	+5FU/IFN	PR	Amplification
10	80	F	C	378	21	III	3 cm, ×3	(−)	(−)	Poor, Mod‡	(−)	CR	Amplification
11	57	M	C	46,835	2,730	IV	14 cm, multiple	Multiple	(−)	Mod	(−)	CR	ND
12	77	M	B	435	71,000	IV	4 cm, multiple	(−)	(−)	Mod	(−)	PR	ND
13	84	M	Non	5,410	847,000*	IV	13 cm, multiple	(−)	(−)	Poor	(−)	PR	ND

Abbreviations: AFP, alpha-fetoprotein; CR, complete response; F, female; IFN, interferon; LN, lymph node; M, male; Mod, moderately differentiated; ND, not done; Non, non-B, non-C; Poor, poorly differentiated; PR, partial response; TAI, transcatheter arterial infusion; Well, well differentiated.

*Warfarin treatment (+).

†HCC with cholangiocarcinoma component.

‡From two different HCC nodules.