Table 2 Univariate analysis of local tumor progression in patients with hepatocellular carcinoma after treatment with RFA

Variables	*n*	*P* value[a]
Age (>65 years), yes/no	186/83	0.051
Gender (male), yes/no	165/104	0.355
Tumor size (>2 cm), yes/no	149/120	0.014
Child–Pugh classification		
Chronic hepatitis/C-P A/C-P B or C-P C	61/167/41	0.561
Cause of liver disease		
Hepatitis B/hepatitis C/nonB nonC	20/213/36	0.263
AST (>40 IU/L), yes/no	189/89	0.935
ALT (>40 IU/L), yes/no	140/129	0.384
ALP (>340 IU/L), yes/no	106/163	0.686
γGTP (>80 IU/L), yes/no	78/191	0.573
Alb (>3.5 g/dL), yes/no	190/79	0.821
T-Bil (>1 mg/dL), yes/no	82/187	0.377
PT (>70%), yes/no	230/39	0.626
Platelets (>10^4/mm^3), yes/no	147/122	0.334
AFP (>100 ng/mL), yes/no	48/221	0.203
DCP (>200 mAU/L), yes/no	42/227	0.007
Post-RFA antiviral therapy, yes/no	26/243	0.221
BMI (>25 kg/m^2), yes/no	78/191	0.657
DM, yes/no	90/179	0.157
BCAA medication, yes/no	45/224	0.113
Contiguous vessels, yes/no	117/152	0.099
R grade, A/B/C/D	49/113/74/33	<0.001

C-P Child–Pugh, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *γGTP* γ-glutamyl transpeptidase, *Alb* albumin, *T-Bil* total bilirubin, *PT* prothrombin time, *AFP* alpha-fetoprotein, *DCP* des-γ-carboxy prothrombin, *RFA* radiofrequency thermal ablation, *BMI* body mass index, *DM* diabetes mellitus, *BCAA* branched chain amino acid, *R* radicality

[a] Log-rank test

Table 3 Multivariate analysis of local tumor progression in patients with hepatocellular carcinoma after treatment with RFA

Variable	Hazard ratio	95% CI	*P* value[a]
Tumor size			
>2 cm	1.000		
≤2 cm	0.754	0.477–1.190	0.255
DCP			
>200	1.000		
≤200	0.718	0.408–1.264	0.251
The R grade			
Grade A	1.000		
Grade B	4.023	0.930–17.406	0.048
Grade C	17.432	4.207–72.226	<0.001
Grade D	26.879	6.218–116.200	<0.001

CI confidence interval, *DCP* des-γ-carboxy prothrombin, *R* radicality

[a] Cox proportional hazard model

Discussion

RFA has several advantages as compared with resection, such as lower invasiveness and liver volume loss, and superior cost-effectiveness [15]. As a result, its use in Japan has been rapidly increasing since its introduction in 1999 and since its inclusion in insurance cover in 2004 [11, 15]. However, it is believed that the local tumor progression rate after RFA is higher relative to that of resection [22, 23]. Therefore, in addition to attaining good local control of HCC, radiologists have been looking for an accurate method to assess RFA treatment efficacy that is strongly correlated with the local tumor progression rate. In our study, the cumulative local tumor progression rates in all patients at 1, 2, and 3 years were 12.8, 23.6, and 36.6%, respectively. These rates were relatively higher than those reported in previous studies [11, 13–17, 24, 25]. One possible reason for this is that our study involved 41 patients (15.2%) with a tumor >3 cm in diameter. Local tumor progression occurred in most of these patients, and in patients that were considered to have insufficient ablative margins (such as Grades C and D); however, additional

RFA was not performed in these patients, because informed consents were not obtained or because these patients were judged to be at high risk if given additional treatment.

Using univariate analysis, the R grade, tumor size (>2 cm), and DCP (>200 mAU/mL) were found to be significant predictive factors for local tumor progression. Kim et al. [13] reported that risk factors for local tumor progression included large tumor size and an insufficient ablative margin. It can be difficult to acquire an ablative margin around the entire circumference of large tumors. In these patients, it might be valuable to use image-supporting methods such as real-time virtual sonography (RVS) and enhanced ultrasonography to detect non-ablated regions when performing additional RFA [11, 26, 27].

In the present study, 42 patients (15.6%) had a DCP value of more than 200 mAU/mL. Kobayashi et al. [28] have reported that high DCP levels reflect the aggressiveness and progression of HCC tumors, and that the DCP level is a predictor of microvascular invasion. These findings seemed to correlate with our results using univariate analysis.

Although age was not found to be a significant factor for predicting local progression ($P = 0.051$) in the present study, the >65-year age group tended to have a higher local tumor progression rate. Using the R Judgment for this age group, 33 patients were classified as Grade A, 75 as Grade B, 48 as Grade C, and 30 as Grade D, and 78 patients did not have an ablative margin around the entire circumference of the tumor. In particular, 30 patients classified as Grade D were expected to require additional treatment. However, in the majority of these patients additional treatment was not performed due to the physical burden of advanced age, decreased liver function, and concerns related to the high incidence of complications due to comorbidities. It was assumed that these factors were linked to the higher local tumor progression rate in patients aged >65 years (local tumor progression occurred in 22 patients (73.3%) out of the 30 patients judged as Grade D).

It has been suggested that the presence of blood vessels contiguous to HCC is related to local tumor progression after RFA, because blood flow reduces the thermal effects of RFA [16]. However, in our study, the presence of contiguous vessels was not a significant factor ($P = 0.099$), possibly because most of the contiguous vessels in the 117 patients with contiguous vessels were hepatic veins rather than portal veins. We performed RFA to tumors adjacent to a hepatic vein, thus destroying the vein. However, hepatic vein injury caused by an RFA electrode does not usually cause serious complications. In fact, Kim et al. [14] reported that aggressive ablation of the portion of the tumor close to a hepatic vein might be useful to prevent local tumor progression.

In our study, antiviral therapy, such as interferon treatment, after RFA was not also found to be a significant factor ($P = 0.221$). This result suggests that antiviral therapy after RFA does not contribute to the suppression of the local tumor progression after RFA, although Ikeda et al. [29] reported that the administration of interferon for two or more years decreased the recurrence rate of early-stage HCC after radical ablation.

We routinely performed arterial infusion of Lipiodol alone, because we believed that Lipiodol accumulation was useful in confirming the tumor border. However, in 96 patients (35.7%) in our study, it was considered difficult to determine the exact location of the tumor because of insufficient Lipiodol accumulation. Exactly how we can measure the ablative margin in such patients will be a challenge that will be met in a future study.

We found a significant difference in the local tumor progression rate more than 1 year after RFA between Grade A and Grade B. In Grade B patients, local tumor progression was observed in 18 patients (15.9%), and in 16 patients, local tumor progression was observed from a site that was <5 mm from the ablative margin of RFA. And in the 18 Grade B patients who showed local tumor progression, the tumor recurred more than 1 year after RFA in 15 patients, in all of whom the tumor recurred <5 mm from the site of the ablative margin. One possible reason that a significant difference in local tumor progression rate was found more than 1 year after RFA between Grade A and Grade B is that, in most Grade B patients who showed local tumor progression, small satellite nodules were already present at a site <5 mm from the tumor border when RFA was performed.

In our study, of the 49 cases judged as Grade A, two had local tumor progression. In these two patients, the intervalsuntil local tumor progression were 5 months and 2.2 years, respectively. When assessing treatment efficacy after RFA at the site of local tumor progression, the ablative margins were found to be 8 and 9 mm in diameter in these two patients. In some HCC patients who underwent surgery, it has been reported that small satellite nodules were found in the lesion within 0.5–1 cm of the main tumor [30]. In the future, further study is needed to determine whether setting the ablative margin at 5 mm is sufficient for adequate assessment.

In our study, in the multivariate analysis, only the R grade was found to be a significant factor for assessing local progression after RFA treatment for HCC. Cases that acquired an ablative margin around the entire circumference, and those that did not were significantly stratified. Using the R grading, all groups (Grades A–D) were significantly stratified. These findings suggest that the proposed R Judgment approach is a valid method for assessing the efficacy of RFA treatment for HCC. Nakazawa et al.

[16] reported that an ablative margin of ≥5 mm was the most important factor in the local control of HCC, while Kim et al. [14] found that an insufficient ablative margin (<3 mm) was the only factor to be significantly associated with local tumor progression. In general agreement with the findings of our study, it has also been reported that, in addition to an insufficient ablative margin, tumor size and age were significant risk factors for local tumor progression, and that patients at high risk of local tumor progression should be closely monitored [24].

In the present study, we used a cohort of patients that was sufficiently large for comprehensive statistical analyses, and we compared the four R grades of our classification system. In addition, we verified the validity of the R Judgment as a method for predicting RFA treatment efficacy and assessing local tumor progression in HCC.

In conclusion, the R Judgment we have proposed is a useful method for predicting local tumor progression after RFA.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Rossi S, Di Stasi M, Buscarini E, et al. Percutaneous RF interstitial thermal ablation in the treatment of hepatic cancer. AJR Am J Roentgenol. 1996;167:759–68.
2. Rossi S, Fornari F, Buscarini E, et al. Percutaneous ultrasound-guided radiofrequency electrocautery for the treatment of small hepatocellular carcinoma. J Interv Radiol. 1993;8:97–103.
3. Rossi S, Di Stasi M, Buscarini E, et al. Percutaneous radiofrequency interstitial thermal ablation in the treatment of small hepatocellular carcinoma. Cancer J Sci Am. 1995;1:73–81.
4. Curley SA, Izzo F, Ellis LM, et al. Radiofrequency ablation of hepatocellular carcinoma in 110 patients with cirrhosis. Ann Surg. 2000;232:1694–702.
5. Allgaier HP, Deibert P, Zuber I, et al. Percutaneous radiofrequency interstitial thermal ablation of small hepatocellular carcinoma. Lancet. 1999;353:1676–7.
6. Lencioni R. Loco-regional treatment of hepatocellular carcinoma. Hepatology. 2010;52:762–73.
7. Higuchi T, Kikuchi M, Okazaki M. Hepatocellular carcinoma after transcatheter hepatic arterial embolization. A histopathologic study of 84 resected cases. Cancer. 1994;73:2259–67.
8. Lin SM, Lin CJ, Lin CC, et al. Randomised controlled trial comparing percutaneous radiofrequency thermal ablation, percutaneous ethanol injection, and percutaneous acetic acid injection to treat hepatocellular carcinoma of 3 cm or less. Gut. 2005;54:1151–6.
9. Shiina S, Teratani T, Obi S, et al. A randomized controlled trial of radiofrequency ablation with ethanol injection for small hepatocellular carcinoma. Gastroenterology. 2005;129:122–30.
10. Lencioni RA, Allgaier HP, Cioni D, et al. Small hepatocellular carcinoma in cirrhosis: randomized comparison of radio-frequency thermal ablation versus percutaneous ethanol injection. Radiology. 2003;228:235–40.
11. Izumi N. Recent advances of radiofrequency ablation for early hepatocellular carcinoma. J Gastroenterol Hepatol. 2011;26(Suppl 1): 115–22.
12. Liu CH, Arellano RS, Uppot RN, et al. Radiofrequency ablation of hepatic tumors: effect of post-ablation margin on local tumor progression. Eur Radiol. 2010;20:877–85.
13. Kim YS, Rhim H, Cho OK, et al. Intrahepatic recurrence after percutaneous radiofrequency ablation of hepatocellular carcinoma: analysis of the pattern and risk factors. Eur J Radiol. 2006;59:432–41.
14. Kim YS, Lee WJ, Rhim H, et al. The minimal ablative margin of radiofrequency ablation of hepatocellular carcinoma (>2 and <5 cm) needed to prevent local tumor progression: 3D quantitative assessment using CT image fusion. AJR Am J Roentgenol. 2010;195:758–65.
15. Ikeda K, Kobayashi M, Saitoh S, et al. Cost-effectiveness of radiofrequency ablation and surgical therapy for small hepatocellular carcinoma of 3 cm or less in diameter. Hepatol Res. 2005;33:241–9.
16. Nakazawa T, Kokubu S, Shibuya A, et al. Radiofrequency ablation of hepatocellular carcinoma: correlation between local tumor progression after ablation and ablative margin. AJR Am J Roentgenol. 2007;188:480–8.
17. Tateishi R, Shiina S, Omata M, et al. Percutaneous radiofrequency ablation for hepatocellular carcinoma. An analysis of 1000 cases. Cancer. 2005;103:1201–9.
18. Takahashi S, Kudo M, Chung H, et al. Initial treatment is essential to improve survival in patients with hepatocellular carcinoma who underwent curative radiofrequency ablation therapy. Oncology. 2007;72(Suppl 1):98–103.
19. Morimoto M, Numata K, Sugimori K, et al. Successful initial ablation therapy contributes to survival in patients with hepatocellular carcinoma. World J Gastroenterol. 2007;13:1003–9.
20. Goldberg SN, Grassi CJ, Cardella JF, Society of International Radiology Technology Assessment Committee, International Working Group on Image Guided Tumor Ablation, et al. Image guided tumor ablation: standardization of terminology and reporting criteria. Radiology. 2005;235:728–39.
21. Goldberg SN, Charboneau JW, Dodd GD 3rd, et al. Image-guided tumor ablation: proposal for standardization of terms and reporting criteria. Radiology. 2003;228:335–45.
22. Arii S, Yamaoka Y, Futagawa S, et al. Results of surgical and nonsurgical treatment for small-sized hepatocellular carcinomas: a retrospective and nation-wide survey in Japan. The Liver Cancer Group of Japan. Hepatology. 2000;32:1224–9.
23. Chen MS, Li JQ, Zheng Y, et al. A prospective randomized trial comparing percutaneous local ablative therapy and partial hepatectomy for small hepatocellular carcinoma. Ann Surg. 2006;242: 36–42.
24. Zytoon AA, Ishi H, Murakami K, et al. Recurrence-free survival after radiofrequency ablation of hepatocellular carcinoma. A registry report of the impact of risk factors on outcome. Jpn J Clin Oncol. 2007;37:658–72.
25. Murakami T, Ishimaru H, Sakamoto I, et al. Percutaneous radiofrequency ablation and transcatheter arterial chemoembolization for hypervascular hepatocellular carcinoma: rate and risk factors for local recurrence. Cardiovasc Interv Radiol. 2007;30: 696–704.
26. Chen MH, Wu W, Yang W, et al. The use of contrast-enhanced ultrasonography in the selection of patients with hepatocellular carcinoma for radio frequency ablation therapy. J Ultrasound Med. 2007;26:1055–63.
27. Osaki Y, Suginosita Y, Kimura T, et al. Usefulness of real-time virtual sonography (RVS) in treatment of hepatocellular carcinoma. Medix. 2005;42:15–20.
28. Kobayashi M, Ikeda K, Kawamura Y, et al. High serum des-gamma-carboxy prothrombin level predicts poor prognosis after radiofrequency ablation of hepatocellular carcinoma. Cancer. 2009;115:571–80.

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Comparison of percutaneous radiofrequency thermal ablation and surgical resection for small hepatocellular carcinoma

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Abstract

Background

The purpose of this investigation was to compare the outcome of percutaneous radiofrequency thermal ablation therapy (PRFA) with surgical resection (SR) in the treatment of single and small hepatocellular carcinoma (HCC).

Methods

We conducted a retrospective cohort study on 231 treatment naive patients with a single HCC ≤ 3 cm who had received either curative PRFA (162 patients) or curative SR (69 patients). All patients were regularly followed up after treatment at our department with blood and radiologic tests.

Results

The 1-, 3- and 5-year overall survival rates after PRFA and SR were 95.4%, 79.6% and 63.1%, respectively in the PRFA group and 100%, 81.4% and 74.6%, respectively in the SR group. The corresponding recurrence free survival rates at 1, 3 and 5 years after PRFA and SR were 82.0%, 38.3% and 18.0%, respectively in the PRFA group and 86.0%, 47.2% and 26.0%, respectively in the SR group. In terms of overall survival and recurrence free survival, there were no significant differences between these two groups. In comparison of PRFA group patients with liver cirrhosis (LC) (n = 127) and SR group patients with LC (n = 50) and in comparison of PRFA group patients without LC (n = 35) and SR group patients without LC (n = 19), there were also no significant differences between two groups in terms of overall survival and recurrence free survival. In the multivariate analysis of the risk factors contributing to overall survival, serum albumin level was the sole significant factor. In the multivariate analysis of the risk factors contributing to recurrence free survival, presence of LC was the sole significant factor. The rate of serious adverse events in the SR group was

significantly higher than that in the PRFA group (P = 0.023). Hospitalization length in the SR group was significantly longer than in the PRFA group (P = 0.013).

Conclusions

PRFA is as effective as SR in the treatment of single and small HCC, and is less invasive than SR. Therefore, PRFA could be a first choice for the treatment of single and small HCC.

Keywords: PRFA, surgical resection, HCC, survival, recurrence

Background

Hepatocellular carcinoma is a major health problem worldwide, with an estimated incidence ranging between 500,000 and 1,000,000 new cases annually. It is the fifth most common cancer in the world and the third most common cause of cancer-related death [1]. The prognosis of HCC is generally poor. Surgical resection (SR) remains the best hope for a cure but is suitable for only 9 to 27% of patients [2,3]. The presence of significant background liver cirrhosis (LC) often precludes hepatic resection in patients with HCC. Recurrence in the liver remnant is also common in patients who have undergone radical hepatic resection.

Currently, local ablative therapy competes with surgical resection and liver transplantation as primary treatment for small HCC. Various locoregional therapies are used to treat patients who are not candidates for surgery because of the severity of the underlying liver disease. Percutaneous radiofrequency thermal ablation (PRFA), a recently developed local ablative technique, has attracted the greatest interest and popularity because of its efficacy and safety [4]. Previous studies have shown PRFA to give good results from the perspective of tumor control, with complete tumor ablation rates of 90 to 95%, and low local tumor progression rates of 5 to 10% [5-8]. Prospective randomized trials have shown PRFA to be better than percutaneous ethanol injection (PEI) in producing a higher rate of complete ablation with fewer numbers of treatment sessions [9]. However, there is still debate with regard to whether PRFA or SR is the most suitable therapy of small HCC.

In the present study, we conducted a retrospective cohort study to compare the results of PRFA and SR in the treatment of small HCC.

Methods

Patients and HCC diagnosis

Between January 2004 and January 2010, 231 patients with single HCC ≤ 3 cm in diameter received curative treatment using PRFA or SR in our department. Before performing PRFA or SR, a full discussion was made between physician and surgeon. After giving enough information including contents of the discussion between physician and surgeon to patients, patients themselves made decisions whether they received PRFA or SR. In patients with the tumor sites extremely difficult to perform PRFA such as the site directly under the hepatic dome or the heart or with poor visibility of the tumor under ultrasonography owing to extreme obesity or impossibility of breath hold when performing PRFA, SR was performed. And in patients whom high rates of complications were expected as when tumors at the site of hepatic hilar lesion were treated by PRFA, SR was performed. In patients whom informed consent could not be obtained upon SR for the reason such as physical burden, PRFA was performed. Even in patients with poor liver function such as Child-Pugh C, if they wished to treat HCC and there were no ascites, treatment for HCC was performed after fully explaining the risk for treatment. PRFA was administered to 162 patients and 69 patients underwent SR. Written informed consent was obtained from all patients. The ethics committee of our department approved the protocols for PRFA and SR. The present study comprised a retrospective analysis of patient records and all treatments were conducted in an open-label manner. The primary end point was overall survival and the secondary end point was recurrence free survival.

HCC was diagnosed using abdominal ultrasound and dynamic computed tomography (CT) scans (hyperattenuation during the arterial phase in all or some part of the tumor and hypoattenuation in the portal-

venous phase) and/or magnetic resonance imaging (MRI), mainly based on the recommendations of the American Association for the Study of Liver Diseases [10]. Arterial and portal phase dynamic CT images were obtained at approximately 30 s and 120 s, respectively, after the injection of the contrast material. Abdominal angiography combined with CT (angio-CT) assistance was performed on all patients before PRFA and SR. This was due to the fact that Yamasaki et al. reported that this technique was useful for detecting small satellite nodules [11]. Then, we confirmed the presence of single HCC ≤ 3 cm in diameter with no vascular invasion using CT during hepatic arteriography (CTHA) and arterial-portography (CTAP). With regard to the diagnosis of liver cirrhosis, resected specimen at surgery was used in the SR group, and biopsy specimen was used in the PRFA group, respectively.

PRFA procedure

We routinely used a cool-tip needle (Radionics Corp., Burlington, MA, USA) while performing PRFA. Using the intercostal or subcostal approach, a 17-gauge, 2 or 3 cm cooled-tip electrode was inserted under real-time ultrasound guidance. The initial treatment was planned with one ablation for tumors of < 2 cm in diameter, and two or more ablations with the overlapping technique for tumors of ≥ 2 cm in diameter. After insertion of the electrode into the tumor, we started ablation at 60 W for the 3-cm exposed tip and 40 W for the 2-cm exposed tip. The power was increased to 120 W at a rate of 10 W/min. The duration of a single ablation was 12 min for the 3-cm electrode and 6 min for the 2-cm electrode. After PRFA exposure, the pump was stopped and the temperature of the needle tip was measured. When the temperature reached > 60°C, additional ablation was not performed. When tumor ablation was complete, thermal ablation was performed along the needle track. All patients were carefully observed for treatment-related complications. All procedures were performed under ultrasound guidance by one of five operators who had at least 3 years of experience of performing PRFA. We used the artificial ascites technique to prevent collateral thermal injury when the anticipated PRFA zone was in contact with a critical organ, such as the hepatic flexure of the colon. We also used this technique to improve visibility when the index tumor was located in the hepatic dome area. In the present study, for all patients who had received PRFA, we confirmed that the ablative margin surrounded the entire circumference of the tumor by using dynamic 16-column multi-detector CT (MDCT) using 3-mm slice scans within 1 week after PRFA and 1 month after PRFA.

Surgical resection procedure

All procedures were performed by one of four surgeons who had at least 10 years of experience of surgical resection. Surgical resection was carried out under general anesthesia using a right subcostal incision with a midline extension. We performed anatomic partial hepatectomy with a resection margin of at least 1 cm over the tumor, based on intraoperative ultrasonography (IOUS) guidance. IOUS was routinely performed to estimate the location, size, number and feeding vessels of the tumor, as well as to give an exact vascular map of liver anatomy. The Cavitron ultrasonic aspiration (CUSA, Valley Lab Corp, USA) was used to dissect the liver tissue. Hemostasis was achieved with dipolar electric coagulation and suturing. The Pringle maneuver was usually used in case of cirrhotic liver, with a clamp/unclamp time of 15 min/5 min policy. When liver function approached normal and adverse events had disappeared after surgical resection, we permitted patient discharge.

Follow-up

Follow-up consisted of monthly blood tests and monitoring of tumor markers, including des-γ-carboxy prothrombin, which was measured using a chemiluminescent enzyme immunoassay (Lumipulse PIVKAII Eisai, Eisai, Tokyo, Japan). Dynamic CT scans were obtained every 3-4 months after PRFA and SR. No patients were lost to follow-up.

Statistical analysis

Differences between the two groups were analyzed using the unpaired t-test for continuous variables, and the categorical variables were analyzed using the χ^2 test or continuity correction method. The overall survival curves and the recurrence-free survival curves were generated using the Kaplan-Meier method and compared using the log-rank test. The relative prognostic significance of the variables in predicting overall survival were

assessed using univariate and multivariate Cox proportional hazards regression models. All variables with a P value < 0.05 evaluated using univariate analysis were subjected to multivariate analysis. Results of the multivariate analysis were presented as the hazard ratio (HR) with a corresponding 95% confidence interval (CI). All statistical tests were two-sided. All data were analyzed using SPSS software, version 9.0 (SPSS Inc., Chicago, IL, USA) for Microsoft Windows. Data are expressed as means ± standard deviation (SD). Values of P < 0.05 were considered to be statistically significant.

Results

Patient characteristics

The baseline characteristics of the two groups are shown in Table 1. Between the two groups, there were significant differences in tumor size (P = 0.001), platelet count (P = 0.004) and PIVKAII value (P = 0.037).

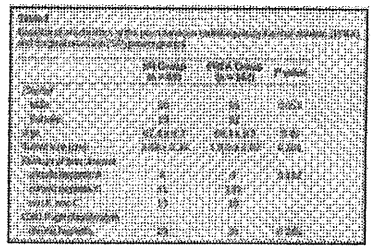


Table 1
Baseline characteristics of the percutaneous radiofrequency thermal ablation (PRFA) and surgical resection (SR) patient groups

SR group

Fifty-four of 69 patients were treated with segmentectomy; 12/69 patients received bisegmentectomy; 3/69 patients underwent hemihepatectomy. The histological diagnoses of 69 patients were as follows: well-differentiated hepatocellular carcinoma (3/69), moderately differentiated hepatocellular carcinoma (36/69) and poorly differentiated hepatocellular carcinoma (30/69). Using dynamic CT performed within 1 month after SR, we confirmed no residual HCC in the liver remnant of all patients.

PRFA group

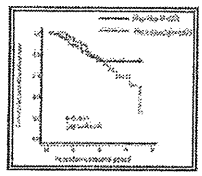
The mean number of treatment sessions for the 162 PRFA treated patients was 1.80 ± 0.37. Target biopsy prior to PRFA was not performed on any of the patients because of the specific complication of tumor seeding. We confirmed that all of the PRFA treated patients achieved complete ablation (ablated zone totally enveloped the tumor without enhancement) using dynamic CT prior to patient discharge and 1 month after PRFA. In the present study, there were 3 patients with Child-Pugh C who underwent PRFA. Their Child-Pugh scores were all 10 points and PRFA was performed safely in these 3 patients.

Patient survival

The median follow-up period was 3.1 years (0.2-7 years) in the PRFA group and 3.3 years (0.7-7 years) in the SR group, respectively. Thirty-three patients (20.4%) in the PRFA group died during the follow-up period. The causes of death were HCC recurrence (24 patients), liver failure (6 patients) and miscellaneous (3 patients). Twelve patients (17.4%) in the SR group died during the follow-up period. The causes of death were HCC recurrence (9 patients), liver failure (2 patients) and miscellaneous (1 patient).

The 1-, 3- and 5-year overall survival rates after PRFA and SR were 95.4%, 79.6% and 63.1%, respectively in the PRFA group and 100%, 81.4% and 74.6%, respectively in the SR group (Figure 1). The corresponding recurrence free survival rates at 1, 3 and 5 years after PRFA and SR were 82.0%, 38.3% and 18.0%, respectively in the PRFA group and 86.0%, 47.2% and 26.0%, respectively in the SR group (Figure 2).

Figure 1
Cumulative overall survival rate. The 1-, 3- and 5-year overall survival rates after percutaneous radiofrequency thermal ablation (PRFA) and surgical resection (SR) were



95.4%, 79.6% and 63.1%, respectively in the PRFA group and 100%, 81.4% and 74.6%, (more ...)

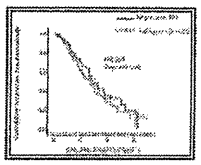


Figure 2
Cumulative recurrence free survival rate. The 1-, 3- and 5-year recurrence free survival rates after percutaneous radiofrequency thermal ablation (PRFA) and surgical resection (SR) were 82.0%, 38.3% and 18.0%, respectively in the PRFA group and 86.0%, (more ...)

In terms of overall survival (P = 0.259) and recurrence free survival (P = 0.324), there were no significant differences between these two groups.

Local tumor progression

We defined local tumor progression as the presence of a hypervascular nodule adjacent to the ablated area of PRFA or the resected area of SR using dynamic CT scan. 20 patients in the PRFA group and 10 patients in the SR group had local tumor progression during the observation period. The 1-, 3- and 5-year local tumor progression rates after PRFA and SR were 2.0%, 14.3% and 28.3%, respectively in the PRFA group and 2.8%, 14.3% and 22.8%, respectively in the SR group. (Figure 3) In terms of local tumor progression, there was no significant difference between these two groups (P = 0.746).

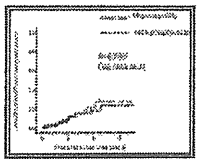


Figure 3
Cumulative local tumor progression rate. The 1-, 3- and 5-year local tumor progression rates after percutaneous radiofrequency thermal ablation (PRFA) and surgical resection (SR) were 2.0%, 14.3% and 28.3%, respectively in the PRFA group and 2.8%, 14.3% (more ...)

Comparison between PRFA group patients with LC and SR group patients with LC

There were 127 patients in PRFA group patients with LC and 50 patients in SR group patients with LC, respectively. The 1-, 3- and 5-year overall survival rates after PRFA and SR were 94.2%, 75.8% and 56.4%, respectively in the PRFA group with LC and 100%, 78.0% and 67.8%, respectively in the SR group with LC. (Figure 4) The corresponding recurrence free survival rates at 1, 3 and 5 years after PRFA and SR were 86.0%, 35.0% and 14.8%, respectively in the PRFA group with LC and 79.5%, 39.3% and 23.8%, respectively in the SR group with LC. (Figure 5) In terms of overall survival (P = 0.521) and recurrence free survival (P = 0.669), there were no significant differences between these two groups.

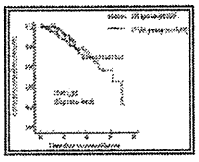


Figure 4
Cumulative overall survival rate between percutaneous radiofrequency thermal ablation (PRFA) group patients with liver cirrhosis (LC) (n = 127) and surgical resection (SR) group patients with liver cirrhosis (LC) (n = 50). The 1-, 3- and 5-year overall (more ...)

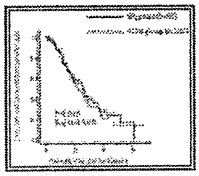


Figure 5
Cumulative recurrence free survival rate between percutaneous radiofrequency thermal ablation (PRFA) group patients with liver cirrhosis (LC) (n = 127) and surgical resection (SR) group patients with liver cirrhosis (LC) (n = 50). The 1-, 3- and 5-year (more ...)

Comparison between PRFA group patients without LC and SR group patients without LC

There were 35 patients in PRFA group patients without LC and 19 patients in SR group patients without LC, respectively. The 1-, 3- and 5-year overall survival rates after PRFA and SR were 96.6%, 87.2% and 74.4%, respectively in the PRFA group without LC and 100%, 95.6% and 95.6%, respectively in the SR group without LC. (Figure 6) The corresponding recurrence free survival rates at 1, 3 and 5 years after PRFA and SR were 93.0%, 52.5% and 22.2%, respectively in the PRFA group with LC and 100%, 75.7% and 30.4%, respectively in the SR group with LC. (Figure 7) In terms of overall survival (P = 0.276) and recurrence free survival (P = 0.258), there were no significant differences between these two groups.

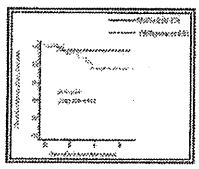


Figure 6
Cumulative overall survival rate between percutaneous radiofrequency thermal ablation (PRFA) group patients without liver cirrhosis (LC) (n = 35) and surgical resection (SR) group patients without liver cirrhosis (LC) (n = 19). The 1-, 3- and 5-year overall (more ...)

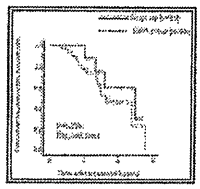


Figure 7
Cumulative recurrence free survival rate between percutaneous radiofrequency thermal ablation (PRFA) group patients without liver cirrhosis (LC) (n = 35) and surgical resection (SR) group patients without liver cirrhosis (LC) (n = 19). The 1-, 3- and (more ...)

Serious adverse events, hospitalization length and mortality

Serious adverse events were significantly more frequent in the SR group than in the PRFA group (6/69 versus 3/162; P = 0.023). Serious adverse events in the SR group were as follows: bile leakage (2 patients); refractory ascites (2 patients); acute respiratory distress syndrome (ARDS) (1 patient); and massive gastrointestinal bleeding (1 patient). Serious adverse events in the PRFA group were as follows: biloma (1 patient); refractory ascites (1 patient); and intra-abdominal bleeding (1 patient).

The hospitalization length was significantly longer in the SR group (18.1 ± 10.4 days) than in the PRFA group (14.7 ± 5.7 days) (P = 0.013). In addition, there was no patient who died within the same hospitalization, making the mortality rate 0% in two groups.

Univariate and multivariate analysis of prognostic factors contributing to overall survival and recurrence free survival

In the univariate analysis of factors contributing to overall survival, hepatitis C virus (HCV) versus non HCV (P = 0.042), serum albumin (g/dL) (> 3.5 versus ≤ 3.5) (P = 0.003), and platelet count (× 10^4/mm^3) (> 10 versus ≤ 10) (P = 0.045) were found to be significant factors (Table 2). However, in the multivariate analyses involving these three factors, serum albumin (g/dL) (> 3.5 versus ≤ 3.5) was the sole significant factor contributing to overall survival.

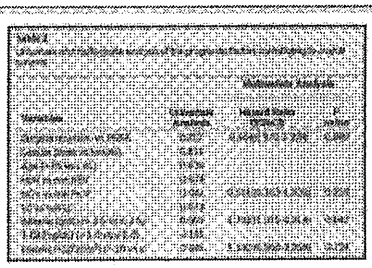


Table 2
Univariate and multivariate analysis of the prognostic factors contributing to overall survival

Similarly, in the univariate analysis of factors contributing to recurrence free survival, HCV versus non HCV (P = 0.022), LC versus non LC (P = 0.002) and platelet count (× 10^4/mm^3) (> 10 versus ≤ 10) (P = 0.005) were found to be significant factors (Table 3). However, in the multivariate analyses involving these three factors, the presence of LC was the sole significant factor contributing to recurrence free survival.

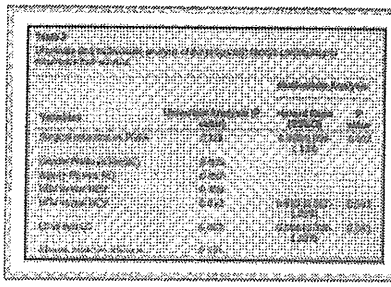


Table 3
Univariate and multivariate analysis of the prognostic factors contributing to recurrence free survival

Discussion

Partial hepatectomy in patients with resectable HCC, who have normal liver function and are in good general condition is still considered the gold standard therapy with the aim of delivering curability [12]. In recent years, it has been possible to reduce perioperative mortality to less than 5% depending on the extent of resection and hepatic reserve [13]. The improved outcome is primarily as a result of advances in surgical and radiologic techniques, perioperative care and more cautious patient selection [14].

Patients not eligible for resection because of their medical condition might be candidates for local ablative therapy, such as percutaneous ethanol injection (PEI) and PRFA. Many clinical trials comparing PRFA and PEI have demonstrated the clear superiority of PRFA over PEI [9,15-17]. However, a major limitation of PRFA is the small volume of tumor that can be treated. The rate of complete ablative necrosis decreases with the size of the tumor, particularly in the case of tumors larger than 3 cm. There is general consensus that complete response to PRFA therapy in patients is associated with improved outcome [18-20]. Therefore, in the present study, objectives were limited to patients with HCC ≤ 3 cm in size.

HCC mainly disseminates through the portal and hepatic veins. The micro-dissemination can invade the tributaries of the portal branches and shed tumor emboli in the neighboring branches of the same liver segment [21-24]. However, in the present study, with regard to recurrence free survival, there was no significant difference between the two treatment groups. One possible reason for this is that a sufficient ablative margin around the tumor when PRFA is administered may suppress the invasion of the micro-dissemination. Previous studies have reported that the initial treatment contributes to the survival of HCC patients treated using PRFA [19,25]. In PRFA therapy, obtaining sufficient ablative margin around the tumor seems to be essential.

The findings of the present study indicated that the overall and recurrence free survivals were the same for patients with a single HCC ≤ 3 cm in diameter treated with either PRFA or SR. In addition, PRFA was demonstrated to have an advantage over SR in causing less serious adverse events and a shorter hospitalization length. Chen et al conducted a randomized control trial (RCT) on 180 patients with a single HCC ≤ 5 cm to receive either PRFA or surgical resection [12], and Lu et al carried out another RCT on 105 patients with early HCC [26]. These two RCTs presented similar findings to those of our study. Additionally, four non-randomized controlled studies also reported similar findings of ours [27-30]. And our study suggests that PRFA is less invasive than SR. It seems that PRFA can be a first choice for the treatment of small HCC. On the other hand, a recent study indicated that surgical resection provided better survival and lower recurrence rates than RFA for patients with HCC that conformed to the Milan criteria for a RCT [31]. However, in comparing their results with ours, the mean age of their patient population was more than 10 years younger than ours. In the etiology of liver disease in their study, patients with chronic hepatitis B were in the majority [31]. However, in our study, patients with chronic hepatitis C were in the majority. Therefore, their study

results did not reflect the actual situation in Japan where Japanese HCC patients consist of many elderly patients, and the etiology of background liver disease involves chronic hepatitis C which accounts for about 80% of Japanese HCC patients. Hence, we should interpret their study results with caution.

Our study had several limitations. First, it was a retrospective cohort study. Patients who had a good hepatic reserve tended to receive surgical resection, and this could have possibly led to bias. Second, we did not assess the histopathologic diagnosis of HCC in the PRFA group. Tateishi et al reported that patients with poorly differentiated HCC had a poorer outcome than patients with well to moderately differentiated HCC after PRFA [32]. Third, our study patients were limited to patients who have undergone curative treatment. These problems should be resolved in a future prospective study.

Conclusion

In conclusion, we demonstrated that PRFA is as effective as SR in the treatment of single and small HCC patients who have undergone curative treatment, and that PRFA is less invasive than SR. Therefore, PRFA can be a first choice for the treatment of single and small HCC.

Competing interests

The authors declare that they have no competing interests. In addition, none of the authors had any financial relationship (within the past 12 months) with a biotechnology manufacturer, a pharmaceutical company, or other commercial entity that has any interest in the subject matter, materials, or processes discussed in the manuscript.

Authors' contributions

YO participated in the design of the study and performed the statistical analysis, and all authors read and approved the final manuscript.

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There is no one who contributed towards the study by making substantial contributions to conception, design, acquisition of data, or analysis and interpretation of data.

References

1. Bosch X, Ribes J, Borras J. Epidemiology of primary liver cancer. *Semin Liver Dis.* 1999;**19**:271–85. doi: 10.1055/s-2007-1007117. [PubMed] [Cross Ref]

2. Liver Cancer Study Group of Japan. Primary liver cancers in Japan. *Cancer.* 1980;**45**:2663–69. doi: 10.1002/1097-0142(19800515)45:10<2663::AID-CNCR2820451030>3.0.CO;2-G. [PubMed] [Cross Ref]

3. Lai EC, Fan ST, Lo CM, Chu KM, Liu CL, Wong J. Hepatic resection for hepatocellular carcinoma: an audit of 343 patients. *Ann Surg.* 1995;**221**:291–98. doi: 10.1097/00000658-199503000-00012. [PMC free article] [PubMed] [Cross Ref]

4. Lau WY, Leung TW, Yu SC, Ho SK. Percutaneous local ablative therapy for hepatocellular carcinoma: a review and look into the future. *Ann Surg.* 2003;**237**:171–79. [PMC free article] [PubMed]

5. Buscarini L, Buscarini E, Di Stasi M, Vallisa D, Quaretti P, Rocca A. Percutaneous radiofrequency ablation of small hepatocellular carcinoma: long term results. *Eur Radiol.* 2001;**11**:914–21. doi: 10.1007/s003300000659. [PubMed] [Cross Ref]

6. Nicoli N, Casari A, Marchiori L. Intraoperative and percutaneous radiofrequency thermal ablation in the treatment of

hepatocellular carcinoma. *Chir Ital.* 2000;**52**:29–40. [PubMed]

7. Wood TF, Rose DM, Chung M, Allegra DP, Foshag LJ, Bilchik AJ. Radiofrequency ablation of 231 unresectable hepatic tumors: indications, limitations and complications. *Ann Surg Oncol.* 2000;**7**:593–600. [PubMed]

8. Rossi S, Buscarini E, Garbagnati F, Di Stasi M, Quaretti P, Rago M, Zangrandi A, Andreola S, Silverman D, Buscarini L. Percutaneous treatment of small hepatic tumors by an expandable RF needle electrode. *Am J Roentgenol.* 1998;**170**:1015–22.

9. Shiina S, Teratani T, Obi S, Sato S, Tateishi R, Fujishima T, Ishikawa T, Koike Y, Yoshida H, Kawabe T, Omata M. A randomized controlled trial of radiofrequency ablation with ethanol injection for small hepatocellular carcinoma. *Gastroenterology.* 2005;**129**:122–30. doi: 10.1053/j.gastro.2005.04.009. [PubMed] [Cross Ref]

10. Bruix J, Sherman M. , Practice Guidelines Committee, American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma. *Hepatology.* 2005. pp. (5): 1208–1236.

11. Yamasaki T, Kurokawa F, Shirahashi H, Kusano N, Hironaka K, Okita K. Percutaneous radiofrequency ablation therapy with combined angiography and computed tomography assistance for patients with hepatocellular carcinoma. *Cancer.* 2001;**91**(7):1342–8. doi: 10.1002/1097-0142(20010401)91:7<1342::AID-CNCR1137>3.0.CO;2-0. [PubMed] [Cross Ref]

12. Chen MS, Li JQ, Zheng Y, Guo RP, Liang HH, Zhang YQ, Lin XJ, Lau WY. A prospective randomized trial comparing percutaneous local ablative therapy and partial hepatectomy for small hepatocellular carcinoma. *Ann Surg.* 2006;**243**:321–28. doi: 10.1097/01.sla.0000201480.65519.b8. [PMC free article] [PubMed] [Cross Ref]

13. Grazi GL, Ercolani G, Pierangeli F, Gaudio MD, Cescon M, Cavallari A, Mazziotti A. Improved results of liver resection for hepatocellular carcinoma on cirrhosis give the procedure added value. *Ann Surg.* 2001;**234**:71–8. doi: 10.1097/00000658-200107000-00011. [PMC free article] [PubMed] [Cross Ref]

14. Rahbari NN, Mehrabi A, Mollberg NM, Müller SA, Koch M, Büchler MW, Weitz J. Hepatocellular carcinoma. Current management and perspectives for the future. *Ann Surg.* 2011;**253**:453–69. doi: 10.1097/SLA.0b013e31820d944f. [PubMed] [Cross Ref]

15. Lencioni RA, Allgaier HP, Cioni D, Olschewski M, Deibert P, Crocetti L, Frings H, Laubenberger J, Zuber I, Blum HE, Bartolozzi C. Small hepatocellular carcinoma in cirrhosis: randomized comparison of radiofrequency thermal ablation versus percutaneous ethanol injection. *Radiology.* 2003;**228**:235–40. doi: 10.1148/radiol.2281020718. [PubMed] [Cross Ref]

16. Lin SM, Lin CJ, Lin CC, Hsu CW, Chen YC. Radiofrequency ablation improves prognosis compared with ethanol injection for hepatocellular carcinoma < or = 4 cm. *Gastroenterology.* 2004;**127**:1714–23. doi: 10.1053/j.gastro.2004.09.003. [PubMed] [Cross Ref]

17. Lin SM, Lin CJ, Lin CC, Hsu CW, Chen YC. Randomised controlled trial comparing percutaneous radiofrequency thermal ablation, percutaneous ethanol injection, and percutaneous acetic acid injection to treat hepatocellular carcinoma of 3 cm or less. *Gut.* 2005;**54**:1151–56. doi: 10.1136/gut.2004.045203. [PMC free article] [PubMed] [Cross Ref]

18. Sala M, Llovet JM, Vilana R, Bianchi L, Solé M, Ayuso C, Brú C, Bruix J. Barcelona Clinic Liver Cancer Group. Initial response to percutaneous ablation predicts survival in patients with hepatocellular carcinoma. *Hepatology.* 2004;**40**:1352–60. doi: 10.1002/hep.20465. [PubMed] [Cross Ref]

19. Takahashi S, Kudo M, Chung H, Inoue T, Ishikawa E, Kitai S, Tatsumi C, Ueda T, Minami Y, Ueshima K, Haji S. Initial treatment response is essential to improve survival in patients with hepatocellular carcinoma who underwent curative radiofrequency ablation therapy. *Oncology.* 2007;**72**:98–103. doi: 10.1159/000111714. [PubMed] [Cross Ref]

20. Guglielmi A, Ruzzenente A, Sandri M. Radiofrequency ablation for hepatocellular carcinoma in cirrhotic patients: prognostic factors for survival. *J Gastrointest Surg.* 2007;**11**:143–149. doi: 10.1007/s11605-006-0082-y. [PubMed] [Cross Ref]

21. Shi M, Zhang CQ, Zhang YQ, Liang XM, Li JQ. Micrometastasis of solitary heaptocellular carcinoma and appropriate resection margin. *World J Surg.* 2004;**28**:376–81. doi: 10.1007/s00268-003-7308-x. [PubMed] [Cross Ref]

22. Regimbeu JM, Kianmanesh R, Farges O, Dondero F, Sauvanet A, Belghiti JSO. Extent of liver resection influences the outcome in patients with cirrhosis and small hepatocellular carcinoma. *Surgery.* 2002;**131**:311–17. doi: 10.1067/msy.2002.121892. [PubMed] [Cross Ref]

23. Hasegawa K, Kokudo N, Imamura H, Matsuyama Y, Aoki T, Minagawa M, Sano K, Sugawara Y, Takayama T, Makuuchi M. Prognostic impact of anatomic resection for hepatocellular carcinoma. *Ann Surg.* 2005;**242**:252–59. doi: 10.1097/01.sla.0000171307.37401.db. [PMC free article] [PubMed] [Cross Ref]

24. Sasaki A, Kai S, Iwashita Y, Hirano S, Ohta M, Kitano S. Microsatellite distribution and indication for locoregional therapy in small hepatocellular carcinoma. *Cancer.* 2005;**103**:299–306. doi: 10.1002/cncr.20798. [PubMed] [Cross Ref]

25. Morimoto M, Numata K, Sugimori K, Shirato K, Kokawa A, Oka H, Hirasawa K, Koh R, Nihommatsu H, Tanaka K. Successful

initial ablation therapy contributes to survival in patients with hepatocellular carcinoma. *World J Gastroenterol.* 2007;**13**:1003–09. [PubMed]

26. Lu MD, Kuang M, Liang LJ, Xie XY, Peng BG, Liu GJ, Li DM, Lai JM, Li SQ. Surgical resection versus percutaneous thermal ablation for early-stage hepatocellular carcinoma: a randomized clinical trial (in Chinese with English abstract) *Zhonghua Yi Xue Za Zhi.* 2006;**86**:801–05. [PubMed]

27. Montorsi M, Santambrogio R, Bianchi P, Donadon M, Moroni E, Spinelli A, Costa M. Survival and recurrences after hepatic resection or radiofrequency for hepatocellular carcinoma in cirrhotic patients: a multivariate analysis. *J Gastrointest Surg.* 2005;**9**:62–7. doi: 10.1016/j.gassur.2004.10.003. [PubMed] [Cross Ref]

28. Hong SN, Lee SY, Choi MS, Lee JH, Koh KC, Paik SW, Yoo BC, Rhee JC, Choi D, Lim HK, Lee KW, Joh JW. Comparing the outcomes of radiofrequency ablation and surgery in patients with a single small hepatocellular carcinoma and well-preserved hepatic function. *J Clin Gastroenterol.* 2005;**39**:247–52. doi: 10.1097/01.mcg.0000152746.72149.31. [PubMed] [Cross Ref]

29. Lupo L, Panzera P, Giannelli G, Memeo M, Gentile A, Memeo V. Single hepatocelluar carcinoma ranging from 3 to 5 cm: radiofrequency ablation or resection? *HPB (Oxford)* 2007;**9**:429–34. doi: 10.1080/13651820701713758. [Cross Ref]

30. Hasegawa K, Makuuchi M, Takayama T, Kokudo N, Arii S, Okazaki M, Okita K, Omata M, Kudo M, Kojiro M, Nakanuma Y, Takayasu K, Monden M, Matsuyama Y, Ikai I. Surgical resection vs. percutaneous ablation for hepatocellular carcinoma: a preliminary report of the Japanese nationwide survey. *J Hepatology.* 2008;**49**:589–594. doi: 10.1016/j.jhep.2008.05.018. [Cross Ref]

31. Huang J, Yan L, Cheng Z, Wu H, Du L, Wang J, Xu Y, Zeng Y. A randomized trial comparing radiofrequency ablation and surgical resection for HCC conforming to the Milan criteria. *Ann Surg.* 2010;**252**:903–12. doi: 10.1097/SLA.0b013e3181efc656. [PubMed] [Cross Ref]

32. Tateishi R, Shiina S, Teratani T Obi S, Sato S, Koike Y, Fujishima T, Yoshida H, Kawabe T, Omata M. Percutaneous radiofrequency ablation for hepatocellular carcinoma. An analysis of 1000 cases. *Cancer.* 2005;**103**:1201–09. doi: 10.1002/cncr.20892. [PubMed] [Cross Ref]

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Genetic Heterogeneity of Hepatitis C Virus in Association with Antiviral Therapy Determined by Ultra-Deep Sequencing

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Abstract

Background and Aims: The hepatitis C virus (HCV) invariably shows wide heterogeneity in infected patients, referred to as a quasispecies population. Massive amounts of genetic information due to the abundance of HCV variants could be an obstacle to evaluate the viral genetic heterogeneity in detail.

Methods: Using a newly developed massive-parallel ultra-deep sequencing technique, we investigated the viral genetic heterogeneity in 27 chronic hepatitis C patients receiving peg-interferon (IFN) α2b plus ribavirin therapy.

Results: Ultra-deep sequencing determined a total of more than 10 million nucleotides of the HCV genome, corresponding to a mean of more than 1000 clones in each specimen, and unveiled extremely high genetic heterogeneity in the genotype 1b HCV population. There was no significant difference in the level of viral complexity between immediate virologic responders and non-responders at baseline ($p = 0.39$). Immediate virologic responders ($n = 8$) showed a significant reduction in the genetic complexity spanning all the viral genetic regions at the early phase of IFN administration ($p = 0.037$). In contrast, non-virologic responders ($n = 8$) showed no significant changes in the level of viral quasispecies ($p = 0.12$), indicating that very few viral clones are sensitive to IFN treatment. We also demonstrated that clones resistant to direct-acting antivirals for HCV, such as viral protease and polymerase inhibitors, preexist with various abundances in all 27 treatment-naïve patients, suggesting the risk of the development of drug resistance against these agents.

Conclusion: Use of the ultra-deep sequencing technology revealed massive genetic heterogeneity of HCV, which has important implications regarding the treatment response and outcome of antiviral therapy.

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Introduction

Hepatitis C virus (HCV) is classified as a member of the Flaviviridae family [1] and has an approximately 9.6-kb single-stranded RNA genome. This RNA genome encodes a large precursor polyprotein, which is cleaved by viral and host proteases to generate at least 10 functional viral proteins; core, envelope (E)-1, E2, p7, nonstructural protein (NS)-2, NS3, NS4A, NS4B, NS5A, and NS5B [2,3]. A strong characteristic of HCV infection is its significant genetic diversity, the consequence of the absence of proofreading activity in RNA-dependent RNA polymerase [4], and the high level of viral replication during its life cycle [5]. The mean frequency of nucleotide alterations occurring in HCV RNA is calculated to be between 1.4×10^{-3} and 1.9×10^{-3} substitutions per nucleotide per year [6,7]. As a result, the infecting HCV clones in each patient invariably show population diversity with a high degree of genetic heterogeneity. The collection of viruses in a population of closely related but non-identical genomes is referred to as a quasispecies [8,9], and the dominant viral population may be evolving as a result of its viral replicative fitness and concurrent immune selection pressures that drive clonal selection.

It is reasonable to assume that the viral pathogenesis and sensitivity to treatment are affected by the generation of escape mutants through immune evasion and the modification of virulence characteristics by anti-viral treatment [10]. Thus, certain viral mutations have important implications for the pathogenesis of the viral disease and the sensitivity to antiviral therapy. Several studies have attempted to associate genetic heterogeneity or

number of mutations with pathogenesis and treatment outcome. However, the abundant diversity and complexity of the chronically-infected HCV has been an obstacle to evaluate the viral genetic heterogeneity in detail. In this respect, the recent introduction of ultra-deep sequencing technology, capable of producing millions of DNA sequence reads in a single run, is rapidly changing the landscape of genome research [11,12]. One application of ultra-deep sequencing was the identification of rare minority drug resistant clones of human immunodeficiency virus, which are not detectable by standard sequencing techniques [13–15]. Moreover, the recent study using 454/Roche pyrosequencing technology clarified the transmission bottlenecks by measuring the population structure within patients with HCV infection [16].

In this study, we used for the first time ultra-deep sequencing with Illumina Genome Analyzer II (Illumina, San Diego, CA) and determined the pictures of viral quasispecies of genotype 1b HCV in patients receiving peg-interferon (IFN) α2b plus ribavirin (RBV) to clarify the significance of the viral genetic complexity in the pathophysiology of HCV infection and the treatment outcome of the current IFN-based therapy for HCV-infected patients. Because our main objective was to determine whether the HCV sequence variation itself is responsible for the sensitivity or resistance to antiviral therapy, we compared the composition of the HCV population complexity 1 week after IFN administration in patients who showed a prompt decrease in HCV viremia with those in whom there was no reduction in the serum HCV RNA levels after the initiation of IFN treatment. We also examined the prevalence of drug-resistant mutations to direct-acting antivirals (DAAs) for HCV in treatment-naïve HCV-infected patients, based on the fact that drug-resistant mutations already exist in treatment-naïve patients with various pathogenic virus infections, such as human immunodeficiency viruses [14,17].

Results

Validation of multiplex ultra-deep sequencing of the HCV genome

We performed a massive parallel ultra-deep sequencing run on the Illumina Genome Analyzer II platform using multiplex tagging methods. First, we conducted a control experiment to validate the efficacy and error rates in ultra-deep sequencing of the viral genome. For this purpose, we used a plasmid encoding full-length HCV [18] as a template and determined the plasmid-derived whole HCV sequence. The ultra-deep sequencing platform provided us the full-length HCV genome information derived from the plasmids with a mean coverage of 1674.3 at each nucleotide site (Table 1). Errors comprised insertions (1.0%), deletions (4.2%), and nucleotide mismatches (94.8%) and the overall error rates by multiplex ultra-deep sequencing were determined to be a mean of 0.0010 per bp. Next we confirmed that the high-fidelity PCR amplification with HCV-specific primer sets followed by multiplex ultra-deep sequencing resulted in no significant increase in the error rates in the viral sequencing data (ranging from 0.0012 to 0.0013 per bp; per-nucleotide error rate, 0.12%–0.13%).

To estimate the accuracy of detecting nucleotide alterations using reads filtered by average base quality and mapping quality, we introduced the plasmid with single point mutations within the wild-type viral sequences with the ratio of 1:99 and 1:999 and assessed the sensitivity and accuracy of quantification with the high-fidelity PCR amplification followed by multiplex ultra-deep sequencing. Duplicate control experiments revealed that mutations present at an input ratio of 0.10% ranged between 0.09 and 0.19%, and the results could be reproducibly quantified (data not shown). Based on these results, we picked up the low abundant mutations that presented at frequency of more than 0.20% among the total viral clones, a level that could rule out putative errors caused by massively-parallel sequencing, in the current platform used in this study.

Table 1. Error frequency of ultra-deep sequencing for the plasmid encoding full-genome HCV sequence.

	PCR amplification	
	(−)*	(+)*
Total read nucleotides	15,118,929	24,158,372
Mean coverage	1674.3	5562.6
Type of errors		
mismatches	14,629 (94.8%)	26,243 (88.6%)
deletions	640 (4.2%)	2510 (8.5%)
insertions	147 (1.0%)	859 (2.9%)
Overall error rate (%)	0.102	0.123

*(−); Ultra-deep sequencing of HCV encoding plasmid
(+); Ultra-deep sequencing of PCR-amplified HCV encoding plasmid.
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Large heterogeneity of viral clones in HCV-infected patients

HCV infection comprises a heterogeneous mixture of viral clones with various mutations. To clarify the landscape of HCV heterogeneity as a quasispecies, we determined the viral full-genome sequences derived from 27 HCV-infected patients by multiplex ultra-deep sequencing and compared the results with those obtained by the direct population Sanger sequencing method. All sequence reads by multiplex ultra-deep sequencing have been deposit in DNA Data Bank of Japan Sequence Read Archive (http://www.ddbj.nig.ac.jp/index-e.html) under accession number DRA000366.

HCV nucleotide sequence reads by ultra-deep sequencing were aligned to the consensus viral sequences in the same serum specimen that were determined by direct population Sanger sequencing. A mean number of 1705-fold coverage on average was achieved at each nucleotide site of the HCV sequences in each specimen. The average frequencies of altered sequences detected in each viral genomic region are summarized in Table 2. Compared with the representative sequence of the population average clone, the mutation frequency was 1.04% of the total viral genomic sequences and 16.1% of the total nucleotide positions on average. Most of the genomic changes observed in viral variants were single base substitutions and unevenly distributed throughout the region of the HCV genome.

Among the viral genomic regions, the nucleotide sequence complexity expressed as the Shannon entropy was smallest in the core region. In contrast, the viral sequence complexity in the E2 region was highest among the HCV genomic regions and significantly greater than the average mutation frequency of the remaining HCV genome ($p = 0.0026$). Similarly, the ratio of the number of mutated nucleotides to the total number of nucleotides analyzed in the E2 region was significantly higher than that of the remaining HCV genome ($p = 5.66 \times 10^{-6}$). These findings clearly confirmed that the quasispecies complexity in E2, which contains hypervariable region1 (HVR1) and HVR2, was prominently larger than that of other viral genomic regions [19].

Table 2. Mean genetic complexity of the genotype1b HCV in chronically infected 27 patients.

Viral genomic Region	Mean number of aligned nucleotides	Mean number of mutated nucleotides	Mean coverage	Mutation frequency (%)	Mean Shannon entropy
Core	779,839	5027	1361	0.61	0.045926
E1	739,220	7902	1360	0.99	0.064884
E2	1,382,907	19,724	1265	1.37	0.088584
p7	217,000	3237	1148	1.44	0.075829
NS2	673,579	8702	1073	1.19	0.075333
NS3	4,958,188	52,204	2619	0.93	0.060767
NS4A	427,677	5604	2640	1.32	0.072217
NS4B	1,209,000	17,485	1544	1.26	0.063190
NS5A	2,034,626	28,820	1518	1.28	0.067398
NS5B	2,720,417	27,449	1681	0.90	0.054805
Total	14,875,801	172,327	1705	1.04	0.062624

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Early dynamic changes of viral complexity after the administration of peg-IFNα2b plus RBV

Among 27 patients enrolled in this study, 8 showed a prompt decrease in their serum HCV RNA levels and 8 showed no significant changes 1 week after initiating treatment with peg-IFNα2b plus RBV. To clarify the changes in the viral quasispecies in response to antiviral therapy, we determined the early dynamic changes in viral complexity before and after 1 week of peg-IFNα2b plus RBV administration in these 8 immediate virologic responders and 8 non-responders. All cases were infected with genotype 1b viruses, and the clinical features, including serum HCV RNA level at baseline, did not significantly differ between immediate virologic responders and non-responders (Table 3). A mean coverage of 1798-fold and 2416-fold were mapped to each reference sequence in immediate virologic responders before and after peg-IFNα2b plus RBV administration, respectively. Similarly, a mean coverage of 1780-fold and 2461-fold were determined in non-responders before and after peg-IFNα2b plus RBV administration, respectively (Table 4 and Table S1).

We then estimated the genomic complexity by calculating the Shannon entropy for each nucleotide position before and after the administration of peg-IFNα2b plus RBV (Table 4). There was no significant difference in the level of viral complexity between immediate virologic responders and non-responders at a baseline (mean Shannon entropy value 0.072 vs 0.075, $p = 0.39$). Immediate virologic responders, however, showed a significant reduction in the nucleotide sequence complexity after the administration of peg-IFNα2b plus RBV (mean Shannon entropy value 0.072 vs 0.049, $p = 0.037$), indicating that the viral quasispecies nature after the peg-IFNα2b plus RBV treatment

Table 3. Characteristics of patients that showed immediate virologic response or non-response to PEG-IFNα2b plus ribavirin combination therapy.

	Immediate virologic responders	Non-responders	P-value
Age†	50.5 (45–68)	60 (55–69)	0.12
Sex (male/female)	5/3	5/3	1
Alanine aminotransaminase† (IU/l)	54 (15–198)	72 (30–143)	0.51
Total bilirubin† (mg/dl)	0.6 (0.4–1.8)	0.8 (0.4–1.4)	0.34
Platelet count† ($\times 10^4$/mm^3)	18.9 (7.1–27.2)	16.7 (11.6–22.5)	0.68
HCV genotype	1b	1b	
HCV viral load† (log IU/ml)			
pre-treatment	6.6 (6.2–7.5)	6.9 (6.1–7.6)	0.43
after treatment	4.6 (4.0–5.2)	6.5 (6.1–6.8)	**0.028**
Final outcome			**0.025**
sustained viral response	6	0	
Relapse	1	1	
non-response	0	6	
withdraw*	1	1	

† Values are median (range).

* The treatment was discontinued in one immediate virologic responder and one non-responder, due to the side effect of IFN and the development of liver cancer, respectively.

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Table 4. Genetic complexity at pre-treatment and 1 week after PEG-IFNα2b plus ribavirin combination therapy in immediate virologic responders and non-responders.

	Immediate virologic responders (N=8)		Non-responders (N=8)	
	Pre-treatment	1 week after IFN therapy	Pre-treatment	1 week after IFN therapy
Mean number of aligned reads	263,452	356,963	256615	354,398
Mean number of aligned nucleotides	16,632,186	22,438,125	16,248,820	22,379,922
Mean coverage	1798	2416	1780	2461
Mutation frequency (%)	0.96	0.63	1.13	1.11
Shannon entropy	0.072*	0.049*	0.075**	0.066**

Wilcoxon rank sum test.
* $p=0.037$.
** $p=0.12$.
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became relatively more homogeneous than at baseline status in this group. In contrast, no significant changes in the nucleotide sequence complexity were observed in non-responder patients before and after treatment with peg-IFNα2b plus RBV (mean Shannon entropy value 0.075 vs 0.066, $p=0.12$). We then examined whether specific nucleotide position might be associated with the response to peg-IFNα2b plus RBV treatment in immediate virologic responders, but complexity was not commonly shared at any specific nucleotide position that changed by more than 50% after peg-IFNα2b plus RBV administration (data not shown), indicating no association between the specific nucleotide position and the response to peg-IFNα2b plus RBV treatment.

Elimination of minor viral clones by peg-IFNα2b plus RBV therapy

Next, we compared the nucleotide complexity in each viral genomic region of the immediate virologic responders with that of non-responders before and after peg-IFNα2b plus RBV administration (Figure 1 and Table S2). In immediate virologic responders, the peg-IFNα2b plus RBV therapy induced a significant reduction in the nucleotide sequence complexity in all viral genomic regions except NS4B. In contrast, non-responders showed no significant change in the viral sequence complexity in any viral genomic region. For example, there was no significant difference in the mean complexity in the E2 region at baseline between the immediate virologic responders and non-responders. The administration of peg-IFNα2b plus RBV significantly reduced the levels of nucleotide sequence complexity in the E2 region in all the immediate virologic responders (mean Shannon entropy value 0.139 vs 0.085, respectively. $p=0.012$, Figure 1 and Table S2). In contrast, no significant changes in the sequence complexity were observed in the E2 (mean Shannon entropy value 0.083 vs 0.082, respectively. $p=0.89$) regions in non-responder cases after treatment with peg-IFNα2b plus RBV.

To examine whether certain viral clones in non-responders showed sensitivity to IFN therapy, we investigated the sequence complexity in HVR1 in the E2 region in detail before and after peg-IFNα2b plus RBV therapy, because the HVR1 region possessed one of the highest complexities among viral genomic regions. In immediate virologic responders, the heterogeneity at each nucleotide position was reduced in response to peg-IFNα2b plus RBV administration (representative nucleotide changes are shown in Figure 2A). In contrast, the ratio of mutated clones among the total sequence reads determined at each nucleotide site in HVR1 showed no significant change before and after the administration of peg-IFNα2b plus RBV in the majority of non-responders (Figure 2B), suggesting that very few viral clones showed sensitivity to peg-IFNα2b plus RBV and were eliminated after the administration of peg-IFNα2b plus RBV.

Detection of viral clones with drug-resistant mutations

Because none of the DAAs for HCV were approved by Japanese health coverage at the time of this study, all patients enrolled into this study were naïve to DAAs for HCV including protease and polymerase inhibitors. Thus, we determined whether the reported drug-resistant mutants exist spontaneously in nature among treatment-naïve HCV-infected patients. For this purpose, we examined the naturally prevalent mutations against HCV protease and polymerase inhibitors in the 27 patients. The drug-resistant mutations examined here included 9 mutations resistant to NS3/4 protease inhibitors, including Telaprevir, Boceprevir, TMC435350, ITMN191/R7227, MK-7009, and BI-201335, and 5 mutations resistant to NS5B polymerase inhibitors, including Filibuvir, BI-207127, and R7128 [20].

The mean number of sequence reads at the nucleotide position comprising mutations resistant to NS3/4A protease and NS5B polymerase inhibitors among the 27 cases were obtained with 1179-fold and 1972-fold coverage, respectively. Based on the detection rate of the low-level viral clones determined by the control experiments, we picked up the drug-resistant mutants that presented at a frequency of more than 0.2% among the total viral clones. Based on these criteria, at least one resistant mutation was detected in all subjects (Table 5). The mean prevalence of the 14 drug-resistant mutations ranged from 0.20% to 99.1% indicating that the proportion of resistant mutations substantially differed in each case. The T54S/A mutation resistant to Teraprevir and Boceprevir in genotype 1b HCV [21] was the most commonly detected (20 of 27 cases, 74.1%). The proportion of T54S/A mutations among the total clones ranged from 0.21% to 86.9% and thus substantially differed between cases. Other mutations resistant to the NS3/4A protease-inhibitor were detected in 16 of 27 cases (59.3%) at V55A and Q80R/K, and 12 of 27 cases (44.4%) at V36A/M. In contrast, no D168A/V/T/H mutation resistant to ITMN191/R7227, MK-7009, TMC435350, and BI-201335 was detectable. Regarding NS5B polymerase inhibitors, the V499A mutation resistant to BI-207127, was most frequently detected and 20 of 27 (74.1%) of subjects possessed the resistant-mutant clones at levels 0.20% to 99.1% at baseline. Only one case had the BI-207127-resistant P496A mutant clones and none had the R7128-resistant S282T clones. Of the 27 subjects, 16 (59.3%) harbored mutations resistant to at least four kinds of NS5B polymerase inhibitors and/or NS3/4A protease-inhibitors. More-

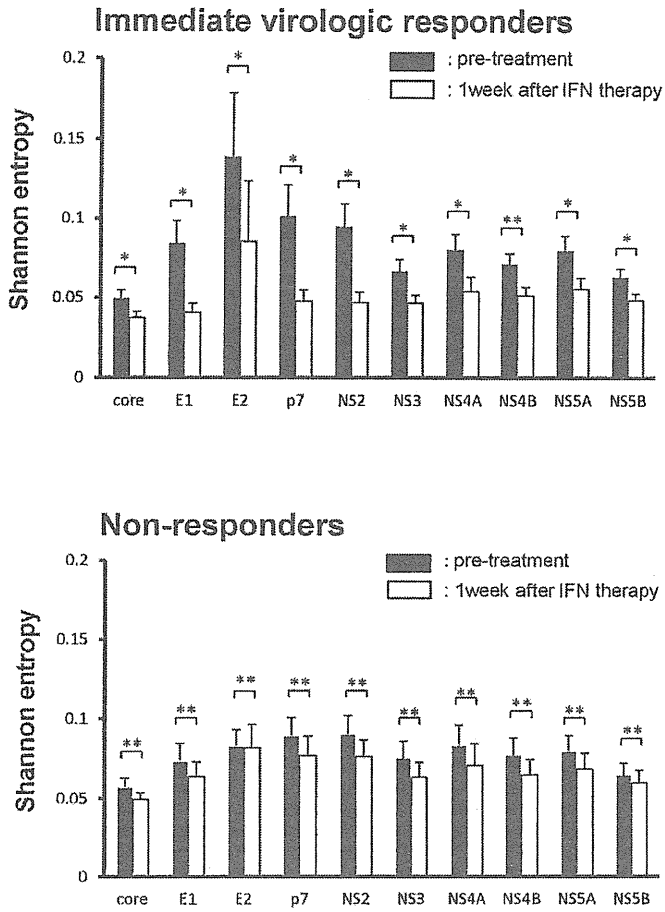

Figure 1. Changes in the genetic complexity of each HCV genomic region before and after the administration of peg-IFNα2b plus RBV. Shannon entropy values at baseline (black bar) and 1 week after initiation of treatment with peg-IFNα2b plus RBV (white bar) in 8 immediate virologic responders (A) and in 8 non-responders (B) are shown. * $p<0.05$, ** not significant. (Mean values ± SD; n=8)
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over, 5 subjects (18.5%) harbored resistance to 6 antiviral drugs. Notably, 3 subjects harbored resistance to 8 of 9 antiviral drugs. There was no significant association between the frequency of drug-resistant mutations and the serum viral load ($r=0.0678$) (Figure S1).

These findings indicate that drug-resistant HCV variants are present in a considerable proportion among the chronically HCV-infected, DAAs-naïve patients.

Discussion

Sequence heterogeneity, so-called quasispecies, is a common feature of RNA viruses, including HCV [22]. Previous studies of the viral genome with conventional Sanger sequencing methods revealed that HCV infection comprises a cloud of closely related sequence variants differing by as little as one nucleotide from a population average sequence [23]. A number of studies have aimed to clarify the significance of viral mutations in association with clinical features, including viral persistency and chronicity, degree of liver damage, response to treatment, and selection of mutants resistant to anti-viral therapy. The quasispecies nature of HCV, however, represents a major obstacle in determining the significance of the viral clone with specific sequence characteristics. Newly developed ultra-deep sequencing analysis allowed us to clarify the whole picture of viral quasispecies present in chronically HCV-infected patients. In the present study, ultra-deep sequencing determined a mean total of more than 10 million nucleotides of the viral genome in each specimen, representing more than 1000 clones infecting each patient, thus demonstrating the abundant genetic complexity of HCV.

It is well recognized that the HCV genome is heterogeneous at the intra-individual level [9,10]. The current ultra-deep sequenc-

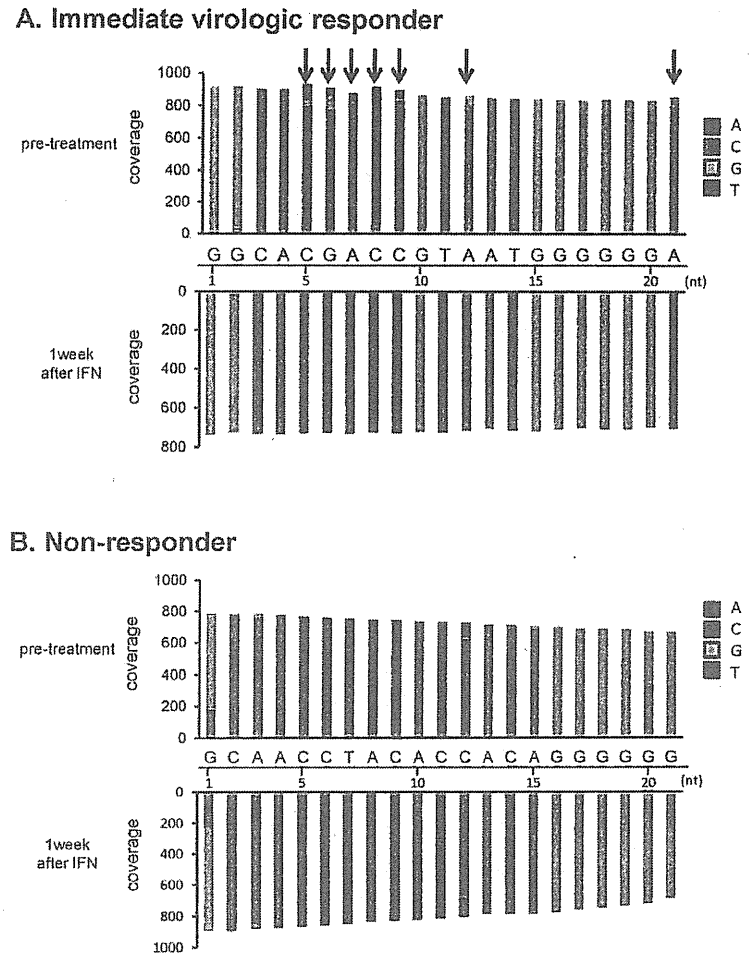

Figure 2. Ratio of mutated nucleotides in the HVR1 region before and after administration of peg-IFNα2b plus RBV therapy. Representative results of a immediate virologic responder (Patient#3) (A) and a non-responder (Patient#9) (B) are shown. The read numbers (coverage) at each nucleotide position of the HVR1 (from 1st nucleotide to 21st nucleotide in E2 region) at pre-treatment (upper graphs) and 1 week after initiating treatment with peg-IFNα2b plus RBV (lower graphs) are shown. Arrows indicate the nucleotide positions that showed the elimination of minor mutant clones after administration of peg-IFNα2b plus RBV.
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ing analyses revealed that the E2 region had the highest sequence heterogeneity, while the core region had the lowest sequence heterogeneity among the viral genomic regions encoding different functional viral proteins. More than 15% of nucleotides in the E2 region were mutated in all cases examined. These findings are consistent with previous conventional Sanger sequencing-based studies showing that HVR1 and HVR2 possess the highest sequence diversity among the HCV genomic regions [19] and that the highest values of mean Shannon entropy at the HCV 1a population level are in the E2 region [24].

Various mutations in the HCV genome are associated with the therapeutic response. For example, a number of mutations within a so-called IFNα sensitivity determining region of NS5A are closely associated with sensitivity to IFN-based anti-viral therapy [25,26]. A recent study also showed that amino acid substitution in the HCV core region could be a useful predictor of the virologic response to peg-IFNα plus RBV combination therapy [27]. Although the findings of these studies suggested that certain mutations in the representative HCV clone could predict treatment outcome, it is unknown whether the specific viral clone comprising those mutations directly displays sensitivity or resistance to anti-viral therapy. In the present study, sequential comparison of the HCV1b genome derived at baseline and at 1 week after the administration of peg-IFNα2b plus RBV demon-

Table 5. Prevalence of anti-HCV drug resistant mutations among the treatment-naïve patients.

Residue and Position	Drugs	Number of patients with mutated clones (%)	Frequency of the mutated clones (%)*
Resistant mutation to NS3/4A protease inhibitor			
T54S/A	Telaprevir Boceprevir	20/27 (74.1%)	0.49 (0.21–86.9)
V55A	Boceprevir	16/27 (59.3%)	0.4 (0.23–1.53)
Q80R/K	TMC435350	16/27 (59.3%)	0.36 (0.24–1.37)
V36A/M	Telaprevir Boceprevir	12/27 (44.4%)	0.47 (0.20–0.88)
V170A/T	Boceprevir	11/27 (40.7%)	0.52 (0.20–1.03)
A156T/V	Telaprevir	7/27 (25.9%)	0.35 (0.20–0.80)
R155K/T/Q	Telaprevir Boceprevir ITMN191/R7227 MK-7009 TMC435350 BI-201335	5/27 (18.5%)	0.42 (0.22–0.62)
A156S	Telaprevir Boceprevir	3/27 (11.15)	0.35 (0.24–0.83)
D168A/V/T/H	ITMN191/R7227 MK-7009 TMC435350 BI-201335	0/27 (0%)	
Resistant mutation to NS5B polymerase inhibitor			
V499A	BI-207127	20/27 (74.1%)	0.59 (0.20–99.1)
M423T/I/V	Filibuvir	12/27 (44.4%)	0.41 (0.21–1.48)
P495S/L/A/T	BI-207127	9/27 (33.3%)	0.37 (0.21–0.87)
P496A/S	BI-207127	1/27 (3.7%)	0.32
S282T	R7128	0/27 (0%)	

* Values are median (range).

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strated that IFN treatment resulted in no selective decrease of the viral clones comprising the previously defined mutational changes that were associated with a response to anti-viral therapy. Moreover, immediate virologic responders showed no common baseline nucleotide alterations that are efficiently eliminated in response to the administration of peg-IFNα2b plus RBV. Thus, our data suggest that an HCV sequence variation itself at a specific single nucleotide position does not directly reflect the virologic features regarding the sensitivity to IFN therapy in each viral clone, at least at the early stage of IFN administration. In contrast, several studies have provided evidence of the pre-existence of viral strains with an inherent resistance to IFN in patients who subsequently experienced a viral breakthrough or relapse [24,28]. Thus, there is room for further investigation to identify IFN-resistant clones by comparing the viral clones at baseline with those at the point of relapse using ultra-deep sequencing technology.

Notably, a distinct pattern of dynamic changes of HCV quasispecies was present between immediate responders and non-responders. Immediate responders showed a significant decrease of genetic complexity spanning all the viral genetic regions, resulting in a more homogeneous viral population after 1 week of peg-IFNα2b plus RBV administration. In contrast, non-responders showed no significant change in the genetic complexity in any of the HCV genomic regions. Our findings are consistent with the previous study showing that the early changes in HCV quasispecies determined by E1/E2 sequences provided prognostic information as early as the first 2 weeks after starting IFN therapy [28]. Moreover, the findings that there is no difference in the level of genetic complexity between early responders and non-responders at baseline and that almost none of the pre-existed HCV clones were eliminated in non-responder cases might suggest that the absence of sensitivity to IFN treatment in non-responders is due to host factors. Consistent with this hypothesis, recent studies revealed that host genetic variations at the IL28B gene are associated with a virologic response to peg-IFNα plus RBV combination therapy [29-32]. Alternatively, it is possible that a particular HCV protein of certain HCV mutants contributed to the strong inhibition of IFN-mediated anti-viral response in the liver of non-responders. Although dynamic changes in HVR1 sequences revealed that the minor viral clones were promptly eliminated in immediate virologic responders, the originally-inhabited major viral clones persisted 1 week after peg-IFNα2b plus RBV administration. Thus, further analyses are required to clarify how viral heterogeneity might be associated with the response to anti-viral therapy.

DAAs are promising drugs that could be more effective than peg-IFNα plus RBV therapy [33]. These DAAs include HCV NS3/4A protease and NS5B RNA-dependent RNA polymerase inhibitors, both of which have currently advanced to phase 1-3 trials. Increasing evidence, however, has clearly revealed that monotherapy with DAAs poses a high risk for the selection of resistant variants because of the high genetic heterogeneity of HCV [20]. Several studies reported the low prevalence of DAAs resistant mutants as the dominant clones in treatment-naïve cases [21,34-36]. For example, Kuntzen et al showed that drug-resistant mutations were detectable by conventional sequencing at individual frequencies between 0.3% and 2.8% in a treatment-naïve genotype 1 HCV-infected population [21]. In sharp contrast, ultra-deep sequencing identified that DAAs-resistant variants are common among treatment-naïve patients. Indeed, ultra-deep sequencing showed that 26 of 27 (96%) treatment-naive Japanese patients enrolled in this study possessed at least two clones resistant to DAAs, while 70.2% of the mutants presented as a very minor population (less than 1%) in each individual. It remains unclear whether these minor drug-resistant mutations have clinical significance, because the DAAs are not yet approved here in Japan. Recent *in vitro* findings, however, showed that minor but preexisting resistant mutants in HCV replicon cells were selected and expanded after DAAs therapy [37]. Lu et al revealed