that M414T mutants preexisting at a frequency of 0.22% and 0.18% in the treatment-naïve replicon population rapidly increased upon treatment with DAAs in a dose-dependent manner, reaching frequencies of 25% and 60% after 4 days of treatment. These findings suggest that those preexisting minor mutants might cause resistance against DAAs through the selection of dominant mutations. Thus, the significance of low-abundance variants in treatment-naïve patients requires further exploration.

The present study raises two limitations of ultra-deep parallel sequencing technology in the analyses of viral quasispecies. First, because the massive parallel ultra-deep sequencing platform is based on multitudinous short reads, it is difficult to separately evaluate the association between nucleotide sites mapped to different viral genome regions in a single viral clone. Indeed, it is difficult to clarify the potential mutational linkage between different viral genomic regions because of the short read length of the shotgun sequencing approach. Second, it is difficult to accurately analyze highly polymorphic regions such as the HVR by ultra-deep sequencing, because mutation findings strongly depend on mapping to the reference genome sequences. Thus, utilization of both conventional and ultra-deep sequencing technology might be necessary to fully clarify the significance and clinical relevance of the prominent HCV genomic heterogeneity.

In summary, using ultra-deep sequencing technology, we clearly demonstrated the extremely large genetic complexity in the genotype1b HCV derived from chronically infected patients. Although there was no significant difference in the level of viral complexity between immediate virologic responders and non-responders at baseline, immediate virologic responders, but not non-responders, showed a rapid reduction in the viral sequence variability at an early phase of peg-IFNα2b plus RBV administration. We also showed that drug-resistant mutants were widely present in treatment-naïve HCV-infected patients, indicating a putative risk for the expansion of resistant clones to DAAs. Further studies with a large number of patients are needed to fully elucidate the significance of viral heterogeneity in the clinical outcome of patients receiving anti-viral therapy.

Materials and Methods

Patients

The participants comprised 27 Japanese adult chronic hepatitis patients with genotype 1b HCV infection and the mean baseline level of serum HCV RNA determined by TaqMan RT-PCR (Applied Biosystems, Foster City, CA) was 6.9 log IU/ml. All patients received conventional peg-IFNα2b plus RBV combination therapy (Schering-Plough, Kenilworth, NJ) at Kyoto University and affiliated hospitals from February 2007 to December 2008. Indications for IFN-based combination therapy included high serum values of alanine aminotransferase and positivity for serum HCV RNA. Patients were treated with peg-IFNα2b (1.5 μg/kg) once per week, combined with daily oral RBV for 48 weeks [38]. The RBV dose was 600 mg/day in patients weighing less than 60 kg, 800 mg/day in those weighing at least 60 kg but less than 80 kg, and 1000 mg/day in those weighing 80 kg or more.

In this study, immediate virologic responders were defined as patients whose serum HCV RNA levels declined by more than 2 log IU/mL after 1 week of treatment with peg-IFNα2b plus RBV, while non-responders were defined as those whose serum HCV RNA levels declined less than 2 log IU/mL after peg-IFNα2b plus RBV administration. Of the original 27 patients, the serum before and 1 week after initiating treatment with peg-IFNα2b plus RBV of 16 cases was available for further analyses, and 8 of these cases were defined as immediate virologic responders and 8 cases were defined as non-responders. Among these non-responder cases, the serum HCV RNA levels in 6 of 8 (75.0%) patients changed by less than 1 log IU/mL after 1 week of treatment. The decline in HCV RNA levels in the remaining 2 cases was slightly over 1 log IU/mL (1.2 and 1.4 log IU/mL).

The ethics committee at Kyoto University approved the studies, and written informed consent for participation in this study was obtained from all patients.

Direct population Sanger sequencing

To define the representative reference sequences of full-length HCV in each clinical specimen, all samples were first subjected to direct population Sanger sequencing using Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA) [39]. Serum samples were obtained before the start and at 1 week after initiation of peg-IFNα2b and RBV combination therapy. Total RNA was extracted from 140 μL of serum using a QIAamp Viral RNA Mini kit (QIAGEN, Valencia, CA) and reverse-transcribed in a volume of 20 μL with the One step RNA PCR Kit AMV (Takara Bio, Ohtsu, Japan).

HCV genomes were amplified using Phusion High-Fidelity DNA polymerase (FINZYMES, Espoo, Finland). Oligonucleotide primers were designed to amplify the first-half (~5,000 bps) and the latter-half (~4,500 bps) of the genotype 1b HCV genome sequences (Table S3).

PCR products purified by the QIAquick Gel Extraction kit (Qiagen) were assayed for direct sequencing [40]. Nucleotide sequences of PCR products were determined using an ABI Prism Big Dye Terminator Ready Reaction Kit (Applied Biosystems). The serum of a healthy volunteer was used as a negative control.

Massive-parallel ultra-deep sequencing

Paired-end sequencing with multiplexed tags was carried out using the Illumina Genome Analyzer II. End-repair of DNA fragments, addition of adenine to the 3′ ends of DNA fragments, adaptor ligation, and PCR amplification by Illumina-paired end PCR primers were performed as described previously [41].

Briefly, the viral genome sequences were amplified with high-fidelity PCR and sheared by nebulization using 32 psi N2 for 8 min and the sheared fragments were purified and concentrated using QIAquick PCR purification Kit (Qiagen). The overhangs resulting from fragmentation were then converted into blunt ends using T4 DNA polymerase and Klenow enzymes, followed by the addition of terminal 3′ adenine-residues. Next, one of the adaptors containing six unique base pair (bp) tags, such as "ATCACG" and "CGATGT" (Multiplexing Sample Preparation Oligonucleotide Kit, Illumina), was ligated to each fragment using DNA ligase. Adaptor-ligated DNAs in the range of 200 to 350 bp were then size-selected by agarose gel electrophoresis. These libraries were amplified independently using a minimal PCR amplification step of 18 cycles with Phusion High-Fidelity DNA polymerase and then purified using a QIAquick PCR purification Kit for a downstream assay. Cluster generation and sequencing was performed for 64 cycles on the Illumina Genome Analyzer II following the manufacturer's instructions. Obtained images were analyzed and base-called using GA pipeline software version 1.4 with default settings provided by Illumina.

Genome Analyzer sequence data analysis

Using the high performance alignment software "NextGene" (SoftGenetics, State College, PA), the 64 base tags obtained from the Genome Analyzer II reads were aligned to the reference HCV RNA sequences of ~9200 bp that were determined by direct population Sanger sequencing in each clinical specimen. Entire reads were removed from the analysis when the median quality value score was below 20 and when containing more than 3 uncalled nucleotides. The low quality bases were trimmed from reads when more than 3 consecutive bases fell below a quality value score of 16. Based on the above criteria, reads with 90% or more bases matching a particular position of the reference sequence were aligned. Each position of the viral genome was assigned a coverage depth, representing the number of times the nucleotide position was sequenced.

Statistical analysis

Results are expressed as mean or median values and range (minimum and maximum). Pretreatment values were compared using the Mann–Whitney U-test. Categorical variables were analyzed by Fisher's exact test. P values of less than 0.05 were considered statistically significant. The viral quasispecies nature was evaluated by analyzing the genetic complexity based on the number of different sequences present in the population. Genetic complexity was determined by Shannon entropy values calculated as follows:

$$Sn = -\frac{\sum_{i=1}^{n} fi(\ln fi)}{N}$$

where n is the number of different species identified, fi is the observed frequency of the particular variant in the quasispecies, and N is the total number of clones analyzed [23,42]. Statistical comparisons of complexity between two groups were made using the Wilcoxon rank sum test or the Mann–Whitney U-test.

Supporting Information

Figure S1 Relationship between serum HCV RNA levels and the number of resistant mutant. No correlation was observed between serum HCV RNA levels (log IU/ml) and the number of resistant mutations against direct-acting antivirals in 27 cases in this study.
(TIF)

Table S1 Aligned reads, nucleotides, and mean coverage of each reference sequence in all patients.
(DOC)

Table S2 Mean genetic complexity in each viral genomic region of the 8 immediate virologic responders and 8 non-responders at pre-treatment and 1 week after IFN therapy.
(DOC)

Table S3 The oligonucleotide primers for PCR amplifying the whole HCV sequences.
(DOC)

Acknowledgments

The following institutions and investigators also participated in the study: Dr. M. Umeda, Dr. T. Kimura, Hyogo Prefectural Amagasaki Hospital; Dr. S. Yazumi, Kitano Hospital; Prof. K. Okazaki, Kansai Medical University; Dr. T. Nakamura, Kansai Denryoku Hospital; Dr. H. Yamada, Shinko Hospital; Dr. H. Kokuryu, Kyoto Katsura Hospital; Dr. A. Nakamura, Sanda City Hospital; Dr. C. Kawanami, Dr. N. Miyake, Ohtsu Red Cross Hospital; and Dr. K. Kajimura, Kishiwada City Hospital.

We thank Prof. A. Sekine for helpful advice, and Dr. Y. Matsumoto, Dr. Y. Endo, Dr. M. Wada, Dr. A. Takai and Ms. C. Hirano for ultra-deep sequencing analysis.

Author Contributions

Conceived and designed the experiments: AN HM. Performed the experiments: AN HM. Analyzed the data: AN HM NN TF FS KS TC YU. Contributed reagents/materials/analysis tools: AN HM YO YY TI TT. Wrote the paper: AN HM KT TC.

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Oncology

Oncology 2011;81(suppl 1):152–157
DOI: 10.1159/000333279

Published online: December 22, 2011

Complete Response of Advanced Hepatocellular Carcinoma with Multiple Lung Metastases Treated with Sorafenib: A Case Report

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Key Words
Hepatocellular carcinoma · Sorafenib · Hepatic necrosis · Lung metastasis · Complete response

Abstract
Sorafenib, an oral multikinase inhibitor, has demonstrated clinical efficacy in patients with advanced hepatocellular carcinoma (HCC). However, in the SHARP trial (Sorafenib HCC Assessment Randomized Protocol trial) and the Asia-Pacific trial (conducted in the Asia-Pacific region), no cases of complete response (CR) were reported. Thereafter, only a relatively small number of CR cases were reported worldwide for sorafenib therapy. We herein report a case of CR in a patient treated with sorafenib for 4 months. The patient had advanced HCC with multiple lung metastases, and there has been no recurrence after 8 months following cessation of administration. To our knowledge, this is the first time a female treated with sorafenib alone for HCC has had a CR.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common type of cancer globally, the third most common cause of cancer-related death, and a major health problem [1]. Surgical and locoregional procedures [especially percutaneous radiofrequency ablation (RFA)] can be curative for early stage HCC. However, because no effective therapies for advanced HCC are available, HCC that is diagnosed at an advanced stage or with progression after locoregional procedures has a dismal prognosis [2]. Recently, two large phase III clinical trials, the SHARP trial (Sorafenib HCC Assessment Randomized Protocol trial) and the Asia-Pacific trial (conducted in the Asia-Pacific region), clearly demonstrated that sorafenib (Nexvar; Bayer Healthcare Pharmaceuticals), an oral multikinase inhibitor, is an active and effective therapy leading to a significant improvement in both progression-free survival and overall survival in patients with unresectable advanced HCC. However, there were no cases of complete response (CR) among the 449 patients treated with sorafenib in these trials [3, 4]. As far as we are aware, there have been few reports of patients achieving CR when treated with sorafenib alone [5–12].

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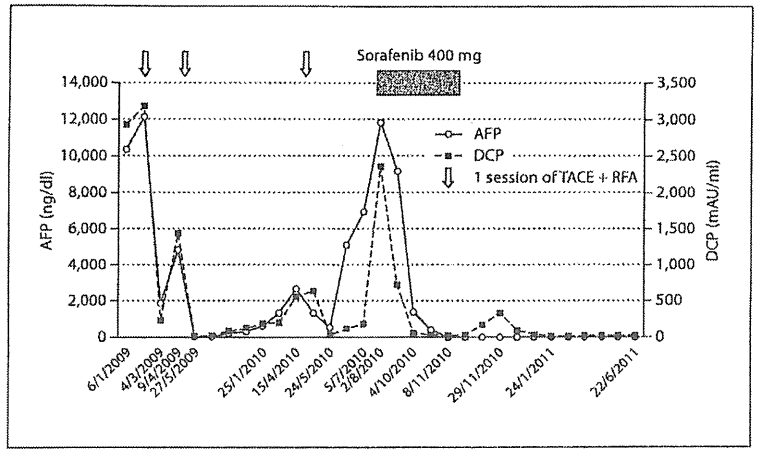

Fig. 1. Changes in AFP and DCP levels. The duration of treatment with sorafenib is indicated by the gray bar. The administration of sorafenib resulted in a dramatic reduction in serum AFP and DCP levels.

We herein report a case of CR in a patient treated with sorafenib for 4 months. The patient had advanced HCC with multiple lung metastases. There was no recurrence for 8 months after the cessation of administration, which was due to the development of hepatic failure as a serious adverse event of sorafenib.

Case Presentation

A 76-year-old Japanese female diagnosed with HCC and hepatitis C virus (HCV)-related liver cirrhosis, and who had previously received percutaneous ethanol injections in 2006, was referred to our department in January 2009 because of elevated α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP) levels. She was 150 cm tall and weighed 53 kg. Based on her history of HCV, elevated tumor markers, and typical radiological findings for classical HCC in the right lobe of the liver on dynamic CT scan, we diagnosed recurrence of HCC without performing a biopsy. The tumor was solitary and 4.5 cm in diameter. The AFP and DCP levels were 10,341 ng/ml and 2,929 mAU/ml, respectively (fig. 1). We performed transcatheter arterial chemoembolization (TACE) for HCC followed by RFA in February 2009 and additional RFA for local tumor progression in April 2009 and April 2010. Subsequently, AFP and DCP levels decreased to 526 ng/ml and 32 mAU/ml, respectively. However, 3 months later, AFP increased to 5,127 ng/ml and DCP to 125 mAU/ml, and CT scan showed the presence of more than 60 HCC-derived lung metastases (fig. 2), even though the intrahepatic tumor was well controlled.

At that time, the significant laboratory test results of the patient were as follows: alanine aminotransferase (ALT) 90 IU/l, aspartate aminotransferase (AST) 131 IU/l, total bilirubin 1.2 mg/dl, albumin 3.0 g/dl, PT INR 1.16, AFP 6,952 ng/ml, and DCP 187 mAU/ml. The patient had no ascites or encephalopathy and had a Child-Pugh score of 6 (Child-Pugh class A) with an Eastern Cooperative Oncology Group (ECOG) performance status of 1. Therefore, oral sorafenib therapy was initiated at 400 mg, once daily (half the standard dosage), for multiple lung metastases of HCC from July 2010.

After 2 weeks, AFP increased to 11,800 ng/ml and DCP to 2,365 mAU/ml. After 3 weeks, the lung metastases were slightly enlarged on chest CT scan. However, after 3 months, both tumor markers markedly decreased (fig. 1) and the lung metastases had almost disappeared on chest CT scan (fig. 2). After 4 months, the lung metastases had completely disappeared and the patient achieved CR (fig. 2).

However, because of hepatic encephalopathy and deterioration of liver function from Child-Pugh class A to C 4 months after starting sorafenib, we were forced to cease sorafenib administration (fig. 3a). Four days following administration cessation, ALT and AST levels suddenly increased dramatically to 1,454 and 1,653 IU/l, respectively. CT scan revealed that multicentric hepatic necroses, not apparent 4 days earlier, had appeared suddenly in the right and left lobes of the liver (fig. 3b). Thereafter, the patient was managed conservatively and after

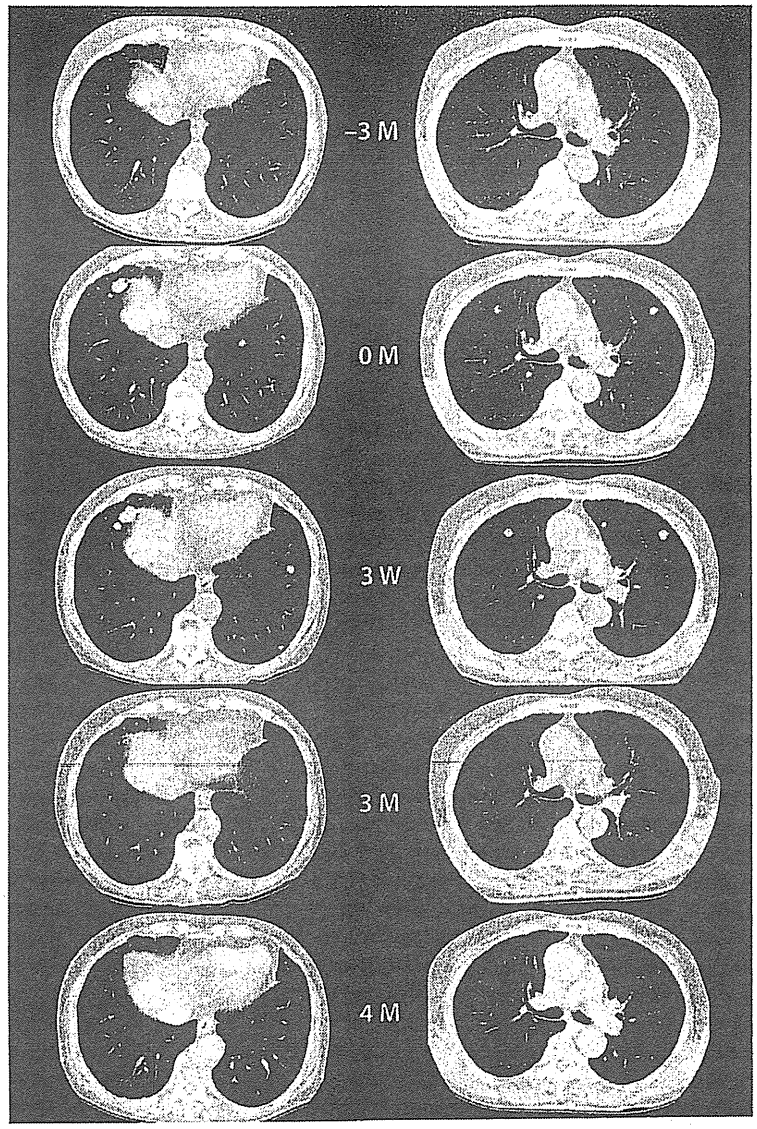

Fig. 2. Changes in chest CT scans. Follow-up CT scans show that multiple lung metastases were slightly enlarged after 3 weeks. After 3 months, they had almost disappeared. After 4 months, they had completely disappeared and the patient achieved radiological CR. The maximum diameter of lung metastases 3 weeks after commencement of therapy was 2 cm.

several weeks her liver function gradually improved. At the most recent follow-up, the patient remained in remission 8 months after the cessation of sorafenib therapy, without clinical or imaging evidence of disease recurrence. Both tumor markers were within the normal range; liver function improved to a Child-Pugh score of 7 (Child-Pugh class B) and her ECOG performance status was 1.

Discussion

Sorafenib is a multikinase inhibitor with reported activity against Raf-1, B-Raf, VEGFR2, PDGFR, and c-Kit receptors, among other receptor tyrosine kinases and serine threonine kinase [13]. It can reduce tumor progression in HCC patients because of its effect on tumor

proliferation and angiogenesis. Two large phase III clinical trials, the SHARP trial and Asia-Pacific trial, clearly demonstrated that sorafenib had efficacy in terms of overall survival in patients with unresectable advanced HCC [3, 4]. According to the SHARP trial, 299 patients had sorafenib therapy; of these, only 2% had a partial response and 71% had stable disease [according to the Response Evaluation Criteria in Solid Tumors (RECIST)]. No patient had a CR [3]. In the Asia-Pacific trial also, among the 150 patients treated with sorafenib, none showed a CR [4]. In a search of the literature, only 10 HCC patients worldwide were found to have achieved a CR with sorafenib (table 1) [5–12]. So et al. [5] first reported a CR in a patient with hemochromatosis and metastatic HCC treated with sorafenib. Wang et al. [7] reported a CR in a patient with HCV and HCC with portal vein tumor thrombosis treated with a reduced dose of sorafenib. Kudo et al. [8], Curtit et al. [11], and Irtan et al. [12] reported a complete histological response in a patient who underwent surgery after sorafenib therapy. All cases were male and most showed an acute decrease in AFP levels before CR was achieved [5–12]. It takes less than 6 months to achieve CR in most cases (table 1). Therefore, it should be possible to determine within 6 months whether sorafenib is effective in most HCC patients when they achieve a dramatic therapeutic response.

Our patient, who was treated with sorafenib alone, achieved CR. This case is especially unique and suggestive for five reasons. First, this is the first time a female patient treated with sorafenib alone for HCC has achieved CR. Second, the elevation of both tumor markers and the CT scan showed disease progression after several weeks of starting sorafenib, but subsequently CR was achieved after 4 months. A recent study has suggested that an early AFP response within the first 4 weeks is a surrogate marker predictive of progression-free survival and overall survival in HCC patients treated with antiangiogenic therapies like sorafenib [14]. However, our case suggests that an early decrease in AFP levels is not the only important factor involved in achieving CR. These findings suggest that 1 month may be insufficient to determine whether HCC patients respond to sorafenib. Third, this patient maintained a CR even after the cessation of sorafenib therapy. This indicates that the tumors completely disappeared due to sorafenib therapy alone. Fourth, this is the second case of CR in HCC patients in which the initial sorafenib dose was reduced to half the standard dosage. Our patient received 400 mg once daily due to her petite build. Even though our patient achieved

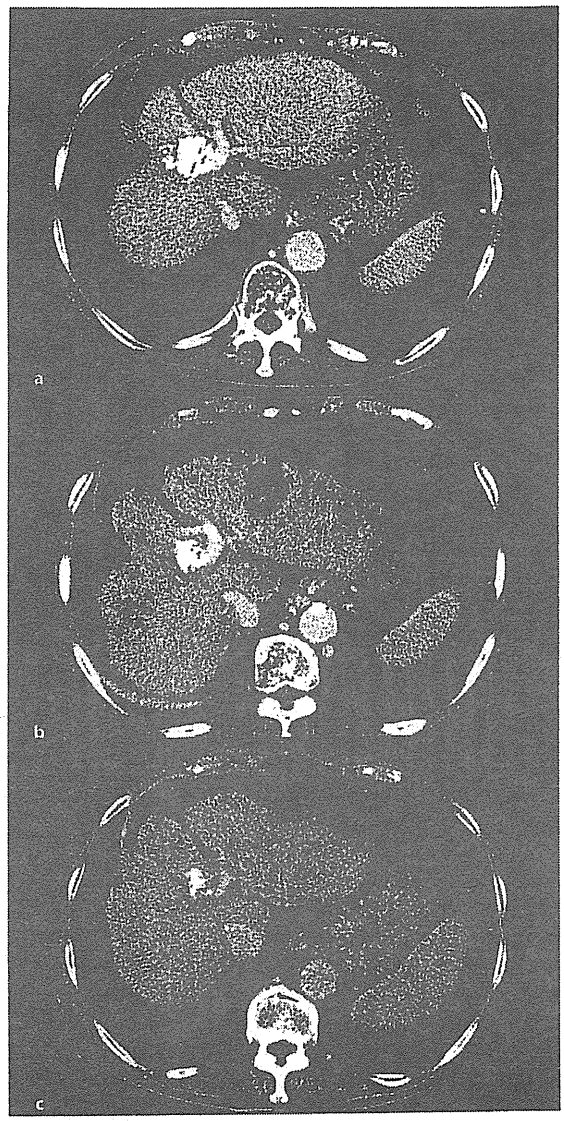

Fig. 3. Changes in abdominal CT scans after cessation of sorafenib therapy. **a** CT taken when sorafenib therapy was suspended because of hepatic encephalopathy and deterioration of liver function. **b** Four days after **a**, ALT and AST levels rose dramatically to 1,454 and 1,653 IU/l, and CT scan revealed the presence of multicentric hepatic necroses in the right and left lobes of the liver. **c** CT at the most recent follow-up revealed slight atrophy of the left lobe of the liver but no recurrence of HCC.

Table 1. CR in patients with HCC treated with sorafenib alone

Case No.	Characteristics[a]	Etiology	Metastasized to	Maximal diameter cm	Initial dose[b] mg	Time to CR months	Time to cessation	Published online	First author	Journal	Comments
1	78, M, USA, unknown	Hemochromatosis	Liver, lung	5.0	400	5	6 months	17/10/08	So [5]	Journal of Hematology & Oncology	First report
2	54, M, USA, Asian	HBV	Lung	4.1	400	18	None	1/9/09	Yeganeh [6]	American Journal of Transplantation	Posttransplant
3	74, M, USA, Caucasian	HCV	Liver (PVTT)	10	200	8	8 months	23/3/10	Wang [7]	Targeted Oncology	Low-dose
4	68, M, Japan, Asian	HBV	Liver, lung	–	400	2	None	8/7/10	Kudo [8]	Oncology	
5	68, M, Japan, Asian	HBV	Liver, lung, lymph node, adrenal gland	5.5	400	1	None				Histological CR
6	69, M, Greece, unknown	HBV + HIV	Liver, lymph node	–	400	6	None	31/8/10	Chelis [9]	Medical Oncology	HIV coinfection
7	84, M, Italy, unknown	HCV	Liver (PVTT)	6.0	400	6	None	17/1/11	Sacco [10]	BMC Gastroenterology	
8	56, M, France, unknown	HCV	Liver	15	400	6	Unknown	24/1/11	Curtit [11]	Journal of Clinical Oncology	Histological CR
9	59, M, France, unknown	Hemochromatosis	Liver (PVTT), lymph node, omentum	10	400	6	Unknown	14/3/11	Irtan [12]	Liver International	Histological CR
10	57, M, France, unknown	HBV	Liver (PVTT)	8	400	12	Unknown				Histological CR

Only 10 HCC patients worldwide have achieved a CR with sorafenib. All cases were male and most showed an acute decrease in terms of AFP levels before CR was achieved. It takes less than 6 months to achieve CR in most cases. PVTT = Portal vein tumor thrombosis.
[a] Presented as age (in years), sex, nationality, race. [b] Dose administered twice daily.

CR on half the usual sorafenib dose, it is unclear whether low-dose sorafenib therapy is as effective as the standard regimen (400 mg twice daily). More studies need to be conducted to clarify the effect of low-dose sorafenib therapy. Fifth, the patient had multicentric hepatic necroses in the right and left lobes of the liver after the cessation of sorafenib. It is assumed that nontumor liver tissues underwent necrosis because there was no evidence of HCC in the liver at that time. To our knowledge, there have been no previous reports of such adverse events in association with sorafenib, while this adverse event could have been caused by antiangiogenic therapies like sorafenib. Further investigation is required to confirm this finding.

In conclusion, we had a rare example of a dramatic therapeutic response in an HCC patient treated with sorafenib. Further studies are needed to elucidate the mechanisms of how CR is achieved at the molecular level and what the molecular biomarkers are in order to identify which patients are most likely to achieve CR. We believe that it is important to make such rare cases known and to search for a breakthrough therapy for advanced HCC.

Disclosure Statement

All authors have no conflict of interest to declare.

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Digestion

Digestion 2011;84(suppl 1):43–49
DOI: 10.1159/000333212

Published online: December 2, 2011

Clinical Characteristics of Non-B Non-C Hepatocellular Carcinoma: A Single-Center Retrospective Study

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Key Words
Liver cancer · Non-B non-C hepatocellular carcinoma · Nonalcoholic steatohepatitis · Alcohol · Diabetes

Abstract
Background/Aims: To clarify risk factors and clinical features of both hepatitis B surface antigen and anti-HCV negative hepatocellular carcinoma (NBNC-HCC). ***Methods:*** HCC patients (n = 1,109) diagnosed at a single center were categorized based on the presence of serum hepatitis B surface antigen and HCVAb. Clinical characteristics of 127 NBNC-HCC patients were evaluated. ***Results:*** NBNC-HCC patients were stratified as those with alcoholic liver disease (ALD-HCC, n = 42) and alcohol-unrelated liver disease (non-ALD-HCC, n = 85). Compared with the ALD-HCC group, the non-ALD-HCC group had a higher prevalence of diabetes (p = 0.015), larger tumor size (p = 0.007), and higher tumor marker levels (p = 0.014). Liver function results were significantly worse in ALD-HCC than in non-ALD-HCC. Although the ALD-HCC group had a higher tendency toward recurrence than the non-ALD-HCC group, survival rates were similar between groups (p = 0.352). ***Conclusion:*** Alcohol consumption was the most common etiologic factor for NBNC-HCC, and diabetes may be related to the development of HCC in non-ALD-HCC patients. Non-ALD-HCC tended to be diagnosed at a more advanced stage, whereas liver function was worse, and tumor recurrence rate was higher in ALD-HCC patients. Further examination of the risk factors and establishment of a precise surveillance system are necessary for early diagnosis and the development of curative therapies for NBNC-HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer-related deaths worldwide [1, 2]. In Japan, chronic hepatitis C virus (HCV) infection is considered to be the most significant risk factor for the development of HCC, and the second most important factor is hepatitis B virus infection. Based on a report by the Liver Cancer Study Group of Japan, approximately 15% of HCC patients in Japan are hepatitis B surface antigen (HBsAg) positive (B-HCC) and 70% are anti-HCV (HCVAb) positive (C-HCC) [3]. Recent progress in the management of patients with viral hepatitis by specific antiviral therapy, including interferon and nucleotide

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analogues, however, has led to better prevention of cancer development and improved disease prognosis [4–7]. On the other hand, the number of patients that are both HBsAg- and HCVAb-negative HCC [non-B non-C HCC (NBNC-HCC)] has increased, and NBNC-HCC is reported to account for 12–20% of all HCC cases in Japan [3, 8].

NBNC-HCC is considered to be associated with several etiologic factors such as alcoholic liver injury, autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), primary sclerosing cholangitis, and nonalcoholic fatty liver disease (NAFLD)/nonalcoholic steatohepatitis (NASH). In addition, a variety of clinical factors are also involved in the development and progression of NBNC-HCC, including age, sex, alcohol consumption, and diabetes mellitus [9, 10]. There are only a few reports, however, on the clinical characteristics of NBNC-HCC, and the actual state of NBNC-HCC has not been fully elucidated [3, 8, 11, 12].

Due to the lack of understanding of the clinical features of NBNC-HCC, neither early detection nor improvement in prognosis has been achieved in patients with NBNC-HCC. Therefore, it is important to understand the complex interactions of the risk factors and clinical features of NBNC-HCC. In this study, we aimed to clarify the clinical characteristics of NBNC-HCC and discuss the etiology of NBNC-HCC.

Methods

Patients

A total of 1,109 HCC patients diagnosed at Osaka Red Cross Hospital from April 1, 2004, to March 31, 2010, were enrolled in the study. The diagnosis of HCC was made based on the presence of both characteristic imaging findings and increases in serum tumor markers; the diagnostic criteria for HCC via imaging was based on previous reports of hyperattenuation at the arterial phase and hypoattenuation at the portal phase in the tumor, determined by dynamic computed tomography and/or magnetic resonance imaging [13]. In addition, increases in serum tumor markers such as α-fetoprotein, des-γ-carboxyprothrombin, or *Lens culinaris* agglutinin-reactive fraction of α-fetoprotein, were also required for the diagnosis of HCC. In doubtful cases, the diagnosis was confirmed by pathologic findings based on liver biopsies obtained under ultrasound guidance.

Classification of HCC according to Etiology

The presence of serum HBsAg and HCVAb was determined in all patients using enzyme immunoassay kits (FUJIREBIO, Tokyo, Japan). Based on the presence of serum antigens/antibodies, the patients were categorized into four groups: B-HCC (HBsAg positive), C-HCC (HCVAb positive), BC-HCC (both HBsAg and HCVAb positive), and NBNC-HCC (both HBsAg and HCVAb negative). AIH was diagnosed based on the simplified diagnostic criteria proposed by the International Autoimmune Hepatitis Group [14], and PBC was diagnosed based on a PBC scoring system [15]. All NBNC-HCC patients, except those with AIH and PBC, were then further divided into two groups, the alcoholic liver disease group (ALD-HCC) and the nonalcoholic group (non-ALD-HCC group) depending on alcohol consumption based on the following criteria: patients whose daily alcohol consumption was over 80 g were included in the ALD-HCC group, and the remaining patients were included in the non-ALD-HCC group. We investigated the background characteristics between the two groups, including age, sex, diabetes, body mass index, hypertension, biochemical test results for liver function, Child-Pugh grade [16], tumor size, tumor number, portal invasion, TNM stage [17], and tumor markers at the time of diagnosis. The diagnosis of diabetes was based on the following criteria: random glucose >200 mg/dl or fasting glucose >126 mg/dl, or hemoglobin A1c >6.5% on two occasions. Hypertension was diagnosed when patients were pharmacologically treated for hypertension or if their arterial pressure was ≥140/90.

The treatment for HCC was performed based on a consensus-based treatment algorithm for HCC proposed by the Japanese Society of Hepatology [2]. Hepatectomy (surgery) or local ablation (radiofrequency ablation, RFA) was performed for 3 or fewer nodules, if the nodules were 3 cm or smaller with no extra-hepatic lesions, good liver function results, and no vascular invasion. Even if the number of nodules was 3 or fewer, if the tumor size exceeded 3 cm, hepatectomy or transcatheter arterial embolization (TACE) was selected. For cases with 4 or more lesions, TACE or transcatheter arterial infusion (TAI) was selected, and resection was considered.

We compared the overall survival rates and cumulative recurrence rates after initial radical treatment between the ALD-HCC and non-ALD-HCC groups. We also studied the factors contributing to recurrence after initial remission and survival for both ALD-HCC and non-ALD-HCC patients. Initial radical treatment (initial remission) was defined as follows: all of the HCC nodules (single/multiple) had disappeared following initial treatment, and no local recurrence or new tumors were detected on computed tomography within 6 months after the initial treatment.

Statistical Analysis

Results are expressed as the mean values with standard deviation or the number (percentage) of patients with each variable. Comparison of background characteristics between ALD-HCC and non-ALD-HCC patients was conducted using Fisher's exact test and Mann-Whitney U test. Overall survival rates and cumulative recurrence rates after initial remission were calculated using the Kaplan-Meier method and the differences between ALD-HCC and non-ALD-HCC patients were examined by log-rank test. The Cox proportional hazards model was used for multivariate analysis for factors that influenced survival and recurrence after the initial remission and performed separately for ALD-HCC and non-ALD-HCC. Statistical data analysis was performed using the SPSS program, version 18.0 (SPSS, Chicago, Ill., USA). All reported p values were two tailed, and statistical significance was set at $p < 0.05$.

Results

Patients

The 1,109 HCC patients in our study comprised 177 NBNC-HCC (16%), 127 B-HCC (11%), 783 C-HCC (71%), and 22 BC-HCC (2%) patients. Of the 177 NBNC-HCC patients, 8 (4 diagnosed with AIH and 4 diagnosed with PBC) were excluded from the study. In addition, 42 other patients were excluded for the following reasons: natural death (n = 15), transfer to other hospitals (n = 18), and missing data (n = 9). We examined the detailed characteristics of the remaining 127 patients. Based on alcohol consumption, 42 patients were included in the ALD-HCC group and the other 85 patients were included in the non-ALD-HCC group.

Clinical Characteristics of Patients with HCC: Comparison between ALD-HCC and Non-ALD-HCC Groups

The clinical features of the 127 NBNC-HCC patients were investigated and compared between the ALD-HCC and non-ALD-HCC groups (table 1). Mean age at the time of diagnosis of HCC was significantly higher in non-ALD-HCC patients than in the ALD-HCC group (71.4 vs. 66.4, respectively; $p < 0.001$). The proportion of men among non-ALD-HCC patients was significantly lower than that among ALD-HCC patients (78 vs. 93%, respectively; $p = 0.034$). The prevalence of diabetes in the non-ALD-HCC group was significantly higher than that in the ALD-HCC group (56 vs. 33%, respectively; $p = 0.015$). Aspartate aminotransferase, alanine aminotransferase, and total bilirubin were significantly higher in the ALD-HCC group than in the non-ALD-HCC group. In addition, albumin levels and prothrombin time were significantly lower in the ALD-HCC group than in the non-ALD-HCC group. Maximum tumor size in diameter (cm) at the time of diagnosis was significantly larger (5.22 vs. 4.20, respectively; $p = 0.007$), and the proportion of patients with α-fetoprotein levels greater than 100 ng/ml was significantly higher (32 vs. 12%, respectively; $p = 0.014$) in the non-ALD-HCC group than in the ALD-HCC group. The differences between groups in body mass index, hypertension, anti-HBc positivity, Child-Pugh grade, number of tumors, portal invasion, the TNM stage (I/II/III/IV), or des-γ-carboxyprothrombin were not significant.

Treatment

According to the consensus-based treatment algorithm for HCC proposed by the Japanese Society of Hepatology [2], initial treatment for NBNC-HCC patients was performed as follows: surgery (n = 7), RFA (n = 21), TACE/TAI (n = 12), and no treatment (n = 2) of the 42 ALD-HCC patients, and surgery (n = 32), RFA (n = 26), TACE/TAI (n = 26), and no treatment (n = 1) of the 85 non-ALD-HCC patients. The proportion of patients receiving surgery as the initial treatment was significantly higher in the non-ALD-HCC group than in the ALD-HCC group (38 vs. 17%, respectively; $p = 0.016$). The proportion of RFA was significantly lower in the non-ALD-HCC group than in the ALD-HCC group (31 vs. 50%, respectively; $p = 0.033$). In total, 22 of 42 ALD-HCC patients and 50 of 85 non-ALD-HCC patients received initial radical treatment, and there was no significant difference (52 vs. 59%, respectively; $p = 0.491$) between groups.

HCC Recurrence Rates after the Initial Radical Treatment

Of the 22 ALD-HCC patients with initial remission, recurrent HCC was detected in 14 patients during a median follow-up period of 28 months (range: 12–83 months). Of the 50 non-ALD-HCC patients with initial remission, recurrent HCC was detected in 19 patients during a median follow-up period of 23 months (range: 9–81 months). The 5-year cumulative recurrence rates in

Table 1. Clinical characteristics of NBNC-HCC

Group	Alcohol (n = 42)	Non-alcohol (n = 85)	p value
Age, years	66.4 ± 7.6	71.4 ± 9.1	<0.001
Males/females	39/3	66/19	0.034
DM +/–	14/28	48/37	0.015
BMI >25/≤25, %	41/59	41/59	NS
Hypertension +/–	13/29	35/50	NS
HBcAb +/–	24/18	37/48	NS
AST, IU/l	59.9 ± 35.5	50.9 ± 49.7	<0.001
ALT, IU/l	44.8 ± 33.3	35.9 ± 34.6	0.042
T-Bil, mg/dl	1.12 ± 0.65	0.92 ± 0.64	0.020
Albumin, g/dl	3.72 ± 0.54	3.92 ± 0.51	0.022
Prothrombin, %	81.4 ± 21.8	90.3 ± 20.3	0.037
Child-Pugh grade A/B/C	28/13/1	67/14/4	NS
Maximum tumor size, cm	4.20 ± 3.67	5.22 ± 3.88	0.007
Single/multiple tumors	21/21	50/35	NS
Portal invasion +/–	5/37	13/72	NS
TNM stage I/II/III/IV	10/16/11/5	6/43/23/13	NS
AFP >100/≤100, ng/ml	5/37	27/58	0.014
DCP >100/≤100, mAu/ml	26/16	58/27	NS

NS = Not significant; AFP = α-fetoprotein; DCP = des-γ-carboxyprothrombin; T-Bil = total bilirubin.

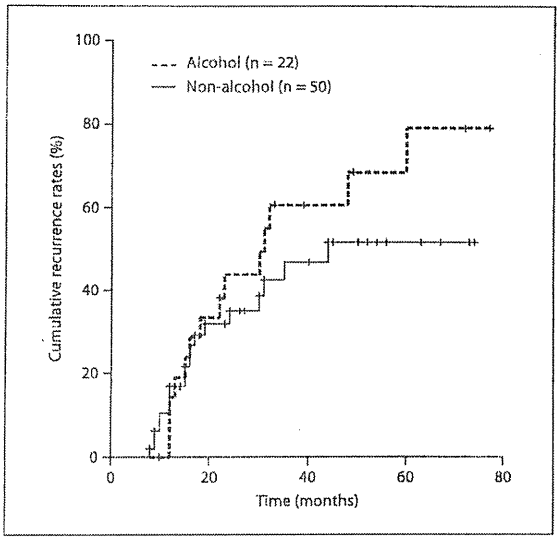

Fig. 1. Cumulative recurrence rates after initial remission. The 5-year cumulative recurrence rates in ALD-HCC and non-ALD-HCC patients were 69 and 52%, respectively. Although the ALD-HCC group was considered to have higher recurrence tendency than the non-ALD-HCC group, the difference was not statistically significant (p = 0.304, log-rank test).

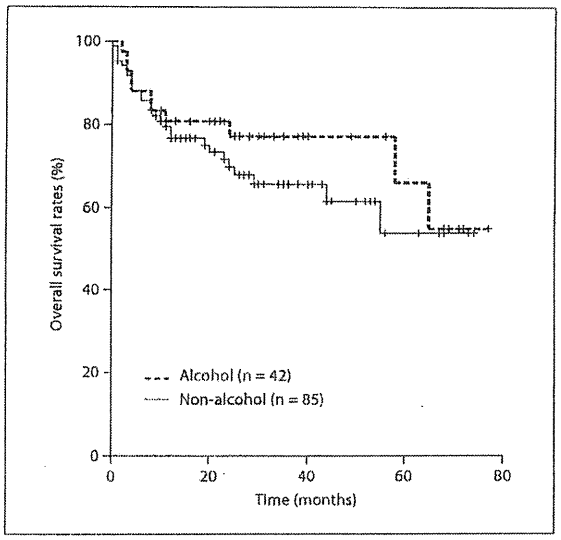

Fig. 2. Overall survival rates. The 5-year survival rates of ALD-HCC and non-ALD-HCC patients were 66 and 53%, respectively, and the difference between groups was not significant (p = 0.352, log-rank test).

ALD-HCC and non-ALD-HCC patients were 69 and 52%, respectively. Although the ALD-HCC group was considered to have higher recurrence tendency than the non-ALD-HCC group, the difference was not statistically significant (p = 0.304, fig. 1).

To identify the factors contributing to the cumulative recurrence of ALD-HCC and non-ALD-HCC, we performed univariate analysis for several factors, but we detected no significant factor responsible for recurrence after the initial radical treatment (data not shown).

Survival Rates

The 5-year survival rates of ALD-HCC and non-ALD-HCC patients were 66 and 53%, respectively, and the difference between groups was not significant (p = 0.352, fig. 2). To identify the factors contributing to the overall survival of ALD-HCC and non-ALD-HCC patients, the Cox proportional hazards model was performed for multivariate analysis for factors that were considered to significantly contribute to survival on univariate analysis (table 2). This analysis revealed that portal invasion of HCC was a significant factor for survival in ALD-HCC patients. On the other hand, a higher α-fetoprotein level (over 100 ng/ml) was a significant factor contributing to survival in non-ALD-HCC patients.

Discussion

The number of patients with hepatic virus-unrelated HCC, i.e. NBNC-HCC, has been increasing annually in Japan [3, 5, 8]. Indeed, the current retrospective cohort study confirmed that the number and proportion of NBNC patients has increased in Osaka Red Cross Hospital; the incidence of NBNC-HCC patients among all HCC patients ranged from 12.8% in 2004 to 22.6% in 2009 (data not shown). The clinical characteristics and prognosis of NBNC-HCC, however, have not been fully elucidated. Therefore, we studied in detail the clinical background and prognosis of a large number of NBNC-HCC patients in a single center, and compared the clinical characteristics between those with alcohol-related and those with alcohol-unrelated HCC.

Table 2. Factors associated with survival according to Cox proportional hazard analysis

Risk factors	Alcohol (n = 42)		Non-alcohol	
	hazard ratio (95% CI)	p	hazard ratio (95% CI)	p
Maximum tumor size >3 cm	NS	NS	NS	NS
Portal invasion	11.9 (0.90–157.4)	0.05	NS	NS
Tumor stage III/IV	NS	NS	NS	NS
AFP >100 ng/ml	NS	NS	4.63 (1.57–13.6)	0.005
DCP >100 mAU/ml	NS	NS	NS	NS

The findings of the present study demonstrated that diabetes was significantly more frequent in the non-ALD-HCC group than in the ALD-HCC group. An earlier large-scale epidemiologic study of 824,263 registered patients showed that among men with diabetes, the risk of HCC is doubled, and this increase in risk is independent of ALD, viral hepatitis, or demographic features [18]. Another population-based study of 8,244 patients reported that diabetes is associated with a 2- to 3-fold increase in the risk of HCC, regardless of the presence of other major HCC risk factors and that diabetes is an independent risk factor for HCC [19]. In Japan, the diagnosis of diabetes continues to increase. Indeed, between 1997 and 2007, people classified as 'strongly suspected of having diabetes' increased from approximately 6.9 to 8.9 million, and those classified as 'people for whom the possibility of diabetes cannot be precluded' increased from approximately 6.8 to 13.2 million [20]. This tendency may be associated with the increased incidence of NBNC-HCC patients.

The pathophysiology underlying the increased risk of HCC with diabetes is not certain. Diabetes is associated with NAFLD, including NASH with specific hepatic insulin resistance [21, 22]. Insulin resistance facilitates peripheral lipolysis and the accumulation of free fatty acids in the liver, thus leading to NAFLD. Hepatocellular injury, inflammation, and, eventually, hepatic fibrosis can result in the occurrence of HCC [18]. A recent study indicated that diabetes-related NAFLD/NASH with elevated liver enzymes is associated with a clinically significant risk of developing end-stage liver disease, including HCC [23]. The annual NAFLD cumulative incidence of HCC is reported to be 2.6% in patients with NASH cirrhosis [24]. Thus, in non-ALD-HCC patients, a higher prevalence of diabetes could contribute to accumulating liver damage as NASH, and eventually HCC.

The diagnosis of NASH is based on following pathologic findings: hepatic steatosis, hepatocellular ballooning, lobular inflammation, pericellular or perisinusoidal fibrosis, and Mallory body formation [25]. It is difficult to evaluate the role of hepatocyte fat deposition in the development of HCC; however, because the fat deposits in hepatocytes tend to disappear as liver fibrosis progresses; this phenomenon is referred to as burnout NASH [26]. Indeed, based on histological analysis of tissues from hepatectomy, it was difficult to confirm pathologic evidence of NASH. In 6 patients with non-ALD-HCC surgically treated, NASH-like pathologic findings, such as hepatic steatosis, hepatocellular ballooning, and Mallory body formation were identified, however, suggesting that some proportion of the non-ALD-HCC group had NASH-based HCC.

In the present study, tumor size was larger, and tumor marker levels were higher in the non-ALD-HCC group than in the ALD-HCC group. This is in part due to the lower opportunity of non-ALD-HCC patients to undergo annual surveillance for chronic liver disease, and the tendency to be diagnosed with definite HCC at a more advanced stage. Surveillance has not been established for patients with NBNC-HCC because the risk factors are not well understood, other than excessive alcohol drinking. As a result, cryptogenic HCC patients, especially those with non-ALD-HCC, are not detected until they reach an advanced stage [27]. Although the general consensus is that metabolic factors are related to the occurrence of HCC, we have not established a method for determining the high risk group. Among patients with metabolic factors, more detailed examination of the possible carcinogenic factors is necessary for earlier detection of HCC.

Liver function results were altogether significantly worse in ALD-HCC patients than in non-ALD-HCC patients. Because liver function in the ALD-HCC group was worse than that in the non-ALD-HCC group, those in the ALD-HCC group were thought to undergo more liver injury, and this cumulative injury may increase the occur-

rence of HCC. Therefore, poor liver function could be the major reason of higher recurrence rate after initial remission of ALD-HCC than non-ALD-HCC. As a result, ALD-HCC and non-ALD-HCC patients had a similar prognosis. The ALD-HCC group was superior to the non-ALD-HCC group in the tumor state, such as tumor size and tumor marker at the time of diagnosis, but inferior to the non-ALD-HCC group in liver function, leading to recurrence. These good and bad points counteract each other and result in an equivalent prognosis between groups.

In conclusion, although the number of patients with NBNC-HCC has been increasing annually, many features of NBNC-HCC remain unknown. Based on the present study, the most common etiologic factor for NBNC-HCC was alcohol, and diabetes may be related to the occurrence of HCC in patients with non-alcohol-related liver disease. The comparison between groups revealed that non-ALD-HCC tended to be detected at a more advanced stage, whereas liver function in ALD-HCC was worse. Finally, the prognosis was equivalent between groups. Further examination of the risk factors for NBNC-HCC and establishing a precise surveillance system are needed to diagnose HCC earlier and develop curative therapies.

Disclosure Statement

All authors disclose no conflicts.

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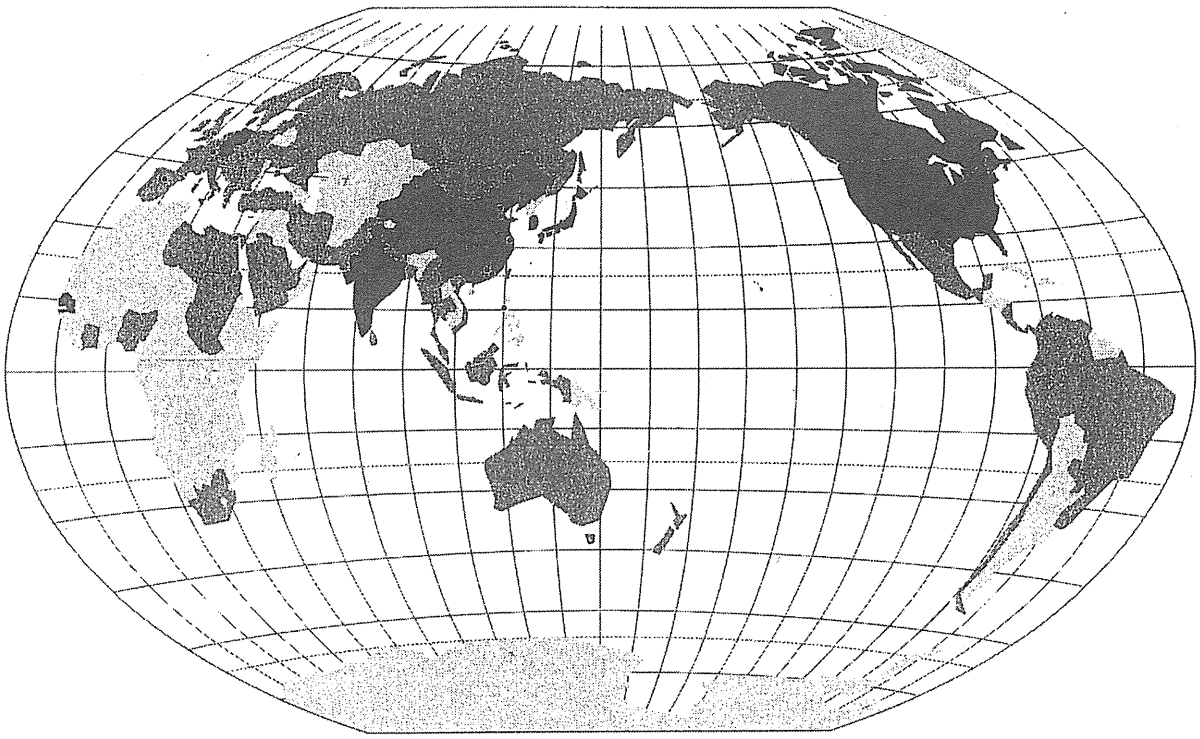

Reprint

J Microwave Surg Vol.29, 2011

Medical Review Co., Ltd.

Original

肝細胞癌に対する外科切除とラジオ波凝固療法 —治療法選択の現状と選択の基準—

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Surgical resection versus radiofrequency ablation

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Abstract

Earlier diagnosis of hepatocellular carcinoma has become possible, and resectable cases have been increasing. However, there are limitations for operation because many cases involve liver cirrhosis, and recurrence rates after operation are high. So radiofreguency ablation (RFA) which is a less invasive treatment is the preferred method. We investigated cases of hepatic resection and RFA to ascertain which should be selected for curative treatment possible cases. The cumulative survival rate showed no difference in both groups for the patients with Child A/B, 3 cm or less in tumor diameter and 3 or less in tumor number. RFA was considered to be first choice of treatment for those cases. However surgical resection tends to present a better long term prognosis, and should be considered for patients who are young with good liver function, and expecting a long term prognosis. Treatment should be selected on a case by case basis while considering expected survival period and QOL.

Key words: hepatocellular carcinoma, radiofrequency ablation, hepatic resection

Accepted on Feb. 18, 2011

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Ⅰ．肝細胞癌診断および治療法の年次推移

一般に癌に対する３大治療法は手術，化学療法，放射線療法である．他方，肝細胞癌（HCC）においてはその多くが肝硬変を合併しており手術に制約があること，根治的治療後も５年約80％という極めて高い再発率を有するなど，他臓器癌とは異なる特徴を持っている[1]．さらに1980年代以降様々な新しい治療法が開発され，HCCにおいては手術，局所療法，カテーテル療法の３つが柱となっている．前２者は局所制御の観点から根治的と考えられる治療法であり，stageⅠ，Ⅱの肝癌が主たる対象となる．1990年代以降，画像診断法は飛躍的に進歩し，肝癌の診断は早期化してきている．1980年代以降当院において診断された肝癌3,493例を対象に（Table 1），診断時のAFP値と肉眼進行度を年代別に示した（Fig. 1, 2）．1980年代には診断時AFP値は100ng/mL以上が60％，診断時肉眼進行度もstageⅠ，Ⅱは37％であったが，2000年代では逆に100ng/mL 以下が70％以上（Fig. 1），肉眼進行度もstageⅠ，Ⅱが60％を超えており（Fig. 2），根治的治療法の対象例が増えている．診断治療におけるこれらの変化を反映し，選択施行される治療法も変化している．Fig. 3に1980年代以降，当院で施行された各種治療件数の年次推移を示した．変化の特徴はまず治療総件数が飛躍的に増えていることである．1980年代は10年間での治療総件数は約800件であったが，近年では１年間の治療件数が1,200件と約15倍となっている．また1980年代にはそのほとんどが肝動脈塞栓術（transcatheter arterial embolization：TAE），肝動脈注入療法（transcatheter arterial infusion：TAI）の経カテーテル治療であったが，1990年代にはエタノール局注療法（percutaneous ethanol injection therapy：PEIT）が最も多くなり，2000年代にはラジオ波凝固療法（radiofrequency ablation：RFA）が中心的な治療法となっている．件数としてはTAIが最も多いがその多くはRFA前に施行した血管造影施行時に抗癌剤とリピオドールの懸濁液を動注したものであり，治療の主体はRFAである．科学的根拠に基づく肝癌診療ガイドラインではChild-Pugh A/Bで腫瘍径３cm以下，３個以下では肝切除と局所療法の両者が推

Table 1　Patients with hepatocellular carcinoma (3493 cases, 1981-2009, Osaka Red Cross Hospital)

Male/Female	2520 (72.1%) / 973 (27.9%)
Age	65.5 ± 9.3 (17-90 year old)
Child-Pugh A/B/C	1922 / 786 / 123
	(67.9%) (27.8%) (4.3%)
A/C/B+C/N.B/N.C	381 / 2305 / 78 / 370
	(12.2%) (73.5%) (2.5%) (11.8%)
LC/CH/Fibrosis/Fatty/etc	2571 / 676 / 19 / 12 / 100
	(76.1%) (20.0%) (0.6%) (0.4%) (3.0%)
Stage Ⅰ/Ⅱ/Ⅲ/Ⅳ-A/Ⅳ-B	542 / 1204 / 974 / 442 / 97
	(16.3%) (36.9%) (29.9%) (13.6%) (3.0%)

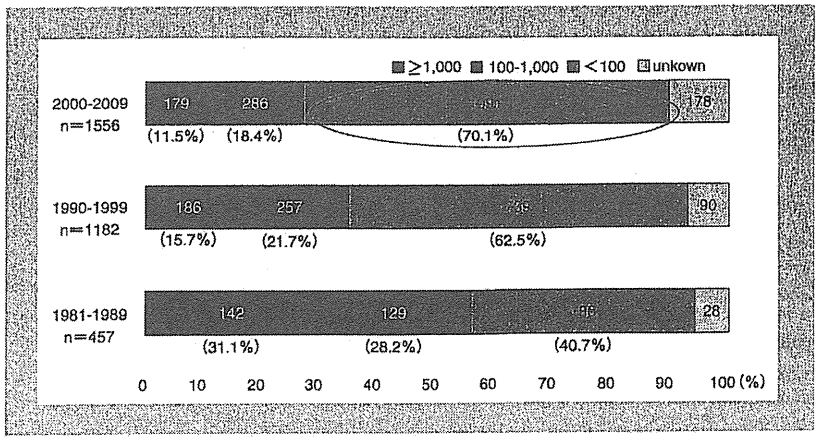

Figure 1　The value of AFP on diagnosis by decade

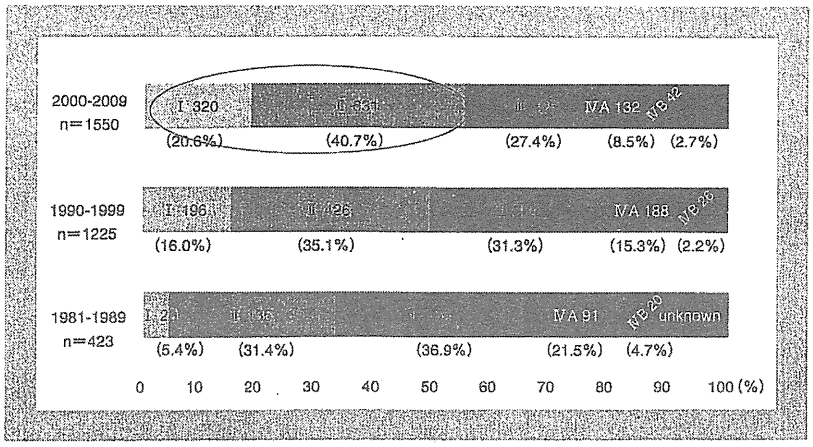

Figure 2 Stages by decade

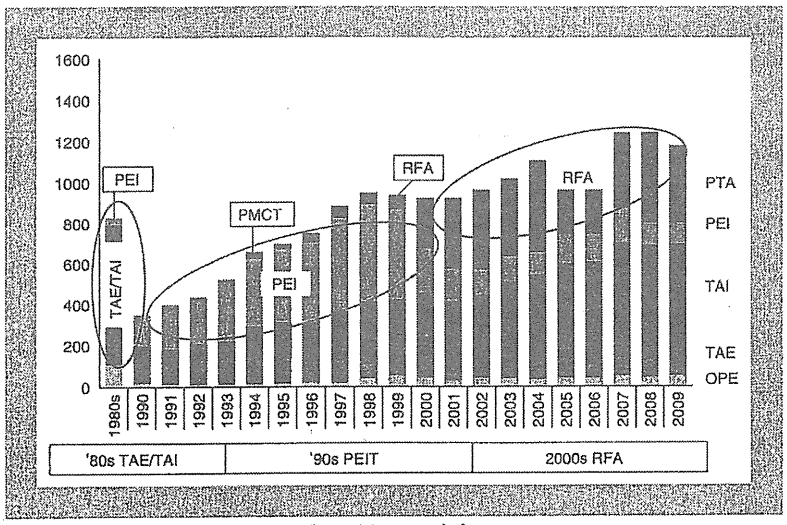

Figure 3 The number of treatments for HCC - annual change -

PTA : percutaneous thermal ablation (RFA, PMCT)
PEI : percutaneous ethanol injection
TAI : transcatheter arterial infusion
TAE : trandcatheter arterial embolization
OPE : operation