Hepatitis B virus (HBV) infection is one of the most common persistent viral infections in humans. Approximately 2 billion people worldwide have been exposed to HBV and 350 million of them remain persistently infected.[1] The incidence of HBV infection and the patterns of transmission vary greatly worldwide among different population subgroups.[2] In Western countries, chronic HBV infection is relatively rare and acquired primarily in adulthood by way of sexual transmission or the use of injectable drugs. Meanwhile, in Asia and most of Africa, the majority of infections are the result of transmission from an infected mother to her newborn. However, very few studies of acute hepatitis B (AHB) in adults have been reported.

HBV genomic sequences vary worldwide and have been classified into at least eight genotypes (A through H) based on an intergroup divergence of 8% or more over the complete nucleotide sequence.[3,4] These genotypes have distinct geographic distributions.[5-7] In particular, genotype A is predominant in Northwestern Europe, the United States, Central Africa, and India.[8, 9] The Japanese have been infected with genotypes B and C since prehistoric times.[10] Recently, many lines of evidence have revealed among the Japanese an increase in acute infection with HBV genotype A following sexual transmission.[11,12] As a result of this increasing transmission of genotype A, the distribution of HBV genotypes in Japan clearly differs among patients with acute and chronic infections.[13] Moreover, recent studies suggest that acute infection with HBV genotype A may be associated with an increased risk of progression to persistent infection.[15] Indeed, the prevalence of HBV genotype A in chronic hepatitis B patients doubled in Japan between 2000-2001 and 2005-2006 (1.7% versus 3.5%).[11]

Human immunodeficiency virus (HIV)-1 infection results in an immunodeficient state, with the virus sharing routes of transmission with HBV. HIV-related immunodepletion influences the natural history of HBV infection, and epidemiological studies have revealed that HIV-positive patients are more likely to have a prolonged acute illness following HBV infection and lower rates of hepatitis B e-antigen (HBeAg) clearance.[16] Therefore, in this study patients with coinfection of HIV were excluded to examine the influence of HBV genotype directly without the confounding influence of HIV.

From 2005 to 2010, a multicenter cohort study was conducted throughout Japan on 212 patients with AHB. The aim of this cohort study was to assess the influence of clinical and virological factors, including HBV genotypes and treatment with nucleotide analogs (NAs), on AHB patients who became persistently infected.

Patients and Methods

Patients With AHB. The multiple-source cohort included 212 randomly selected AHB patients without coinfection of HIV. From 2005 through 2010, the study participants were recruited from 38 liver centers throughout Japan. The cohort included patients who were admitted to the hospital because of AHB and who visited the hospital every month after being discharged. The diagnosis of AHB was contingent on the rapid onset of clinical symptoms accompanied by elevated serum alanine aminotransferase (ALT) levels, the detection of serum hepatitis B surface antigen (HBsAg), and a high-titer antibody to hepatitis B core antigen (anti-HBc) of the immunoglobulin M (IgM) class. Patients with initial high-titer anti-HBc (>10.0 S/CO) were diagnosed as having an exacerbation of chronic hepatitis B and were excluded. If the patient had been tested previously, the absence of serum HBsAg and anti-HBc before admission was verified from the medical record to discriminate a new infection from an acute exacerbation of a persistent infection. Patients with acute hepatitis A, hepatitis C, and drug- or alcohol-induced acute hepatitis were also excluded; hepatitis D virus infection was not determined because of its extreme rarity in Japan. The study protocol conformed to the 1975 Declaration of

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Table 1. Characteristics of Patients With Genotype A or a Non-A Genotype Acutely Infected With Hepatitis B Virus

Features	Genotype A (n = 107)	Non-A Genotypes (n = 105)*	*P* Value
Age (years)	36.3 ± 12.0	40.7 ± 14.3	0.032
Male sex	102 (95.3)	75 (71.4)	<0.001
HBeAg positive	104 (97.2)	79 (75.2)	<0.001
ALT (IU/L)	1210 ± 646	2225 ± 2851	0.045
Total bilirubin (mg/dL)	9.9 ± 9.4	7.5 ± 6.7	0.115
HBV DNA (log copies/mL)	7.0 ± 1.5	5.8 ± 1.5	<0.0001
Duration until disappearance of HBsAg (month)	6.7 ± 8.5	3.4 ± 6.5	<0.0001
Persistence of HBsAg positivity more than 6 months	25 (23.4)	9 (8.6)	0.003
Persistence of HBsAg positivity more than 12 months	8 (7.5)	1† (0.9)	0.018
Sexual transmission	81/84 (96.4)‡	71/79 (89.9)§	0.095
Treatment with NAs	61 (57.0)	42 (40.0)	0.013

Data are presented as n (%), mean ± standard deviation. HBV, hepatitis B virus; HBeAg, hepatitis B e-antigen; ALT, alanine aminotransferase; NAs, nucleotide analogs.

*Non-A genotypes include genotypes B, C, D, F and H (n = 25, 77, 1, 1, and 1, respectively).

†One patient had genotype C.

‡Transmission routes were unknown for 23 patients.

§Transmission routes were unknown for 26 patients.

Helsinki and was approved by the Ethics Committees of the institutions involved. Every patient gave informed consent for this study.

Serological Markers of HBV Infection. HBsAg, HBeAg, antibodies to HBsAg (anti-HBs), HBeAg (anti-HBe), and HBcAg, and anti-HBc of the IgM class were tested by a chemiluminescent enzyme immunoassay (CLIA) by ARCHITECT (Abbott Japan, Tokyo, Japan). HBV DNA measurements were performed using a real-time polymerase chain reaction (PCR) assay (Cobas TaqMan HBV Auto; Roche Diagnostics, Tokyo, Japan).

Genotyping of HBV. The six major HBV genotypes (A through F) were determined serologically by enzyme immunoassay (EIA) using commercial kits (HBV GENOTYPE EIA; Institute of Immunology, Tokyo, Japan). This method is based on the pattern of detection by monoclonal antibodies of a combination of epitopes on preS2-region products, which is specific for each genotype.[17,18] Samples for which EIA could not determine the genotype were examined by direct sequencing of the pre-S2/S gene, followed by phylogenetic analysis.

Treatment With NAs. Treatments with NAs were performed using lamivudine or entecavir for more than 3 months. The individual clinicians determined if NAs were administered to patients, and when the treatment was to be started. The time to onset of treatment with NAs was measured in days from onset of AHB.

Statistical Analysis. Categorical variables were compared between groups by the chi-squared test and noncategorical variables by the Mann-Whitney *U* test. A *P* value less than 0.05 was considered significant. Multivariate analysis was performed using a backward stepwise logistic regression model to determine independent factors for viral persistence following AHB. Variables in the multivariate analysis were selected based on variables that were marginally significant with $P < 0.1$ in univariate analysis. Maintenance of HBsAg positivity was analyzed using the Kaplan-Meier method and significance was tested with the log-rank test. STATA Software (StataCorp, College Station, TX) v. 11.0 was used for analyses.

Results

Comparison of Characteristics Between Genotype A and Non-A Genotype AHB Patients. A total of 107 AHB patients (50.5%) were infected with genotype A while 105 AHB patients (49.5%) were infected with non-A genotypes, including genotypes B (25 [11.8%]), C (76 [35.8%]), D (1 [0.5%]), F (1 [0.5%]), and H (1 [0.5%]). Compared to those infected with non-A genotypes, genotype A patients were significantly younger (36.3 ± 12.0 versus 40.7 ± 14.3 years, $P = 0.032$), predominantly men (95.3% versus 71.4%, $P < 0.001$), and more frequently positive for HBeAg (97.2% versus 75.2%, $P < 0.001$). Moreover, genotype A patients had a lower peak ALT levels (1,210 ± 646 versus 2,225 ± 2,851 IU/L, $P = 0.045$) and a higher peak level of HBV DNA (6.7 ± 8.5 versus 3.4 ± 6.5 log copies/mL, $P < 0.0001$). A significantly higher percentage of genotype A patients were treated with NAs (57% versus 40%, $P = 0.013$). These data are summarized in Table 1.

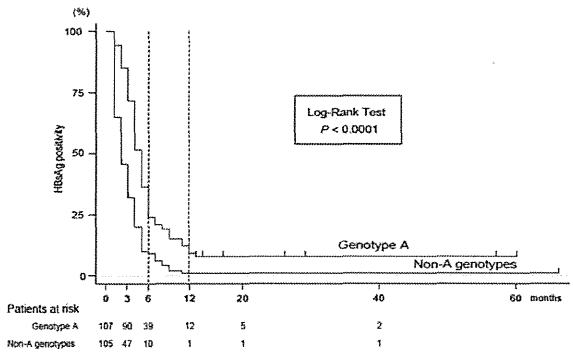

Fig. 1. Comparison of the cumulative proportion of AHB patients maintaining HBsAg positivity between genotype A and non-A genotypes, analyzed using the Kaplan-Meier test. $P < 0.0001$, genotype A: red line, non-A genotypes: blue line.

Cumulative Maintenance of HBsAg Positivity During Follow-up in Patients With Genotype A and Non-A Genotypes. In the patients infected with genotype A and non-A genotypes, the mean durations of HBsAg positivity maintenance were 6.7 ± 8.5 and 3.4 ± 6.5 months, respectively ($P < 0.0001$; Table 1, Fig. 1). For 6 months after AHB onset, the number of patients with genotype A and non-A genotypes maintaining HBsAg positivity were 39/107 (36.4%) and 10/105 (9.5%), respectively ($P < 0.001$). However, in many patients HBsAg disappeared between 7 and 12 months after AHB onset; that is, HBsAg disappeared in 31/107 (29.0%) of patients with genotype A and in 9/105 (8.6%) of patients with non-A genotypes during this time period. However, in some patients HBsAg never disappeared after persisting for more than 12 months following AHB onset. When chronicity after AHB was defined as the persistence of HBsAg for more than 12 months, chronicity developed in 7.5% (8/107) of patients with genotype A and in 0.9% (1/105) of patients with non-A genotypes ($P = 0.018$).

Comparison of Characteristics Between Patients in Whom HBsAg Persisted More Than 6 or 12 Months and Those With Self-Limited AHB Infection. Table 2 compares the demographic and clinical characteristics between patients in whom HBsAg disappeared within 6 months and those in whom HBsAg persisted for more than 6 months from AHB. The peak ALT levels ($1,882 \pm 2,331$ versus $1,018 \pm 696$ IU/L, $P = 0.0024$) and peak HBV DNA levels (6.3 ± 1.6 versus 7.4 ± 1.6 mg/dL, $P = 0.0004$) were significantly higher and lower in the former group than in the latter group, respectively. Moreover, marked differences were present in the distribution of genotypes between the two groups. The percentage of the HBV genotype A (46.1% versus 73.5%, $P = 0.003$) was significantly higher among patients in whom HBsAg was persistent for more than 6 months. In addition, we compared the demographic and clinical characteristics between patients in whom HBsAg disappeared within 12 months and those in whom HBsAg persisted for more than 12 months from AHB. Peak ALT ($1,787 \pm 2,118$ versus 775 ± 513 IU/L, $P = 0.0089$) and peak total bilirubin (8.7 ± 8.2 versus 3.8 ± 6.6 mg/dL, $P = 0.0039$) levels were significantly higher in the former group than in the latter group. In contrast, the peak HBV DNA levels (6.4 ± 1.6 versus 7.9 ± 1.4 mg/dL, $P = 0.0046$) were significantly lower

Table 2. Comparison Between Patients With Chronicity Following Acute Hepatitis B and Those With Self-Limited Acute Infections Determined by the Persistence of HBsAg for More Than 6 or 12 Months

Features	Disappearance of HBsAg Within 6 Months (n = 178)	Persistence of HBsAg for More Than 6 Months From AHB (n = 34)	P Value	Disappearance of HBsAg Within 12 Months (n = 203)	persistence of HBsAg for More Than 12 Months From AHB (n = 9)	P Value
Age (years)	38.2 ± 13.1	40.0 ± 14.5	0.454	38.1 ± 13.2	46.7 ± 14.0	0.061
Male sex	147 (82.6)	30 (88.2)	0.416	169 (83.3)	8 (88.9)	0.677
HBeAg positive	150 (84.3)	32 (94.1)	0.131	175 (86.2)	8 (88.9)	0.815
ALT (IU/L)	1882 ± 2331	1018 ± 696	0.0024	1787 ± 2118	775 ± 513	0.0089
Total bilirubin (mg/dL)	8.6 ± 7.5	8.7 ± 11.3	0.137	8.7 ± 8.2	3.8 ± 6.6	0.0039
HBV DNA (log copies/mL)	6.3 ± 1.6	7.4 ± 1.6	0.0004	6.4 ± 1.6	7.9 ± 1.4	0.0046
HBV genotype						
Non-A	96 (53.9)	9 (26.5)		104 (51.2)	1 (11.1)	
A	82 (46.1)	25 (73.5)	0.003	99 (48.8)	8 (88.9)	0.018
Sexual transmission	128/137 (93.4)*	24/26 (92.3)†	0.711	146/157 (93.0)‡	6/6 (100.0)§	0.356
NAs treatment (+)	82 (46.1)	21 (61.8)	0.093	98 (48.3)	8 (88.9)	0.017

Data are presented as n (%) and mean ± SD. HBsAg, hepatitis B surface antigen; AHB, acute hepatitis B, HBeAg, hepatitis B e-antigen; ALT, alanine aminotransferase; HBV, hepatitis B virus; NAs, nucleotide analogs.

*Transmission routes of 41 patients were unknown.

†Transmission routes of 8 patients were unknown.

‡Transmission routes of 46 patients were unknown.

§Transmission routes of 3 patients were unknown.

Table 3. Multivariate Analysis of Factors Independently Associated With Persistence of HBsAg Positivity Following Acute Hepatitis B

	Persistence of HBsAg More Than 6 Months From AHB		
Factors	Odds Ratio	95% CI	*P* Value
ALT (per 1 IU/L increase)	1.000	0.999-1.000	0.035
HBV DNA (per 1 log copy/mL increase)	1.176	0.931-1.484	0.173
Genotypes			
Non-A	1.00		
A	4.224	1.853-9.631	0.001

95% CI, 95% confidence interval; ALT, alanine aminotransferase; HBV, hepatitis B virus.

in the former group than in the latter group. The percentages of HBV genotype A (48.8% versus 88.9%, $P = 0.018$) and NAs treatment (+) (48.3% versus 88.9%, $P = 0.017$) were significantly higher among patients in whom the HBsAg persisted for more than 12 months.

Factors Independently Associated With Viral Persistence Following AHB. A stepwise logistic regression model was used to perform multivariate analysis which explains relationships between some factors and persistence of HBsAg positivity more than 6 months following AHB. Peak ALT level, peak HBV DNA level, genotype A, and treatment with NAs were retained in the final multivariate logistic model in a backward stepwise manner ($P < 0.1$). For predicting the persistence of HBsAg for more than 6 months, only genotype A was independently associated with progression of AHB to the persistence of HBsAg (odds ratio [OR]: 4.224, $P = 0.001$, Table 3).

Characteristics of Patients Who Progressed to Chronicity That Was Defined as the Persistence of HBsAg for More Than 12 Months Following Acute Hepatitis B. Table 4 shows the clinical and virological characteristics of nine patients who progressed to chronicity defined as the persistence of HBsAg for more than 12 months following AHB. Among the nine patients who progressed to chronicity from AHB, eight (88.9%) were men and eight (88.9%) were HBeAg-positive. In general, among the patients who progressed to chronicity following AHB, the peak HBV DNA levels were high, and the peak total bilirubin and ALT levels were low. In eight (88.9%) patients, entecavir was administered; however, the duration until the onset of NA treatment from AHB onset was long (75-570 days).

Early Onset of Treatment With NAs Was Able to Prevent Viral Persistence After AHB Caused by Genotype A. The cumulative proportion maintaining HBsAg positivity during follow-up, expressed in terms of time after AHB onset, were significantly longer in patients with NAs treatment than in those without NAs treatment ($P = 0.046$, Fig. 2A). Table 5 shows the percentages of patients in whom HBsAg persisted for more than 6 or 12 months among patients categorized based on the period of time (i.e., duration) until the onset of NAs treatment. For patients in whom the onset of NAs treatment was less than 4 weeks from the onset of AHB, 12.7% of the patients showed persistent HBsAg for more than 6 months, while none showed HBsAg positivity for more than 12 months. For patients in whom the onset of NAs treatment was at 5-8 weeks, 37.5% of the patients showed persistent HBsAg for more than 6 months, whereas none showed persistent HBsAg for more than 12 months. For all groups, the period of HBsAg positivity in patients starting NAs treatment within 8 weeks from AHB onset was significantly shorter than that in patients beginning NAs treatment after more than 8 weeks from AHB onset ($P < 0.0001$, Fig. 2B). Patients starting NAs treatment within 8 weeks from AHB onset never progressed to chronicity after AHB caused by genotype A.

Table 4. Characteristics of Patients Who Progressed to Chronicity Following Acute Hepatitis B

Case	Age	Gender	HIV	HBeAg	HBV DNA (log copies/mL)	Total Bilirubin (mg/dL)	ALT (IU/L)	Observation Period (Months)	NAs Treatment	Duration Until NAs Treatment (Days)	Transmission Routes	Genotype
1	23	Male	(−)	(+)	7.6	1.7	1271	26	ETV	570	Heterosexual	A
2	40	Male	(−)	(−)	8.8	1.4	568	13	ETV	240	Heterosexual	A
3	45	Male	(−)	(+)	7.7	0.9	867	57	ETV	135	Heterosexual	A
4	37	Male	(−)	(+)	7.6	3.4	384	29	ETV	75	Unknown	A
5	54	Male	(−)	(+)	9	2	455	17	ETV	155	Homosexual	A
6	45	Male	(−)	(+)	4.8	21.2	512	60	(−)	(−)	Homosexual	A
7	61	Male	(−)	(+)	9.1	1.5	804	17	ETV	88	Unknown	A
8	56	Male	(−)	(+)	9.0	1.1	1820	14	ETV	118	Unknown	A
9	31	Female	(−)	(+)	7.4	0.8	296	66	ETV	150	Blood transfusion	C

HIV, human immunodeficiency virus; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; NAs, nucleotide analogs; ETV, entecavir.

Table 5. Proportion of Patients in Whom HBsAg Persisted for More Than 6 or 12 Months Among Patients Categorized Based on the Number of Weeks Until the Onset of NAs Treatment

Duration Until Onset of NAs Treatment (Weeks)	Persistence of HBsAg for More Than 6 Months	Persistence of HBsAg for More Than 12 Months	Total Patients
<4 weeks (n, %)	9 (12.7)	0 (0)	71
5-8 weeks (n, %)	6 (37.5)	0 (0)	16
9-12 weeks (n, %)	1 (33.3)	1 (33.3)	3
13-16 weeks (n, %)	4 (100)	1 (25.0)	4
>17 weeks (n, %)	9 (100)	6 (66.7)	9
Total	29	8	103

HBsAg, hepatitis B surface antigen; NAs, nucleotide analogs.

Discussion

A multicenter nationwide study was conducted throughout Japan to evaluate the influence of clinical and virological factors on chronic outcomes in Japanese patients who contracted AHB in adulthood. The study was feasible in Japan, where a universal vaccination program for HBV has not been implemented because of the extremely high efficacy of the immunoprophylaxis that is given to babies born to carrier mothers. The implementation of this program has resulted in a decrease in the persistent HBV carrier rate from 1.4% to 0.3%.[19] Selective vaccination means that Japanese are more likely to be infected with HBV by way of horizontal transmission since the percentage of the population possessing anti-HBs is much lower than that in countries in which universal vaccination programs have been established.[20] In addition, Japan is faced with the ever-increasing impacts of globalization: as many as 17 million Japanese travel abroad and over 7 million people visit Japan from overseas each year. This "population mixing" may help to explain the increased prevalence in Japan of AHB due to genotype A, which is transmitted through indiscriminate sexual contact. Consequently, Japan may be the only country in the world where the influences of HBV genotypes, including genotype A (as is predominant in Western countries) and genotypes B and C (as are predominant in Asian countries), on chronic outcomes after AHB can be compared.

Currently, the persistence of HBsAg in serum for more than 6 months is considered to represent a progression to chronic infection.[21] However, our data showed that HBsAg frequently disappeared between 7 to 12 months after the onset of AHB in patients with genotype A (31/107 [29.0%]) and non-A genotypes (9/105 [8.6%]) (Fig. 1). These patients were considered to exhibit prolonged cases of AHB, rather than persistent infection. This finding reflects the higher sensitivity of the most up-to-date assays for HBsAg as compared with previous methods. In the present study, HBsAg was measured by CLIA, which has been reported to be about 150 times more sensitive in the detection of HBsAg than reverse passive hemagglutination (RPHA)-HBsAg, which has been used for the last 30 years in Japan.[22] The use of a more sensitive assay for HBsAg results in a longer period during which HBsAg may be detected. In this study, HBsAg did not disappear in nine patients after remaining continuously detectable for more than 12 months. Therefore, the persistence of HBsAg for more than 12 months, as measured with a highly sensitive method for detecting HBsAg, may be suitable for defining the progression of AHB to chronicity; however, further study is necessary to determine whether this definition is appropriate worldwide.

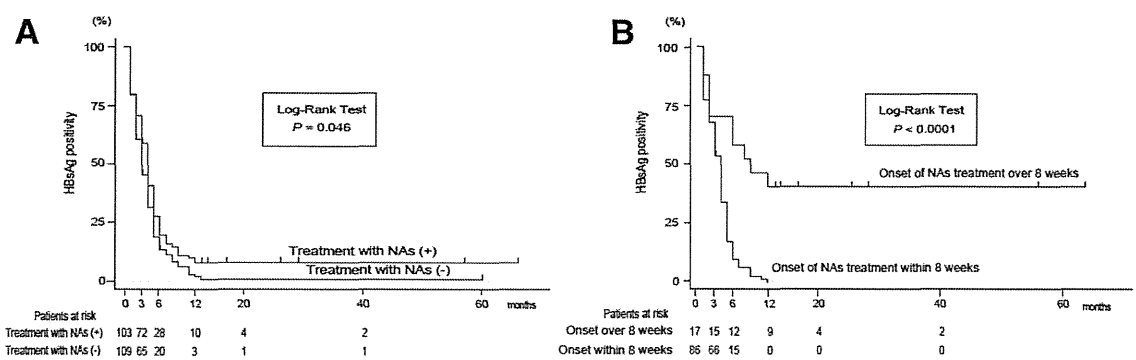

Fig. 2. (A) Comparison of the cumulative proportion of AHB patients maintaining HBsAg positivity between treatment with NAs (+) and treatment with NAs (−), as analyzed using the Kaplan-Meier test. $P = 0.046$, treatment with NAs (+): red line, treatment with NAs (−): blue line. (B) Comparison of the cumulative proportion of AHB patients in genotype A maintaining HBsAg positivity between treatment onset with NAs within 8 weeks and treatment onset with NAs over 8 weeks after onset of AHB, as analyzed using the Kaplan-Meier test. $P < 0.0001$, treatment onset with NAs over 8 weeks: red line, treatment onset with NAs within 8 weeks: blue line.

It has been reported that ~10% of patients who contract HBV as adults do not clear HBsAg from their serum and become carriers.[23] Meanwhile, a wide variation has been seen in the rate of persistence after AHB infection in adults. For example, viral persistence following AHB was seen in 0.2% (1/507) of adults in Greece,[24] 7.7% (5/65) of adult Alaskan Eskimos, and 12.1% (7/58) of adults in Germany.[25] The difference in the proportion of patients progressing from AHB to chronicity in different regions may be attributable to virological and host factors. In this study, 4.2% (9/212) of patients progressed to chronicity after AHB: 7.5% (8/107) of those infected with genotype A and 0.9% (1/105) of those infected with non-A genotypes. The non-A genotypes included genotypes B, C, D, F, and H (n = 25, 77, 1, 1, and 1, respectively). Genotypes B and C are predominant in eastern Asian countries, where the majority of those infected with HBV acquired the virus during the perinatal period by way of vertical transmission.[26] On the other hand, genotype A is predominant in Western countries, where the main route is horizontal transmission later in life.[26,27] Because HBeAg persists long after the infection in the genotype C as compared to other genotypes, this genotype has been shown to be a risk factor for perinatal and horizontal transmission in newborns and children.[28] The predominance of genotype A in Western countries may be attributable to a higher chronicity rate following AHB by way of horizontal transmission in adults.

In this study the characteristics of AHB associated with genotype A were a higher peak level of HBV DNA and a lower peak level of ALT. These findings were similar to those for patients with HBV-HIV coinfection.[29] Such characteristics of genotype A or coinfection with HIV are assumed to be attributable to milder hepatitis associated with weaker cellular immune responses. More slowly replicating viruses have been reported to evoke weaker cellular responses, enhancing the likelihood of persistence.[30] Indeed, our prior study showed that the replication of genotype A was significantly slower than that of genotype C in immunodeficient, human hepatocyte chimeric mice.[31] Moreover, variation among genotypes in the expression pattern of HBeAg may affect the progression of AHB to chronicity. Another previous study of ours revealed that a single form of HBeAg was detected by western blot analysis in serum samples from patients infected with genotypes B through D, but that two additional larger forms of HBeAg were detected in patients with genotype A.[32] Milich and Liang[33] reported that HBeAg may modulate the host immune response as a tolerogen to promote chronicity. Therefore, the different expression pattern of HBeAg by genotype A HBV may contribute to chronicity following AHB.

Early NAs initiation appeared to enhance the viral clearance across genotypes, although treatment with NAs did not show any overall benefit in duration of HBsAg. Previous studies examining the efficacies of NAs for preventing progression to chronic infection after AHB have reported conflicting results. Some small-scale studies have suggested the efficacy of lamivudine and entecavir in preventing the progression of AHB to chronic hepatitis.[34,35] Another study showed a lower seroconversion rate of HBsAg in lamivudine users.[36] Further, a randomized placebo-controlled trial showed no significant difference in clinical outcomes.[37] However, these previous studies did not mention the prevalence of HBV genotypes in the respective study populations. Although this was a retrospective study, our study included data on the prevalence of HBV genotypes. Additionally, our findings suggested that larger prospective randomized studies for every HBV genotype should be performed to determine whether early treatment with NAs prevented the progression of AHB to a chronic state.

In conclusion, in Japan genotype A was an independent risk factor for progression to chronic infection following AHB in adults. Confirmation of this association in patients with AHB in other countries is desirable and may provide insight into the pathogenetic mechanisms underlying this association. Early NA treatment appeared to reduce the likelihood of chronicity but this potentially important intervention needs to be prospectively studied before recommendations can be made.

Appendix

Members of the Japanese AHB Study Group include Yasuharu Imai (Ikeda Municipal Hospital), Norie Yamada, Hideaki Takahashi (St. Marianna University School of Medicine), Koji Ishii (Toho University School of Medicine), Hideyuki Nomura (Shin-Kokura Hospital), Jiro Nishida (Tokyo Dental Collage Ichikawa General Hospital), Shigeru Mikami (Kikkoman Hospital), Tsuneo Kitamura (Juntendo University Urayasu Hospital), Akihito Tsubota (Kashiwa Hospital Jikei University School of Medicine), Noritomo Shimada (Shinmatsudo Central General Hospital), Tetsuya Ishikawa (Nagoya University Graduate School of Medicine), Yoshiyuki Ueno (Tohoku University Graduate School of Medicine), Tomoyoshi Ohno (Social Insurance Chukyo Hospital), Etsuro Orito (Nagoya

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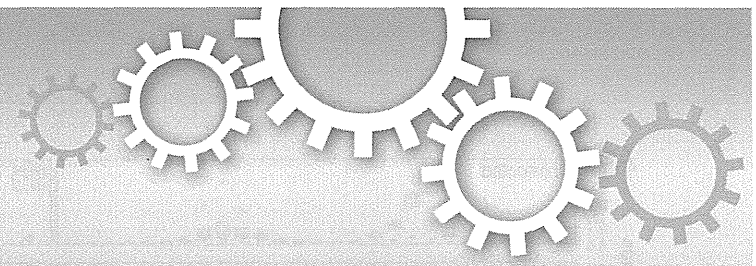

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Small tRNA-derived RNAs are increased and more abundant than microRNAs in chronic hepatitis B and C

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Persistent infections with hepatitis B virus (HBV) or hepatitis C virus (HCV) account for the majority of cases of hepatic cirrhosis and hepatocellular carcinoma (HCC) worldwide. Small, non-coding RNAs play important roles in virus-host interactions. We used high throughput sequencing to conduct an unbiased profiling of small (14-40 nts) RNAs in liver from Japanese subjects with advanced hepatitis B or C and hepatocellular carcinoma (HCC). Small RNAs derived from tRNAs, specifically 30–35 nucleotide-long 5′ tRNA-halves (5′ tRHs), were abundant in non-malignant liver and significantly increased in humans and chimpanzees with chronic viral hepatitis. 5′ tRH abundance exceeded microRNA abundance in most infected non-cancerous tissues. In contrast, in matched cancer tissue, 5′ tRH abundance was reduced, and relative abundance of individual 5′ tRHs was altered. In hepatitis B-associated HCC, 5′ tRH abundance correlated with expression of the tRNA-cleaving ribonuclease, angiogenin. These results demonstrate that tRHs are the most abundant small RNAs in chronically infected liver and that their abundance is altered in liver cancer.

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are phylogenetically unrelated non-cytopathic viruses that infect the liver[1]. While HBV is a DNA virus, and HCV is a positive-strand RNA virus, both have the capacity to persist for years in some infected individuals. Hundreds of millions of people worldwide are chronic carriers of HBV or HCV, 30–50% of whom have chronic liver disease[2]. Together, these viral infections are responsible for ~60% of liver cirrhosis and ~80% of hepatocellular carcinoma (HCC), a leading cause of cancer-related deaths worldwide. Numerous studies suggest that microRNAs (miRNAs), small 21–23 nt non-coding RNAs are important in the pathogenesis of these infections, modulating viral replication as well as host responses and possibly influencing the risk of carcinogenesis[3]. For example the HBV X protein represses expression of miR-148a, potentially enhancing tumorigenesis[4]. In contrast, HCV infection is associated with higher expression of miR-21, which targets key components of Toll-like receptor signaling pathways, possibly facilitating viral evasion of innate immune responses[5]. miR-122 stabilizes HCV RNA and promotes its replication[6,7], and the importance of this interaction is reflected in the clinical development of an anti-miR-122 antagomir (miravirsen) as an antiviral therapeutic[8].

Somewhat larger, 30–35 nt RNAs derived from the 5′ half of tRNA (5′ tRHs) represent a second major class of small non-coding RNA[9]. Increased expression of 5′ tRHs has been associated with viral and rickettsial infections in animals[10,11], and may serve to prevent apoptosis and promote cell survival[12]. However, they have not been studied previously in the context of viral hepatitis. To our knowledge, only one study has described unbiased profiling of small RNAs in the liver during chronic viral hepatitis[13], but the analysis was restricted to miRNAs. We sequenced small (14–40 nts) RNAs in liver biopsies from subjects with chronic hepatitis and HCC, examining both non-tumor and matched cancer tissue, and found a surprisingly high proportion of reads representing 5′ tRHs[9]. Our results document their presence in human tissue, demonstrate that they are the most highly abundant

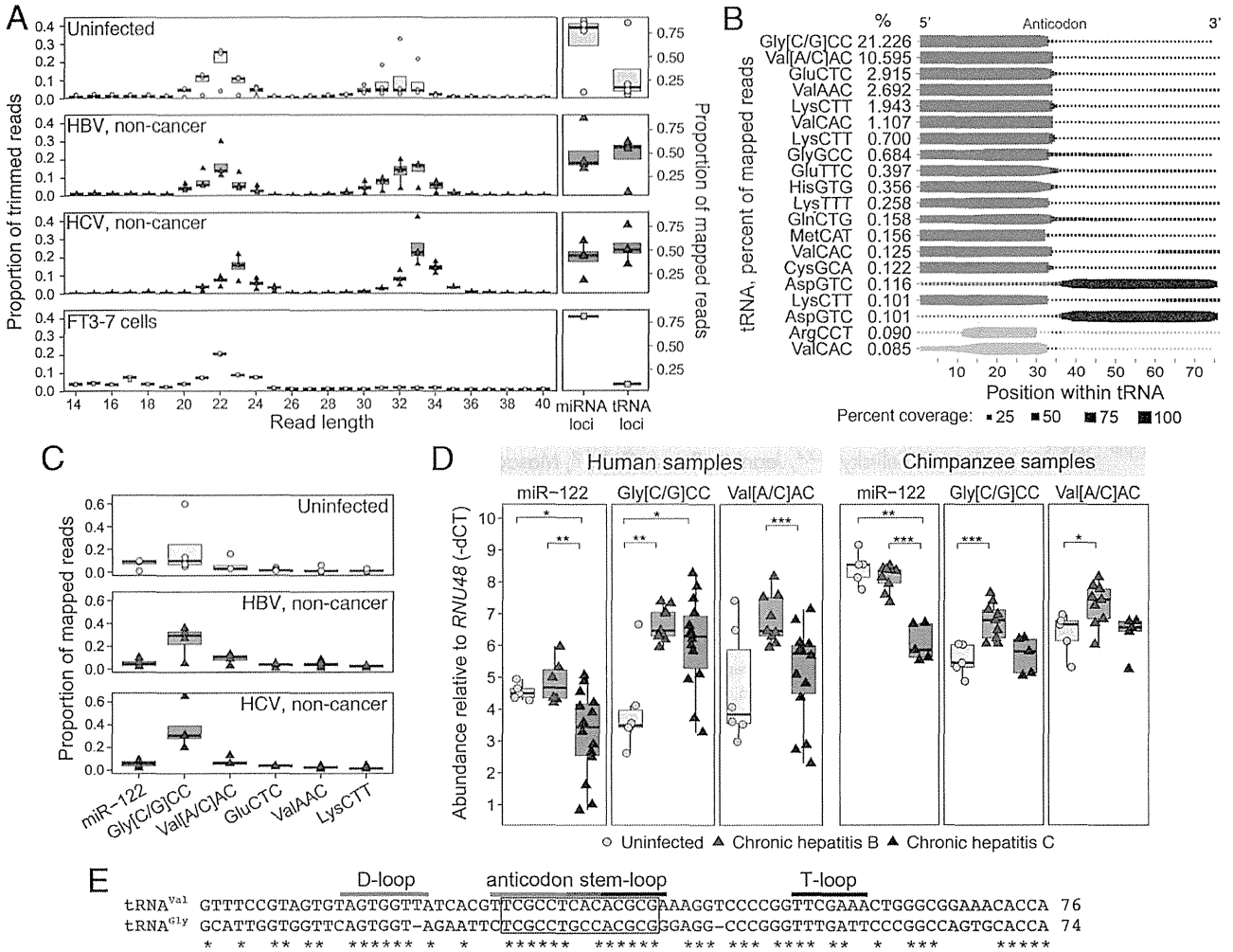

Figure 1 | tRH abundance in HBV- and HCV-infected liver. (a) (left) Read length distribution of 14–40 nt RNAs in non-malignant liver from uninfected, HBV-, or HCV-infected subjects (n=4 each), and FT3-7 cells (n=3 replicates). (right) Proportion of reads mapping to miRNA versus tRNA loci. Boxes represent median ± 1.5 * interquartile range. (b) tRNA coverage plot from the average of the 20 non-cancer samples. Dot size represents percent of reads mapping at each base position within each tRNA (top 20 by average abundance). The anticodon is red, with 5′ bases green and 3′ bases blue. Gray: bases of RNAs that are non-tRHs. See Supplemental Figure 1. (c) Proportion of mapped reads aligning to miR-122 versus the five most abundant tRNA-derived RNAs. (d) (left) Expression levels (RT-qPCR) of miR-122, 5′ tRH^{Gly} ("Gly[C/G]CC") and 5′ tRH^{Val} ("Val[A/C]AC") in uninfected (n=5–6), HBV-infected (n=6–9) and HCV-infected (n=14) human liver. Numbers of samples differ due to limited RNA. (right) Similar results from uninfected (n=5), HBV-infected (n=9), and HCV-infected C (n=5) chimpanzees. *RNU48* was used as a normalizer. *P < 0.05; **P < 0.01; ***P < 0.005 by Mann-Whitney U-test. (e) ClustalW[43] multiple sequence alignment of representative tRNA^{Gly} and tRNA^{Val} genes from which 5′ tRH^{Gly} and 5′ tRH^{Val} could originate (see Supplemental Figure 3). tRNAs regions are highlighted according to the color scheme in panel (b). The box identifies a unique conserved sequence motif described in the text. "Mapped reads" represents all reads aligning to miRNAs or tRNAs (see Methods).

small RNAs in virus-infected liver, and show that their abundance is altered in various disease states including hepatocellular carcinoma.

Results

tRNA-half abundance is significantly increased in chronic viral hepatitis. We employed high-throughput sequencing to characterize the small RNA transcriptome in liver tissue from Japanese adults with advanced hepatitis B or hepatitis C and concomitant HCC (see Supplemental Table 1 for patient information; see Supplemental Tables 2–4 for summary statistics on RNA, qRT-PCR, and sequencing). Initial studies focused on non-malignant tissue from 4 subjects with hepatitis B (mean age 53 ± 4 yrs s.e.m.), 4 with hepatitis C (63 ± 2 yrs), and 4 uninfected individuals undergoing resection of metastatic tumors (60 ± 10 yrs)[14]. A large proportion of the sequencing reads were 19–25 nts in length (median 38%, range 10–73%), as expected for miRNAs[15] (Figure 1a, left). However, we detected an equal or greater abundance of 30–35 nt reads in HBV- and HCV-infected liver (median 54%, range 14–80%). These larger RNAs were less abundant in uninfected tissue (median 21%, range 14–84%) and in human hepatoma (FT3-7) cells (median 9%, range 8.7–9.3%).

Most (~65%) of the 30–35 nt reads in infected samples aligned perfectly to the region 5′ of the anticodon triplet in annotated tRNA genes[16] (Figure 1b, Supplemental Figure 1, Supplemental Table 5 and 6). We refer to these as "5′ tRNA-halves" (5′ tRHs)[9]. Many of the remaining 30–35 nt reads also aligned to the 5′ end of tRNAs, particularly tRNA^{Gly}, but with one or more nucleotide deletions. Also present were 3′ tRHs (~36–39 nts) mapping to the region 3′ of the anticodon, including the 3′ terminal CCA (Figure 1b, Supplemental Figure 1). Additionally, we identified shorter reads derived from 3′ or 5′ tRNA termini, referred to previously as "tRNA fragments"[9] (tRFs), or the region immediately 5′ or 3′ of the anticodon loop, but these

were much less frequent. In 6 of 8 infected livers, more reads mapped to tRNA loci[16] than to known miRNAs[17] (see Methods), while the opposite was true in 3 of 4 uninfected tissues as well as FT3-7 cells (Figure 1a, right).

There are 625 annotated tRNA genes in the human genome (hg19) encoding 458 unique tRNA sequences. We identified reads mapping to 348 of these 458 sequences. Notably, in 11 of the 12 subjects, the same five 5′ tRHs comprised >80% of tRNA-derived reads (Supplemental Figure 2a). The two most abundant 5′ tRHs were Gly[C/G]CC ("5′ tRH^{Gly}"), which could be derived from any of 10 $tRNA^{Gly}$ genes with identical 5′ sequence, and Val[A/C]AC ("5′ tRH^{Val}"), which could originate from any of 15 $tRNA^{Val}$ genes (Figure 1b and C, Supplemental Figure 1 and 3, and Supplemental Table 5)[16]. 5′ tRH^{Gly} accounted for 54 ± 9% (s.d.) and 5′ tRH^{Val} 17 ± 9% of all tRNA-derived RNA reads (Supplemental Figure 2a). Remarkably, 5′ tRH^{Gly} abundance exceeded that of miR-122, one of the most abundant liver miRNAs[13], in 7 of 8 virus-infected tissues.

We used real-time reverse transcription quantitative PCR (RT-qPCR) to validate these results and compare 5′ tRH^{Gly}, 5′ tRH^{Val} and miR-122 abundance in liver tissue from 22 additional subjects (Supplemental Table 1–3)[14]. These analyses confirmed that 5′ tRH^{Gly} abundance was increased in HBV- and HCV-infected liver compared with uninfected tissues ($P<0.01$ and $P<0.05$, respectively) (Figure 1d, left). A similar trend was observed for 5′ tRH^{Val} (HBV $P=0.07$; HCV $P=0.7$). 5′ tRH^{Gly} and 5′ tRH^{Val} were more abundant than miR-122 in HBV- and HCV-infected liver (5′ tRH^{Gly}, $P<0.005$ for both HBV and HCV; 5′ tRH^{Val}, $P<0.005$ for HBV and $P<0.01$ for HCV) (Figure 1d left). Notably, 5′ tRH^{Val} abundance was higher in HBV- than in HCV-infected tissues ($P<0.005$).

Chimpanzees (*Pan troglodytes*) recapitulate many aspects of HBV and HCV infections in humans[18,19], and are free of potential confounding variables (e.g., alcohol intake, smoking) that are difficult to control in human cohorts. Similar to humans, we found that intrahepatic 5′ tRH^{Gly} and 5′ tRH^{Val} abundance was increased in archived liver tissue from chimpanzees chronically infected with HBV compared to uninfected animals ($P<0.005$ and $P<0.05$, respectively) (Figure 1d, right, and Supplemental Table 7). However, 5′ tRH abundance was not increased in chronically HCV-infected chimpanzee liver.

In human tissues, the relative abundance of specific tRNA-derived RNAs correlated with codon usage (codon frequency in DNA sequence) (Spearman's $rho=0.32$, $P=0.01$) and the number of possible tRNA genes from which each could originate ($rho=0.41$, $P=0.001$) (Supplemental Figure 2b). However, tRNAs representing potential sources of the five most abundant tRHs were not the most highly ranked by gene number or codon usage, suggesting that additional factors likely determine tRH biogenesis (Supplemental Figure 4). Interestingly, those tRNAs from which 5′ tRH^{Gly} and 5′ tRH^{Val} are potentially derived share a unique sequence motif in the anticodon stem-loop region (Figure 1e) not present in other tRNAs (Supplemental Figure 3).

tRNA-half abundance is altered in viral hepatitis associated cancer. In HCC tissue from HBV-infected subjects, RT-qPCR analysis showed that 5′ tRH^{Gly} and 5′ tRH^{Val} abundance was significantly reduced ($P<0.005$ for both) (Figure 2a). Similar reductions were evident in HCV-associated cancer tissue, but significant only for 5′ tRH^{Val} ($P<0.05$). We then sequenced small RNAs in cancer tissue from 4 HBV- and 4 HCV-infected subjects. The proportion of reads mapping to tRNA genes was reduced in 4 of 7 samples for which a paired analysis with non-malignant liver was possible, and relatively unchanged in the other 3 (Figure 2b). Although tRNA-derived RNA expression profiles were similar across non-malignant tissues from different subjects, there was substantial variation when compared to cancer tissues (Figure 2c). This suggests that the relative abundance of specific tRNA-derived RNAs is altered in HCC. Notably, the relative abundance of 5′ tRH^{Gly} was reduced by ~50–60% in both HBV- and HCV-associated cancer (Figure 2d).

tRNA-half abundance correlates with angiogenin levels in HBV-associated cancer. Angiogenin (encoded by the gene *ANG*) is best known for its role in angiogenesis, but several studies suggest its RNase activity contributes to tRH biogenesis[20,21]. Consistent with this, analysis of previous microarray data obtained from these tissues[14] revealed that *ANG* mRNA was reduced in both HBV- and HCV-associated cancer compared to non-malignant tissue ($P<0.01$ and $P<0.005$, respectively) or uninfected liver ($P<0.005$ and $P=0.01$) (Figure 3a). Analysis of data from The Cancer Genome Atlas (https://tcga-data.nci.nih.gov/tcga/) also indicates that *ANG* expression is reduced in HCC compared to non-malignant tissue, although the difference is significant only for HBV-associated cancer (HBV $P<0.005$, HCV $P=0.12$) (Supplemental Figure 5). *ANG* mRNA abundance correlated strongly with 5′ tRH expression in the HBV-infected subjects we studied (5′ tRH^{Gly}: Spearman's $rho=0.67$, $P<0.01$; 5′ tRH^{Val}: $rho=0.74$, $P<0.005$) (Figure 3b). Quantitative immunoblot analyses (Supplemental Figure 6) confirmed a correlation between ANG protein abundance and 5′ tRH expression in HBV-associated cancer (5′ tRH^{Gly}: $rho=0.83$, $P<0.005$; 5′ tRH^{Val}: $rho=0.87$, $P<0.005$) (Figure 3c). ANG was expressed within the cytoplasm of hepatocytes (Figure 3d), and although its expression varied substantially in different tumors (Figure 3e), reductions in ANG expression likely explain the reduced tRH abundance we observed in most HBV-associated cancers. Unfortunately, however, the available tissue sections from these subjects were insufficient to power a formal analysis of the correlation between cytoplasmic versus nuclear expression of ANG and tRH abundance. ANG expression correlated poorly with tRH abundance in HCV-infected livers, suggesting that other factors determine tRH biogenesis.

Discussion

Recent advances in high-throughput sequencing technology have unveiled the complexity and diversity of functional small RNAs. We found that small RNAs derived from tRNAs, specifically 5′ tRNA-halves[9] (5′ tRHs, ~30–35 nts), are abundant in liver, significantly increased during chronic viral infection, and altered in abundance in liver cancer associated with these infections. We do not believe that these tRNA-halves are products of stochastic endonuclease cleavage of tRNAs for several reasons. First, the same tRNA-halves were found to be increased in chronic viral hepatitis across all individuals. Second, each tRNA-half family exhibited a uniform length distribution (e.g., 5′ tRH^{Gly} was represented primarily by reads of length 32–34 in every individual). Third, tRNA-halves were preferentially induced in chronic HBV infection (as compared to chronic HCV infection) in both human and chimpanzee tissue, indicating biological specificity. Finally, tRH abundance was correlated with disease state (cancer versus non-cancer), indicating reproducible sensitivity to the cellular environment.

Several models of disease have been shown to exhibit an increase in tRH abundance, including cultured human airway cells infected with respiratory syncytial virus[22], mice infected with spotted-fever group rickettsia[23], and rats treated with cisplatin[24]. While their function is not well understood, previous work in cell culture suggests that some tRHs promote cell survival, are anti-apoptotic[12], reduce translation[25], and promote the formation of stress granules[26]. Preliminary studies in our laboratory do not support a role for 5′ tRH^{Gly} or 5′ tRH^{Val} in the regulation of global protein translation in human hepatoma cells (Supplemental Figure 7–8); however, more detailed investigation is required to uncover the potential functions of tRHs. It has also been suggested that tRHs may alter the immune response due to their enrichment in mouse lymphoid organs[27], high

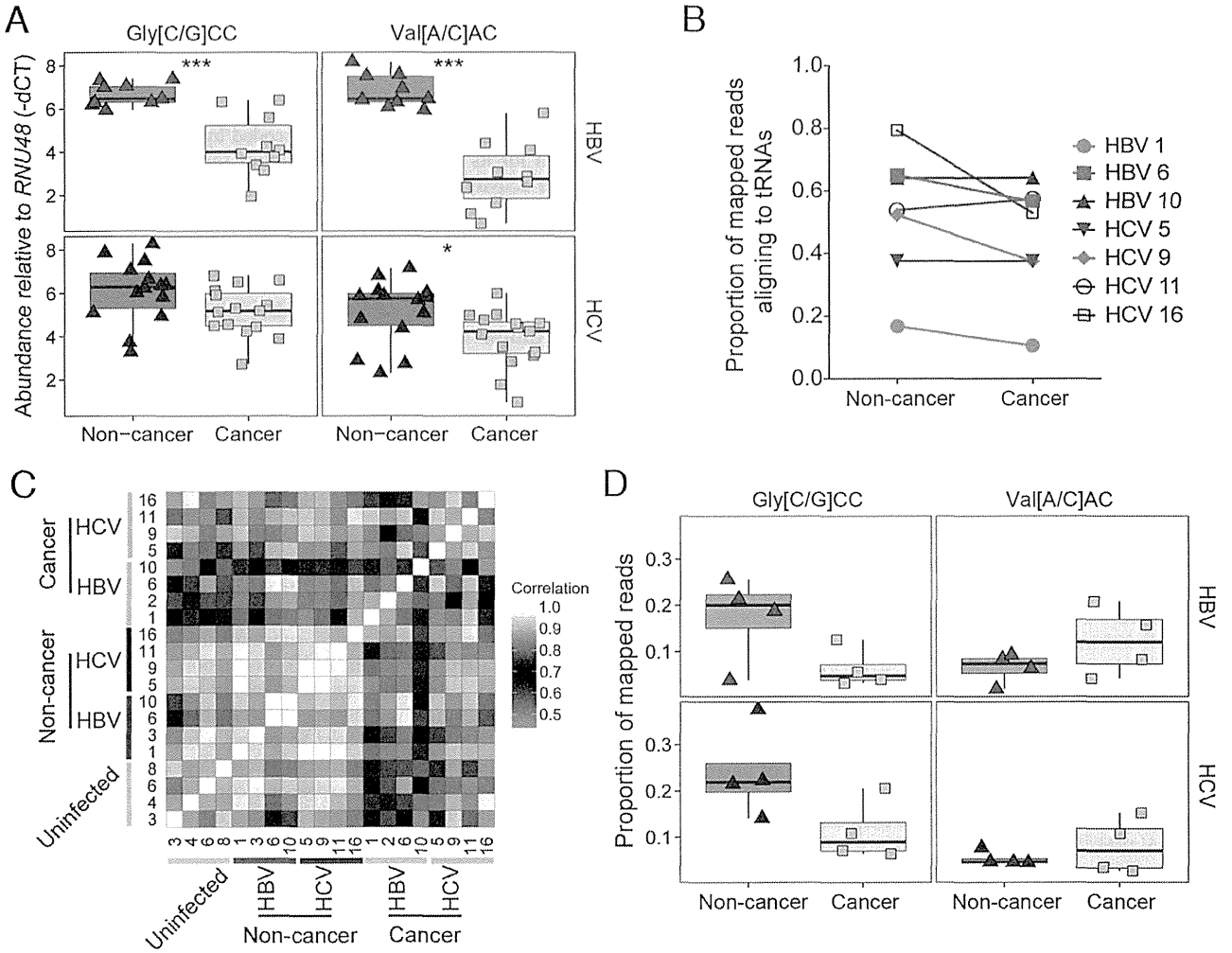

Figure 2 | 5′ tRH abundance in HBV- and HCV-associated hepatocellular carcinoma. (b) Abundance (RT-qPCR) of (left) 5′ tRH^{Gly} (Gly[C/G]CC) and (right) 5′ tRH^{Val} (Val[A/C]AC) in (top) non-malignant (n=9) and cancer tissue (n=10) from HBV-infected subjects, and (bottom) non-malignant (n=14), and cancer tissue (n=15) from HCV-infected subjects. Box and whisker plots are overlaid with data from each sample; whiskers extend to 1.5 * interquartile range. P-values calculated using Mann-Whitney U-test. *P < 0.05; **P < 0.01; ***P < 0.005. (b) Proportion of mapped reads aligning to tRNAs for the paired cancer and non-cancer tissue from subjects with chronic hepatitis B (n=3) and hepatitis C (n=4) (c) Correlation heatmap of tRNA-derived RNA expression profiles determined by small RNA sequencing. The colors of the cells represent Spearman's rank correlation coefficients of the relative levels of the 10 most abundant tRNA-derived RNAs between all pairs of tissue samples sequenced (n=20). (d) Proportion of mapped reads that align to 5′ tRH^{Gly} and 5′ tRH^{Val} in non-malignant and cancer tissue from (top) HBV-infected and (bottom) HCV-infected subjects. "Mapped reads" represents all reads aligning to miRNAs or tRNAs (see Methods).

abundance in seminal exosomes (considered to be immunosuppressive)[28], and roles in facilitating *Trypanosoma cruzi* infection in human cells and altering host gene expression[29].

There is good evidence that the abundance of these small non-coding RNAs increases in response to specific kinds of cellular stress. For example, tRHs are induced in cell culture by the addition of sodium arsenite, exposure to UV, nutrient starvation, hypoxia, hypothermia and heat, but not by exposure to etoposide, γ-radiation, caffeine[30,31]. This strongly suggests that the formation of tRHs is a regulated process, rather than due to general degradation of tRNAs in response to stress. In the nucleus angiogenin is involved in promoting angiogenesis[32] and in the cytoplasm, when not bound to RNH1, it acts as a tRNA-processing RNase[33,34], cleaving tRNAs at the anticodon loop and producing tRHs[30,31]. The cellular localization of angiogenin and its ribonuclease activity depend on the intracellular conditions and are regulated by RNH1[33]. The differences we observed in correlations between ANG expression and tRH abundance in chronic hepatitis B, hepatitis C and associated liver cancer may be a result of differences in angiogenin localization and function in these disease states. Non-tumor and tumor tissues from patients with chronic hepatitis C tend to show more evidence of angiogenesis than the in chronic hepatitis B[35,36]. This could mean that in chronic hepatitis C angiogenin is primarily nuclear, and therefore not exclusively involved in tRH production. Finally, it must also be noted that factors other than ANG may be critical to tRH biogenesis in different cell types or in response to different types of cellular stress. Much more remains to be uncovered about the specific mechanisms that lead to tRH accumulation.

Chronic infections with HBV and HCV typically lead to more severe liver disease in human patients than in the chimpanzee model[37,38]. Disease severity may account for the differences we observed in tRH abundance between liver tissue from human subjects with chronic HCV infection and the chimpanzee samples. Interestingly, however, humans and chimpanzees exhibited similar increases in tRH abundance in chronic hepatitis B, suggesting that

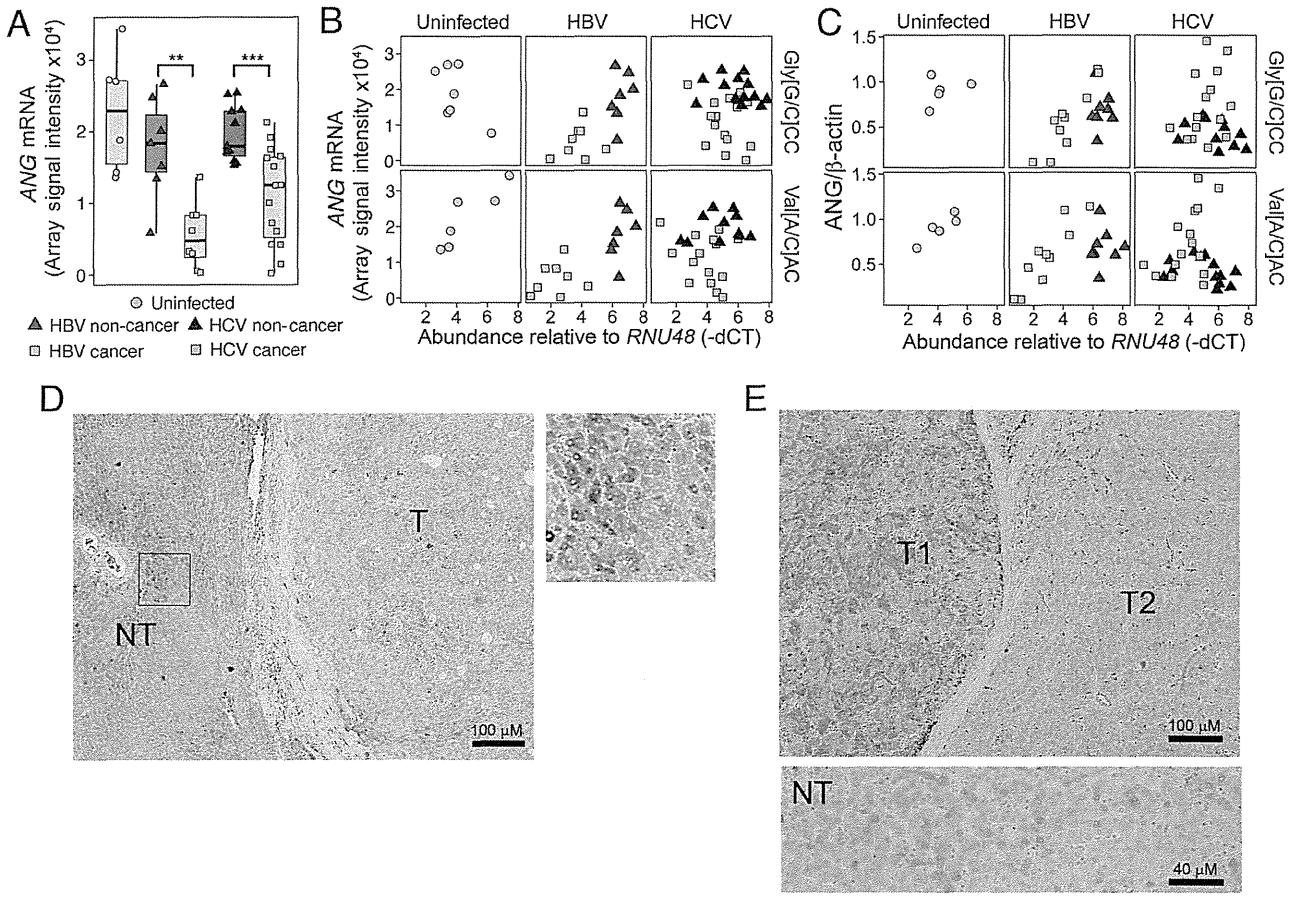

Figure 3 | Angiogenin expression in viral hepatitis and hepatocellular carcinoma. (a) Normalized *ANG* mRNA levels from previously generated liver microarray data[14] from uninfected subjects (n=6), non-malignant (n=7) and liver cancer (n=8) tissue from HBV-infected subjects, and non-malignant (n=11) and cancer tissue (n=15) from HCV-infected subjects. **P < 0.01; ***P < 0.005, calculated by Mann-Whitney *U*-test. (b) Scatter plot of the levels of 5′ tRHs (RT-qPCR, -dCT normalized to *RNU48*) and *ANG* mRNA (microarray). 5′ tRH^{Gly} ("Gly[C/G]CC"): uninfected subjects (n=7), non-cancer (n=7) and cancer (n=8) liver tissue from chronic hepatitis B subjects, and non-cancer (n=11) and cancer (n=15) liver tissue from chronic hepatitis C subjects; 5′ tRH^{Val}: uninfected subjects (n=6), non-cancer (n=7) and cancer (n=8) liver tissue from chronic hepatitis B subjects, and non-cancer (n=11) and cancer (n=15) liver tissue of chronic hepatitis C subjects. (c) Scatter plot of the levels of 5′ tRHs (RT-qPCR, -dCT normalized to *RNU48*) and ANG protein expression (normalized to β-actin) determined by immunoblot analysis. (d) Immunohistochemistry staining for ANG in formalin-fixed non-tumor (NT) and tumor tissue (T) from HBV-infected subject #10. (Right) Magnified view of non-tumor (NT). (e) ANG staining in adjacent tumor nodules (T1 and T2) and in non-tumor (NT) tissue from HCV-infected subject #7.

there may be a primary HBV-specific mechanism that directly regulates tRH biogenesis.

Our study has some technical limitations. tRNAs are subject to many different chemical modifications[39], several of which could impede library preparation and sequencing. This may have biased which tRNA-derived RNAs we detected. Also, we do not know how tRH abundance varies among the diverse cell types that populate the liver. Given that these small RNAs have not previously been studied in human tissue, we also have little appreciation of what functions these small non-coding RNAs have within the liver. Nonetheless, our finding that the intrahepatic abundance of tRHs is substantially increased in chronic viral infections of the liver and altered in HCC suggest that tRHs may have important, yet to be determined roles in liver disease. Thus, this study may have implications for disease pathogenesis and novel therapeutic strategies.

Methods

The methods were carried out in accordance with the approved guidelines.

Human subjects. Written informed consent was obtained from all human subjects. Ethics approval was obtained from the Ethics Committee for Human Genome/Gene Analysis Research at Kanazawa University Graduate School of Medical Science.

Chimpanzee liver tissue. The chimpanzee samples used in this study were archived from previous studies and were collected prior to December 15, 2011. Chimpanzees were housed and cared for at the Southwest National Primate Research Center (SNPRC) of the Texas Biomedical Research Institute. The animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the Institutional Animal Care and Use Committee. SNPRC is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. SNPRC operates in accordance with the NIH and U.S. Department of Agriculture guidelines and the Animal Welfare Act. Animals were sedated for all procedures. Animals are group-housed with indoor and outdoor access and an environmental enrichment program is provided by a staff of behavioral scientists.

Small RNA-sequencing. RNA was isolated as described previously[14]. RNA purity was assessed with Nanodrop 2000 (Thermo Scientific) and integrity was determined with an Agilent 2100 Bioanalyzer (Agilent). RNA integrity and sequencing quality were comparable for all specimens (Supplemental Table 2–4). Small RNA libraries were generated using Illumina TruSeq Small RNA Sample Preparation Kit (Illumina, San Diego, CA). Sequencing was performed on Illumina HiSeq 2000 platform. Bioinformatic analysis: Sequencing reads were trimmed using Cutadapt (parameters O -10 e 0.1) and were further analyzed in two different ways: (i) Mapped trimmed reads allowing no mismatches to all tRNA sequences (except pseudo-tRNAs and undefined tRNAs) downloaded from GtRNAdb[16] (Figure 1b,c; Figure 2c,d; Supplemental Figure 1,2,4,7); (ii) Mapped trimmed reads to genomic regions spanning annotated miRNA s[40] (+/− 20 nts) and tRNA sequences (+/− 40 nts) using Bowtie 0.12.7[41] allowing for no mismatches. Next, reads that did not map

without mismatches were aligned to the same regions using SHRiMP2.2.2[42]. SHRiMP2.2.2 seeds were set based on the length of the read allowing 1 mismatch anywhere in the body and up to 3 mismatches at the 3′ end of the read (based on the length of the read). (Figure 1a right, Figure 2b, Supplemental Figure 7, Supplemental Table 4). Small RNA-sequencing data was deposited on GEO (GSE57381).

AGO2-RNA Co-immunoprecipitation. FT3-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) and supplemented with 10% fetal calf serum and 2 mM GlutaMAX (Life Technologies, Carlsbad, CA). Cells were cultured in a humidified incubator at 37°C and 5% CO_2. Three technical replicates of 1×10^7 FT3-7 cells were harvested in lysis buffer [150 mM KCl, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1% Triton X-100, 5 mM DTT, Complete protease inhibitor mixture (Roche), and 100 U/mL RNaseOUT (Life Technologies)]. Lysates were centrifuged for 30 min at 17,000 × g at 4°C and filtered through a 0.22-um filter. Filtrates were incubated with anti-human AGO2 mAb (RN003M, MBL International, Woborn, MA) or isotype control IgG (Abcam, Cambridge, England) at 4°C for 2 h, followed by addition of 30 μL of Protein G Sepharose (GE Healthcare) for 1 h. The Sepaharose beads were washed three times in lysis buffer and RNA extracted using the miRNeasy Mini Kit (Qiagen, Hilden, Germany).

Small RNA real time quantitative PCR (RT-qPCR). Complementary DNA (cDNA) was synthesized using TaqMan MicroRNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's instructions. Real time PCR amplification was performed using TaqMan Universal Master Mix (Life Technologies) on the Bio-Rad CFX96 real time PCR detection system. *U6*, miR-24, let-7a, let-7f, *RNU48*, and *RNU66* were all evaluated as potential housekeeping small RNAs for purposes of normalization. *RNU48* was selected because it was the most consistent across disease groups. RT-qPCR reactions for human samples were performed in triplicate. RT-qPCR reactions for chimpanzee samples were performed in duplicate. The following TaqMan assays were purchased from Life Technologies: miR-122 (product number 4427975; 002245) and *RNU48* or *SNORD48* (product number 4427975; 001006). Primers for the custom TaqMan assays (5′ tRH-^{Gly} and 5′ tRH-^{Val}) were designed using 5′-GCAUUGGUGGUUCAGUGGUAGAAU-UCUCGCCU-3′ for 5′ tRH-^{Gly} and 5′-GUUUCCGUAGUGUAGUGGUUAUCAC-GUUCGCCU-3′ for 5′ tRH^{Val}.

Metabolic Radiolabeling and Measurement of Nascent Protein Synthesis. Huh7 cells were seeded onto the wells of 6-well cell culture plates at a density of 2×10^5 cells/well and incubated overnight to allow cell attachment. Cells were transfected with 50 nM and 100 nM of 5′ tRH^{Gly} (5′-GCAUUGGUGGUUCAGUGGUAGAAU-UCUCGCCU-3′), 5′ tRH^{Val} (5′-GUUUCCGUAGUGUAGUGGUUAUCACGUU-CGCCU-3′), or scramble (5′-GCAUUCACUUGGAUAGUAAAUCCAAGC-UGAA-3′)[21] (all from Integrated DNA Technologies, Coralville, IA) oligonucleotide after replacing cell culture medium with methionine- and cysteine-deficient DMEM (Life Technologies) and cultured for further 12 hrs. Cells were then metabolically radiolabeled for 12 hrs with 200 μCi/well of Express Protein Labeling Mix containing [^{35}S]methionine and [^{35}S]cysteine (PerkinElmer, Waltham, MA) in the presence or absence of 50 μg/ml puromycin and lysed with lysis buffer (20 mM Tris-HCl [pH 7.4] containing 150 mM NaCl, 1% Triton X-100, 0.05% SDS, and 10% glycerol) supplemented with 50 mM NaF, 5 mM Na_3VO_4, and a protease inhibitor cocktail (Complete; Roche, Mannheim, Germany). The protein concentration of cell lysates was determined by the Bio-Rad Protein Assay (Bio-Rad), and 10 μg (total protein) of cell lysates was subjected to SDS-PAGE followed by staining gels with the Sypro Ruby Protein Gel Stain (Bio-Rad, Hercules, CA) and autoradiography.

Immunohistochemistry (IHC). Staining was performed by immunoperoxidase technique with an Envision kit (DAKO Japan). Primary antibodies used were against β-actin (Cell signaling technology, #4967, Beverly, MA) and Human Angiogenin Affinity Purified Polyclonal Ab (R and D Systems, AF265, Minneapolis, MN).

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Author contributions

The experiments were designed by S.R.S., P.S. and S.M.L. The data were analyzed by S.R.S., J.B., T. Shirasaki, M.H., P.S. and S.M.L. Experiments were performed by S.R.S., D.Y., T.M., E.E.F., B.G. and T. Shirasaki. M.H., T. Shimakami, S.K., R.E.L., S.M.L. and P.S. contributed resources. The manuscript was written by S.R.S., P.S. and S.M.L.

Additional information

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Original Article

Feasibility and efficacy of hepatic arterial infusion chemotherapy for advanced hepatocellular carcinoma after sorafenib

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Aim: Sorafenib is the standard treatment for advanced hepatocellular carcinoma (HCC). However, although there is no proven therapeutic procedure following the termination of sorafenib, hepatic arterial infusion chemotherapy (HAIC) may be a treatment option in advanced HCC. The aim of this study was to evaluate feasibility and efficacy of HAIC for patients with advanced HCC as subsequent therapy.

Methods: We retrospectively evaluated 27 consecutive patients with advanced HCC who were treated with HAIC following sorafenib between June 2009 and December 2012 at our hospital. Cisplatin (20 mg/m^2 per day) was administered via the hepatic artery for 10 min, prior to the continuous administration of 5-fluorouracil (330 mg/m^2 per day) over 24 h from days 1–5 and 8–12 and the s.c. administration of pegylated interferon α-2b (1 μg/kg) on days 1, 8, 15, and 22. A treatment cycle consisted of 28 days of drug administration followed by 14 days of rest.

Results: The toxicity profile showed that hematological toxicities were common, and grade 3/4 neutropenia and thrombocytopenia were observed (51.9% and 48.1%, respectively). Five patients (18.5%) experienced device-related complications. No unexpected adverse reactions and no treatment-related deaths were observed. Partial response was obtained in eight patients (29.6%), and stable disease was noted in nine patients (33.3%). Median progression-free survival and median survival time from initiation of HAIC were 4.0 and 7.6 months, respectively.

Conclusions: Because HAIC was well tolerated and exhibited moderate antitumor activity, it is a potentially useful treatment procedure in patients with advanced HCC even after failure of sorafenib.

Key words: hepatic arterial infusion chemotherapy, hepatocellular carcinoma, sorafenib

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is the sixth most common cancer and the third leading cause of cancer-related mortality worldwide.[1] A variety of new techniques of imaging modalities have enabled the detection of HCC at an early stage,[2] and advances in various therapeutic procedures have improved its curability.[3,4] However, the number of patients with HCC who can be treated curatively is limited because of impaired hepatic function and frequent recurrence even after curative therapy. The prognosis of patients with advanced HCC where tumor has spread over the liver or invaded major vessels remains extremely poor.[5]

Sorafenib, an oral multikinase inhibitor that blocks tumor cell proliferation and angiogenesis, is the only systemic therapy that has shown survival benefit for patients with advanced HCC,[6,7] and it is recognized worldwide as standard first-line therapy in advanced HCC.[8,9] Alternative systemic chemotherapies using cytotoxic agents or novel targeted drugs have been attempted in patients with advanced HCC;[10,11] however, to date none have proven effective, except sorafenib. Moreover, following sorafenib therapy most patients are not suitable candidates for subsequent therapy because of the progressive nature of their disease, poor general condition, and impaired hepatic function.

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Compared with systemic chemotherapy, hepatic arterial infusion chemotherapy (HAIC) is based on theoretical advantages such as higher concentrations of drugs delivered directly to tumors[12] and first-pass effect reducing systemic toxicity.[13] Although few reports have recorded the survival benefits of HAIC, HAIC in combination with interferon (IFN) has been reported to be a useful treatment procedure in patients with advanced HCC.[14,15] Although an optimal protocol of HAIC has not been established, the clinical benefits of HAIC regimen consisting of 5-fluorouracil (5-FU) and cisplatin with IFN were reported in a randomized phase II study.[15] However, it remains unclear whether HAIC is also safe and effective in patients with advanced HCC who were previously administered sorafenib.

The aim of the present study was to evaluate the feasibility and efficacy of HAIC in patients with advanced HCC after failure of sorafenib therapy. This approach provides useful information in determining treatment strategies for sorafenib-refractory patients with HCC.

METHODS

Patients

ALL OF 68 consecutive patients with unresectable advanced HCC who had received sorafenib monotherapy at Kanazawa University Hospital and for whom this therapy was subsequently stopped because of tumor progression or/and unacceptable adverse effects between June 2009 and December 2012 were considered for enrollment. HCC was diagnosed by either histological confirmation or typical radiological findings, which showed hyperattenuation in the early phase and hypoattenuation in the late phase on dynamic computed tomography (CT).[16] All patients underwent dynamic CT to assess the extent of the cancer, and their hepatic and major organ functions were evaluated by physical examination and laboratory findings. We reviewed patients' medical records and investigated their backgrounds, treatment courses, and outcomes.

Sorafenib

The following were the inclusion criteria for sorafenib at our institution: patients with advanced HCC involving macroscopic vascular invasion, extrahepatic lesions and/or intrahepatic multiple lesions considered unsuitable for surgical resection, locoregional therapy or transarterial chemoembolization; all patients with an Eastern Cooperative Oncology Group performance status score of 2 or less and with appropriate function of major organs, such as bone marrow, kidney and heart; and patients categorized as Child–Pugh A in terms of hepatic function.

HAIC

The inclusion criteria for HAIC at our institution is nearly same as that of sorafenib. Patients with extrahepatic lesions were also considered eligible if these lesions were mild, and intrahepatic lesions were considered as prognostic factors. With regard to hepatic function, patients categorized as Child–Pugh A or B were eligible.

The reservoir system implantation technique was the same as described previously.[15] Catheters were introduced through the right femoral artery, and angiography from the celiac artery was initially performed to localize the HCC and evaluate intra- and extrahepatic vascularization. We then inserted a catheter with a side vent into the gastroduodenal artery, positioning the vent in the common hepatic artery using an image-guided procedure. The gastroduodenal artery, right gastric artery and other arteries presumed to supply the gastroduodenal region were embolized as far as possible to prevent gastrointestinal mucositis. The other end of the catheter was connected to an injection port that was subcutaneously implanted in the right lower abdomen. Finally, blood flow redistribution was confirmed.

Hepatic arterial infusion chemotherapy was initiated approximately 5 days after implantation of the reservoir, and the following protocol was then implemented: 5-FU (330 mg/m^2 per day) was continuously administrated via the hepatic artery using an infuser pump over 24 h from days 1–5 and 8–12, and cisplatin (20 mg/m^2 per day) was also administrated via the hepatic artery for 10 min prior to 5-FU administration. Pegylated IFN-α-2b (1.0 μg/kg) was s.c. administrated on days 1, 8, 15, and 22. A treatment cycle consisted of 28 days of drug administration followed by 14 days of rest. The treatment protocol was approved by the Ethics Committee of Kanazawa University, and informed consent for participation in the study was obtained from each subject. The study conformed to the guidelines of the 1975 Declaration of Helsinki.

Evaluation

Tumor staging was assessed according to the criteria of the Liver Cancer Study Group of Japan.[17,18] The efficacies of HAIC and sorafenib were assessed every 4–6 weeks by dynamic CT, and response to chemotherapy was assessed according to the Response Evaluation Criteria in Solid Tumors ver. 1.1.[19] Response rate was defined as

the sum of complete and partial response rates. Similar to an approach adopted in a recent report, the causes of progression after sorafenib therapy (progression pattern) were classified as follows: intrahepatic growth, extrahepatic growth, new intrahepatic lesion or new extrahepatic lesion and/or vascular invasion.[20] Adverse effects, including both hematological and non-hematological toxicities, were assessed by the Common Terminology Criteria for Adverse Events version 4.0.

Statistical analysis

Progression-free survival (PFS) was calculated from the first day of HAIC until either the date of radiological progression, the date of death or the last day of the follow-up period. Overall survival (OS) was calculated from the first day of HAIC until either the date of death or the last day of the follow-up period. A χ^2-test was used to analyze the predictive factor for the response to HAIC. To compare prognosis according to response to chemotherapy and the progression pattern, cumulative survival was calculated using the Kaplan–Meier method[21] and any differences were evaluated using the log-rank test. $P < 0.05$ were considered to be statistically significant, and all tests were two-sided. All statistical analyses were performed using the SPSS statistical software program package (version 11.0 for Windows; SPSS, Chicago, IL, USA).

RESULTS

Patients

OF 68 PATIENTS, 41 were not treated with HAIC because of either poor general condition ($n = 12$), massive extrahepatic lesions ($n = 9$), inadequate major organ function ($n = 8$), treatment with HAIC prior to sorafenib therapy ($n = 7$) or refusal to be treated with HAIC ($n = 5$). Finally, 27 patients who had been treated with HAIC were analyzed in this study, all of whom had previously received sorafenib monotherapy. The response and tumor control rates for sorafenib therapy were 7.4% and 44.4%, respectively. In 22 patients (81.5%), sorafenib therapy was terminated because of tumor progression and in five (18.5%) because of unacceptable adverse effects. The median period of sorafenib therapy was 2.4 months (range, 0.1–18.0).

Patient characteristics at commencement of treatment with HAIC are summarized in Table 1. Because hepatic function was impaired in more than half of the patients in this study, 18 patients (66.7%) were classified as Child–Pugh class B or C. Macroscopic vascular invasion and extrahepatic metastasis were observed in 25.9% and 44.4% of the patients, respectively.

Table 1 Patient characteristics

	($n = 27$)
Age, years	
Median, range	68, 44–84
Sex, *n* (%)	
Male	23 (85.2)
ECOG PS†, *n* (%)	
0	24 (88.9)
1	3 (11.1)
HBs antigen‡, *n* (%)	
Positive	9 (33.3)
HCV antibody§, *n* (%)	
Positive	15 (55.6)
Child-Pugh class at start of HAIC, *n* (%)	
A	9 (33.3)
B	16 (59.3)
C‡‡	2 (7.4)
Child-Pugh class at start of sorafenib, *n* (%)	
A	21 (77.8)
B§§	6 (22.2)
Ascites, *n* (%)	
Presence	18 (66.7)
Albumin, g/dL	
Median, range	3.2, 2.1–3.9
Prothrombin consumption test, %	
Median, range	82, 37–112
LCSGJ¶ tumor stage, *n* (%)	
II, III	12 (44.4)
IVA	4 (14.8)
IVB	11 (40.7)
Macroscopic vascular invasion, *n* (%)	
Yes	7 (25.9)
Extrahepatic spread, *n* (%)	
Yes	12 (44.4)
AFP††, ng/mL	
Median, range	404, <10–175560

†ECOG PS: Eastern Cooperative Oncology Group performance status.
‡HBs antigen: hepatitis B surface antigen.
§HCV antibody: hepatitis C virus antibody.
¶LCSGJ: Liver Cancer Study Group of Japan.
††AFP: α-fetoprotein.
‡‡Child-Pugh class B at decision making of HAIC.
§§Child-Pugh class A at decision making of sorafenib.

Treatment

A total of 60 courses were administered to 27 patients, with a median number of 2 (range, 0–5). All but two patients completed at least one course of HAIC. The

median duration between cessation of sorafenib therapy and commencement of HAIC was 1.2 months (range, 0–9.0). The median observation period from commencement of HAIC was 7.0 months (range, 0.8–48.0). Treatment with HAIC was terminated in 25 patients due to radiological tumor progression (20 patients), symptomatic tumor progression (one patient) or change in the treatment procedure (four patients); however, there were no patients in whom HAIC was terminated because of adverse effects. HAIC was continued in the remaining two patients until the last day of the follow-up period.

Safety

All 27 patients were assessed for adverse effects, and the toxicity profile of HAIC is summarized in Table 2. Hematological toxicities were common, particularly grade 3/4 neutropenia and grade 3/4 thrombocytopenia, which were observed in 14 (51.9%) and 12 (48.1%) patients, respectively, even though no serious complication such as sepsis or bleeding were observed and all toxicities were tolerable and reversible. Mild and low-frequency nonhematological toxicities were observed, except in one patient who had grade 3 diarrhea. Although 5 patients (18.5%) had device-related complications (3 catheter obstruction, 1 hepatic artery occlusion, and 1 hepatic arteritis), all issues were satisfactorily resolved by either exchanging the reservoir or conservative therapy. No unexpected adverse reactions were noted, and no treatment-related deaths were observed.

Table 2 Hepatic arterial infusion chemotherapy toxicities

	All grade *n* (%)	Grade 3 *n* (%)	Grade 4 *n* (%)
Hematological toxicities			
Leukocytopenia	20 (74.1)	10 (37.0)	0 (0)
Neutropenia	21 (77.8)	10 (37.0)	4 (14.8)
Anemia	12 (44.4)	1 (3.7)	1 (3.7)
Thrombocytopenia	22 (88.9)	13 (48.1)	0 (0)
Nonhematological toxicities			
Anorexia	7 (25.9)	0 (0)	0 (0)
Fever	5 (18.5)	0 (0)	0 (0)
Diarrhea	4 (14.8)	1 (3.7)	0 (0)
Fatigue	4 (14.8)	0 (0)	0 (0)
Hiccoughs	3 (11.1)	0 (0)	0 (0)
Gastric ulcer	3 (11.1)	0 (0)	0 (0)
Creatinine increased	2 (7.4)	0 (0)	0 (0)
Mucositis oral	2 (7.4)	0 (0)	0 (0)
Nausea	1 (3.7)	0 (0)	0 (0)
Ascites	1 (3.7)	0 (0)	0 (0)
Edema	1 (3.7)	0 (0)	0 (0)
Abdominal pain	1 (3.7)	0 (0)	0 (0)
Hypokalemia	1 (3.7)	0 (0)	0 (0)
Encephalopathy	1 (3.7)	0 (0)	0 (0)
Device-related complications			
Catheter obstruction	3 (11.1)	0 (0)	0 (0)
Hepatic artery occlusion	1 (3.7)	0 (0)	0 (0)
Vasculitis	1 (3.7)	0 (0)	0 (0)

Response to treatment and patient outcomes

Of the 27 patients, one died due to tumor progression and hepatic failure before radiological assessment could be performed; however, the remaining 26 were assessable for response to treatment. Tumor responses to HAIC are shown in Table 3. Although no patient achieved complete response, eight patients (29.6%) achieved partial response (PR) and nine (33.3%) achieved stable disease (SD); therefore, the response rate to HAIC was 29.6%. These results were independent of the Child–Pugh class, the response to previous sorafenib therapy and the progression pattern (Table 3), and none of the tested factors were found to be a significant predictive factor for response to HAIC (Table S1).

The median PFS of patients from commencement of HAIC was 4.0 months (Fig. 1). The median survival time (MST) of all patients was 7.6 months, with a 1-, 2-, and 3-year survival rate of 29.4%, 24.5% and 16.4%, respectively (Fig. 2a). The MST of patients who achieved PR were 36.7 months, which was significantly better than that of patients who achieved SD/progressive disease/not evaluable, namely, 6.6 months ($P < 0.01$; Fig. 2b). Patient prognosis did not differ according to the progression pattern (Fig.S1).

DISCUSSION

THE DEVELOPMENT OF a safe and effective alternative therapy is essential because sorafenib, which represented a breakthrough in the treatment of advanced HCC, had a low response rate and frequent adverse effects, often leading to a cessation of treatment.[22,23] An increasing number of emerging agents, including novel molecular targeted drugs, have been attempted in sorafenib refractory HCC. Nevertheless, their efficacy was found to be limited (response rate, 0–4.3%; time to progression, 1.6–2.7 months).[24–26]

The first aim of this study was to investigate the feasibility of HAIC in advanced HCC after the failure of sorafenib therapy. In this study, the frequency of

Table 3 Tumor response

Response to HAIC†	All, *n* (%)	Child-Pugh class[b]		Response to sorafenib				Progression pattern[c]		
		A	B or C	PR	SD	PD	NE	IHG§§	NIH¶¶	NEH[a]
CR‡	0 (0)	0	0	0	0	0	0	0	0	0
PR§	8 (29.6)	1	7	0	4	3	1	3	0	1
SD¶	9 (33.3)	5	4	1	5	3	0	7	2	0
PD††	9 (33.3)	3	6	0	4	4	1	6	2	0
NE‡‡	1 (3.7)	0	1	1	0	0	0	1	0	0
Total	27 (100)	9	18	2	13	10	2	17	4	1

†HAIC: hepatic arterial infusion chemotherapy.†, ‡, §, ¶, ††, ‡‡, §§ and ¶¶.
‡CR: complete response.
§PR: partial response.
¶SD: stable disease.
††PD: progressive disease.
‡‡NE: not evaluable.
§§IHG: intrahepatic growth.
¶¶NIH: new intrahepatic lesion.
[a]NEH: new extrahepatic lesion.
[b]At decision-making of HAIC.
[c]At termination of sorafenib therapy.

hematological toxicity, particularly neutropenia and thrombocytopenia, was high. One of the possible causes of these toxicities was pre-existing pancytopenia derived from liver cirrhosis in most patients, and another was the concurrent administration of IFN added to 5-FU and CDDP.[15] All of the patients recovered immediately after the end of treatment and no additional complications were noted. Moreover, the frequencies of leukocytopenia, neutropenia and thrombocytopenia observed in this study (74.1%, 77.8% and 88.9%, respectively) were very similar to those of patients who were not pretreated by sorafenib and underwent HAIC with the same protocol, including 5-FU/cisplatin/IFN (75.4%, 77.2% and 89.5%, respectively),[15] which suggested that prior administration of sorafenib did not have an additional impact on hematological toxicities. With regard to non-hematological toxicities, most of them were less frequent than those in a previous report,[15] and there were no unexpected adverse reactions. These favorable results may be derived from newly available drugs such as a second-generation 5-hydroxytryptamine 3 receptor antagonist and neurokinin-1-receptor antagonist or active supportive therapy. These findings suggested that HAIC was considered tolerable even for those patients who were previously administered sorafenib.

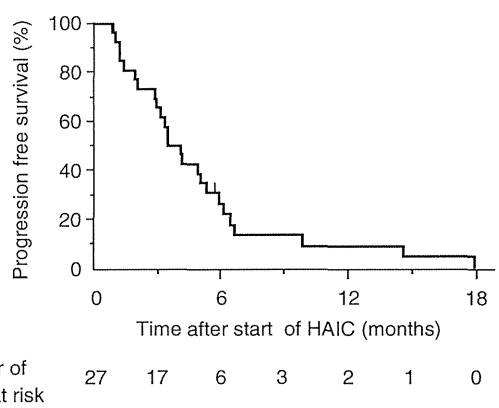

Figure 1 Kaplan–Meier plot of progression-free survival (PFS) since commencement of hepatic arterial infusion chemotherapy (HAIC). Median PFS was 4.0 months.

The response rate obtained in the present study (29.6%) appears to be low compared with that of previous reports.[14,15] Although it is difficult to compare the response rates among studies, possible reasons include variation in patients' hepatic function, the criteria used to evaluate responses, the effect of previous administration of sorafenib, and the relatively small number of patients. In addition, the proportion of patients with extrahepatic lesions may have been a meaningful factor because it was higher (44.4%) in this study than that of the previous study (0–14%)[14,15] and the response rate was reported to be lower in patients with HCC having extrahepatic metastases than in those without.[27] We could not identify any significant