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Table 1 Demographic and clinical characteristics of patients with acute hepatitis who were infected with HBV of different genotypes (1991–2009)

		HBV genotypes			
Feature	Total (n=547)	A (n=137)† (25.0%)	B (n=48) (8.8%)	C (n=359) (65.6%)	Others (n=3)‡ (0.5%)
Age (years)	35.6±14.8	35.2±12.2	39.6±15.6	35.1±15.5	49.7±13.6
Male	367 (67.1%)	129 (94.2%)¶ * †† ***	38 (79.2%)†† *	201 (56.0%)	3 (100%)
ALT (IU/l)§	2553±1563	2289±1069	2557±1412	2342±1728	3333±2406
T-Bil (mg/dl)§	7.8±6.7	9.6±7.6††*	7.7±7.4	7.1±6.2	9.0±2.5
PT (%)§	74.6±22.6	75.2±15.9	73.8±24.5	74.7±24.5	15.8‡‡
Severe hepatitis	34 (6.2%)	4 (2.9%)	4 (8.3%)	26 (7.2%)	0 (0.0%)
Fulminant hepatitis	10 (1.8%)	0 (0.0%)	1 (2.1%)	8 (2.2%)	1 (33.3%)
Mortality	8 (1.5%)	0 (0.0%)	1 (2.1%)	7 (1.9%)	0 (0.0%)
HBsAg persisting >6 months	5/514 (1.0%)	5/123 (4.1%)†† *	0/46 (0.0%)	0/342 (0%)	0/3 (0.0%)
PreC/CP mutations					
PreC	43/461 (9.3%)	1/109 (0.9%)¶ * †† *	6/39 (15.4%)	34/310 (11.0%)	2/3 (66.7%)
CP	69/461 (15.0%)	3/109 (2.8%)†† ***	0/39 (0.0%)†† *	63/310 (20.3%)	3/3 (100%)
PreC and/or CP	92/461 (20.0%)	4/109 (3.7%)¶ * †† ***	6/39 (15.4%)	79/310 (25.5%)	3/3 (100%)
Transmission route					
Homosexual	32 (5.9%)	29 (21.2%)¶ ** †† ***	0 (0.0%)	3 (0.8%)	0 (0.0%)
Heterosexual	217 (39.5%)	52 (38.0%)	25 (52.1%)	139 (39.6%)	1 (33.3%)
Medical procedure	16 (2.9%)	2 (1.5%)	2 (4.2%)	12 (3.3%)	0 (0.0%)
Other	10 (1.8%)	1 (0.7%)	1 (2.1%)	7 (1.9%)	1 (33.3%)
Undetermined	272 (49.7%)	53 (38.7%)†† *	20 (41.7%)	198 (55.2%)	1 (33.3%)
Anti-HIV	14/53 (26.4%)	11/35 (31.4%)	0/3 (0.0%)	3/15 (20.0%)	0/0

Values are mean±SD or number (%).
†One patient with genotype A was simultaneously infected with genotype G.
‡Each patient was infected with genotype D, E or H.
§Highest values during the clinical course are shown for ALT and T-Bil, and lowest values for PT.
Statistical analysis was performed to compare genotypes A, B and C.
¶Significantly different compared with genotype B.
††Significantly different compared with genotype C.
*p<0.05, **p<0.001, ***p<0.0001.
‡‡Data from the patient with genotype E only.
ALT, alanine aminotransferase; CP, core promoter; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; PreC, precore; PT, prothrombin time; T-Bil, total bilirubin.

for 6% (9/150) in the 1st period, 15.4% (19/123) in the 2nd, and 39.4% (89/226) in the 3rd, with significant differences between 1st and 2nd ($p<0.05$), 2nd and 3rd ($p<0.0001$), and 1st and 3rd ($p<0.0001$). Conversely, AHB associated with genotype C decreased through three periods with significant differences, while AHB associated with genotype B did not change appreciably.

On the basis of these results, the yearly incidence in each of the three 6-year periods is calculated to be: 25.0 cases including 1.5 with genotype A in the 1st period; 20.5 cases including 3.2 with genotype A in the 2nd; and 37.7 cases including 14.8 with genotype A in the 3rd. Hence, the incidence of AHB had not changed markedly over the 12 years from 1991 to 2002, but increased thereafter until 2008. Of the increment in the 3rd period of 17.2 (37.7 minus 20.5) cases, there were 11.6 (14.8 minus 3.2) with genotype A; they accounted for 67% (11.6/17.2) of the recent increase in AHB.

Regional distributions and longitudinal changes in genotype A

Among the 183 patients from the capital region, the genotype was A in 65 (35.5%), B in 22 (12.0%), C in 94 (51.4%), E in one (0.5%), and H in one (0.5%) (table 2). Of the remaining 364 (66.5%) patients from other regions, by contrast, the genotype was A in 72 (19.8%), B in 26 (7.1%), C in 265 (72.8%), and D in one (0.3%). Genotype A was significantly more common in the capital than in other regions (35.5% vs 19.8%, $p<0.0001$). In the capital region, genotype A accounted for 4.8% (2/42) in the 1st period, 29.3% (12/41) in the 2nd, and 50.0% (42/84) in the 3rd. There were significant differences between the 1st and 2nd periods ($p<0.05$), 2nd and 3rd ($p<0.05$), and 1st and 3rd ($p<0.0001$). In other regions, by contrast, genotype A accounted for 6.5% (7/108) in the 1st period, 8.5% (7/182) in the 2nd, and 33.1% (47/142) in the 3rd. For the first time in other regions, genotype A increased in the 3rd period, in comparison with the 1st and 2nd (1st vs 3rd, $p<0.0001$; 2nd vs 3rd, $p<0.0001$).

Subgenotypes of genotype A

Of the 137 genotype A isolates, amplification and sequencing of HBV DNA were feasible in 114 (83.2%); the isolate from the single patient with genotypes A and G was excluded. A phylogenetic tree was constructed, on the entire preS1/S2/S genes of ~1.2 kb, for these 114 isolates along with 34 genotype A isolates retrieved from the database (figure 3).

Of the 114 isolates in this study, 101 (88.6%) were subgenotype A2, and the remaining 13 (11.4%) were subgenotype A1. In a pair-wise comparison, the sequence divergence among the 101 subgenotype A2 isolates was 0–1.3%, and that among the 13 subgenotype A1 isolates spanned 0% to 2.3%. The sequence divergence between subgenotype A2 and A1 isolates ranged from 2.6% to 4.7%.

A sequence of 1203 nucleotides was possessed in common by three of the 101 (3%) isolates of subgenotype A2. For convenience, the group comprising these three isolates was labelled 'identical group I'. Likewise, an additional six 'identical groups' were found, and numbered from 'II' to 'VII'. They comprised 35 (35%), seven (7%), two (2%), three (3%), 12 (12%) and three (3%) of the 101 isolates of subgenotype A2. In contrast, only one identical group, designated 'VIII', was constructed by three of the 13 (23%) isolates of subgenotype A1.

Some isolates of subgenotype A1 and A2 were obtained from patients who had travelled to foreign countries in the recent past (5/13 (38.5%) patients with A1 to Africa, Philippines, Myanmar and China; and 5/101 (5.0%) patients with A2 to Europe, Thailand, Brazil and the USA).

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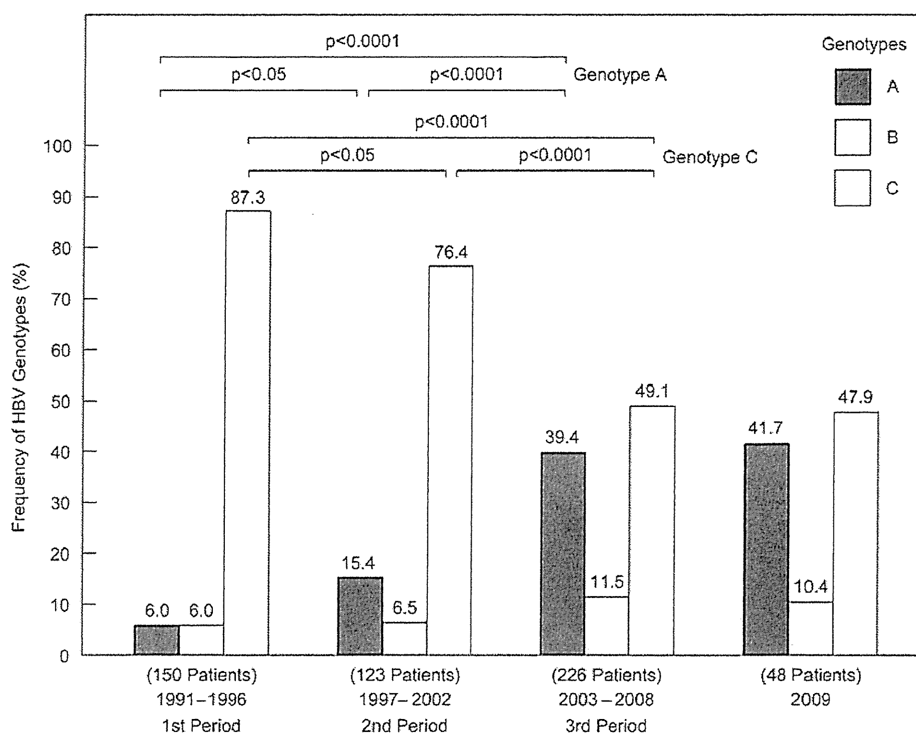

Figure 2 Distribution of hepatitis B virus (HBV) genotypes in three periods.

Subgenotypes of genotype B

Of the 48 isolates of genotype B, subgenotyping was feasible in 43 (90.0%). A phylogenetic tree was constructed on preS1/S2/S-gene sequences from these 43 isolates, along with those from 25 isolates of genotype B retrieved from the database (figure 4). Of the 43 isolates in this study, 10 (23.3%) were subgenotype B1, 28 (65.1%) were B2, two (4.7%) were B3, and three (7.0%) were B4. In a pair-wise comparison, the sequence divergence among 10 subgenotype B1 isolates ranged from 0.4% to 1.4%, and that among 28, two and three isolates of subgenotypes B2, B3 and B4 spanned 0–1.7%, 0.5% and 0.6–0.8%, respectively. The inter-subgenotype divergence among B1–B4 ranged from 0.6% to 4.4%.

One 'identical group' made up of five isolates was detected among the 28 of subgenotype B2; it was named 'IX'. In contrast, no 'identical group' was found in 10, two or three isolates of subgenotype B1, B3 or B4.

Some isolates of subgenotypes B2, B3 and B4 were obtained from patients who had travelled to foreign countries in the recent past (7/28 (25.0%) patients with B2 to China and other countries; 1/2 (50.0%) patients with B3 to a country unknown; and 1/3 (33.3%) patients with B4 to Vietnam). However, none of the 10 subgenotype B1 isolates was associated with travel to foreign countries.

Identical groups

The proportion of isolates that shared a sequence in identical groups was higher for subgenotype A2 (64.4%) than for A1, B1, B2, B3 or B4 (23.1%, 0%, 17.9%, 0% or 0%, respectively (A2 vs A1, $p<0.001$; A2 vs B1, $p<0.0001$; A2 vs B2, $p<0.0001$)).

Homosexual activity was more common in patients belonging to the seven identical groups than the non-identical group of subgenotype A2 (17/65 (26.2%) vs 3/36 (8.3%), $p<0.05$). Among the isolates in the seven identical groups of subgenotype A2, those in groups I, III and VII clustered locally during short periods of 2–7 years. In contrast, subgenotype A2 isolates in groups II and VI were scattered widely over longer periods of 11–16 years.

DISCUSSION

In Japan, as in most Asian countries, the persistent HBV carrier state had been established mainly through perinatal transmission from mother to baby and horizontal infection during infancy. In 1986, a national prevention programme was launched in Japan with selective vaccination of babies born to carrier mothers with hepatitis B e antigen (HBeAg). In 1995, this was extended to babies born to HBeAg-negative carrier mothers. As a result, the prevalence of HBsAg among younger people born since 1986 has decreased dramatically.[28 29] However, there are an

Table 2 Changes in the distribution of genotype A compared between the capital region and other regions over three periods

Area	n	1st Period (1991–1996)	2nd Period (1997–2002)	3rd Period (2003–2008)	2009
Capital region	65/183 (35.5%)†***	2/42 (4.8%)‡* §***	12/41 (29.3%)†* §*	42/84 (50.0%)†*	9/16 (56.3%)
Other regions	72/364 (19.8%)	7/108 (6.5%)§***	7/82 (8.5%)§***	47/142 (33.1%)	11/32 (34.4%)
Total	137/547 (25.0%)	9/150 (6.0%)‡* §***	19/123 (15.4%)§***	89/226 (39.4%)	20/48 (41.7%)

Statistical analysis of the differences between the capital and other regions was performed, as well as through the 1st, 2nd and 3rd periods.
†Significantly different compared with other regions.
‡Significantly different compared with the 2nd period.
§Significantly different compared with the 3rd period.
*$p<0.05$, ***$p<0.0001$.

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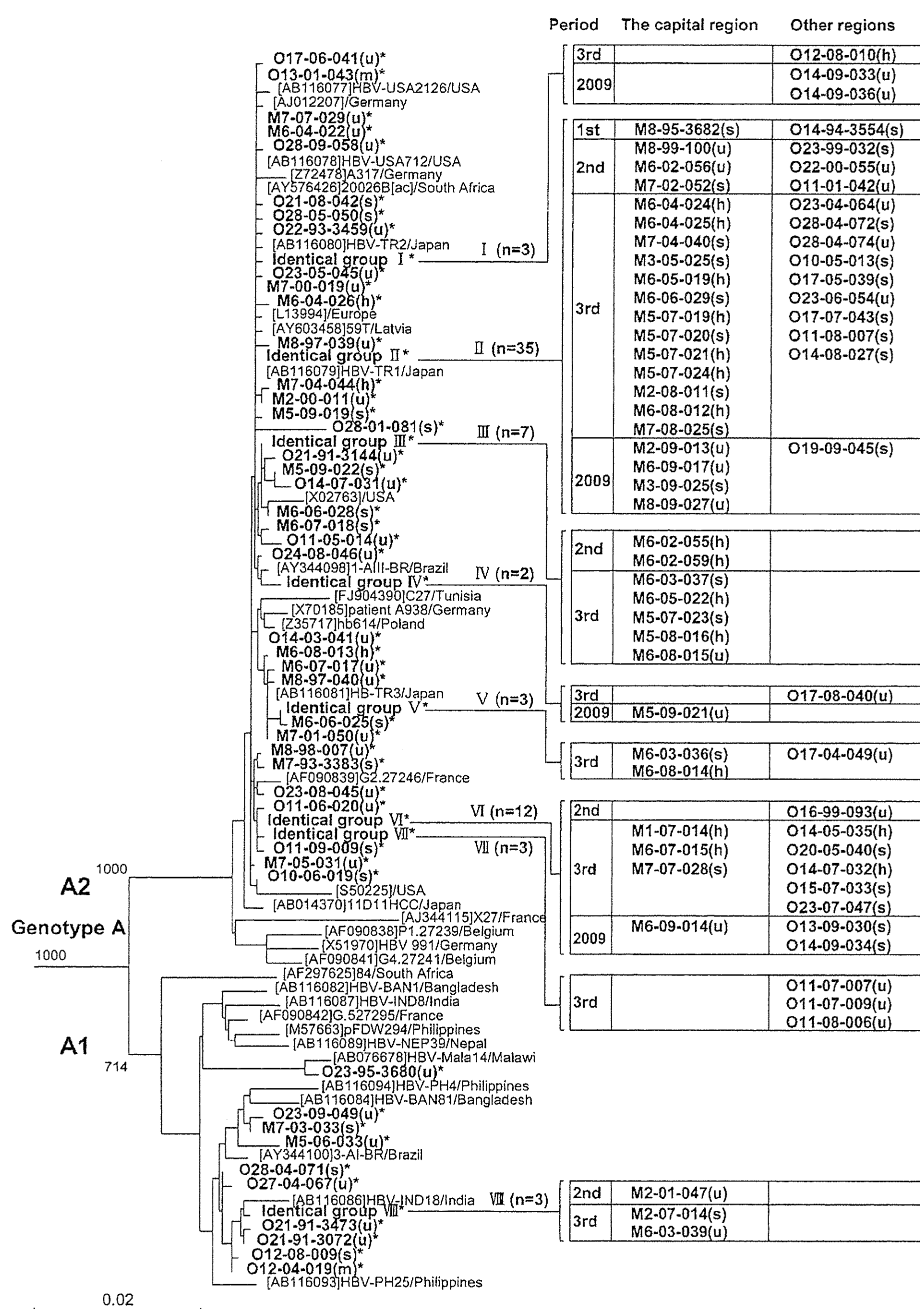

Figure 3 Phylogenetic analysis of genotype A strains by the neighbour-joining method. Isolates obtained in this study are shown in bold with asterisks. Hospitals in the capital region are labelled M1—M8 and those in other regions O9—O28 (corresponding to those in figure 1). Year of onset is indicated by the last two digits after the first hyphen. Numbers after the second hyphen represent the identification numbers of patients in each year (not always consecutive). Transmission routes are shown in lower-case letters in parentheses: h, homosexual; s, heterosexual; m, medical procedure; o, others; and u, undetermined. Isolates with identical sequences are bracketed in 'Identical groups I through VIII' on the tree. Each bracket is divided by areas and periods. Reference hepatitis B virus (HBV) isolates, including 12 of subgenotype A1 and 22 of subgenotype A2, were obtained from the database and specified by their accession numbers, isolate names and countries of origin. Bootstrap values are indicated on major phylogenetic branches.

estimated one million HBV carriers in Japan at present.[30] Furthermore, many Japanese remain at increased risk of horizontal infection with HBV, because they have not received selective vaccination and therefore do not have the antibody to HBsAg. Because AHB is extremely under-reported and no national surveillance data are available in Japan, the incidence has not been determined accurately. In the USA, the incidence of AHB has decreased markedly since the adoption of a comprehensive immunisation strategy in 1991.[31 32]

In the present study over 1991—2009, we conducted a nationwide, sentinel surveillance on AHB in Japan. In the 547 patients recruited over 19 years, genotype C was the most prevalent (65.6%), followed by genotype A (25.0%) and genotype B (8.8%). Demographic and clinical differences were observed among patients with genotypes A, B and C (table 1). The proportion of men reached 94.2% for genotype A infection, higher than that for genotype B (79.2%) or C (56.0%) infection. In the analysis of the route of transmission, homosexual activity was reported by 21.2% of patients with genotype A; all were male. In general, sexual activity tends to be higher in men than women. The predominance of genotype A in men may be attributable to a high frequency of homosexual activity among men.

Although adult-acquired HBV infection persists at a high frequency of ~10% in European countries and the USA,[33] it rarely, if ever, becomes chronic in Japan. Recent studies suggest that the chance of a chronic outcome of AHB may differ by HBV genotype[21 34]; it is more common for genotype A than other genotypes.[22 35 36] In the present study, HBV infection persisted in 4.1% of patients with genotype A, in comparison with 0% of

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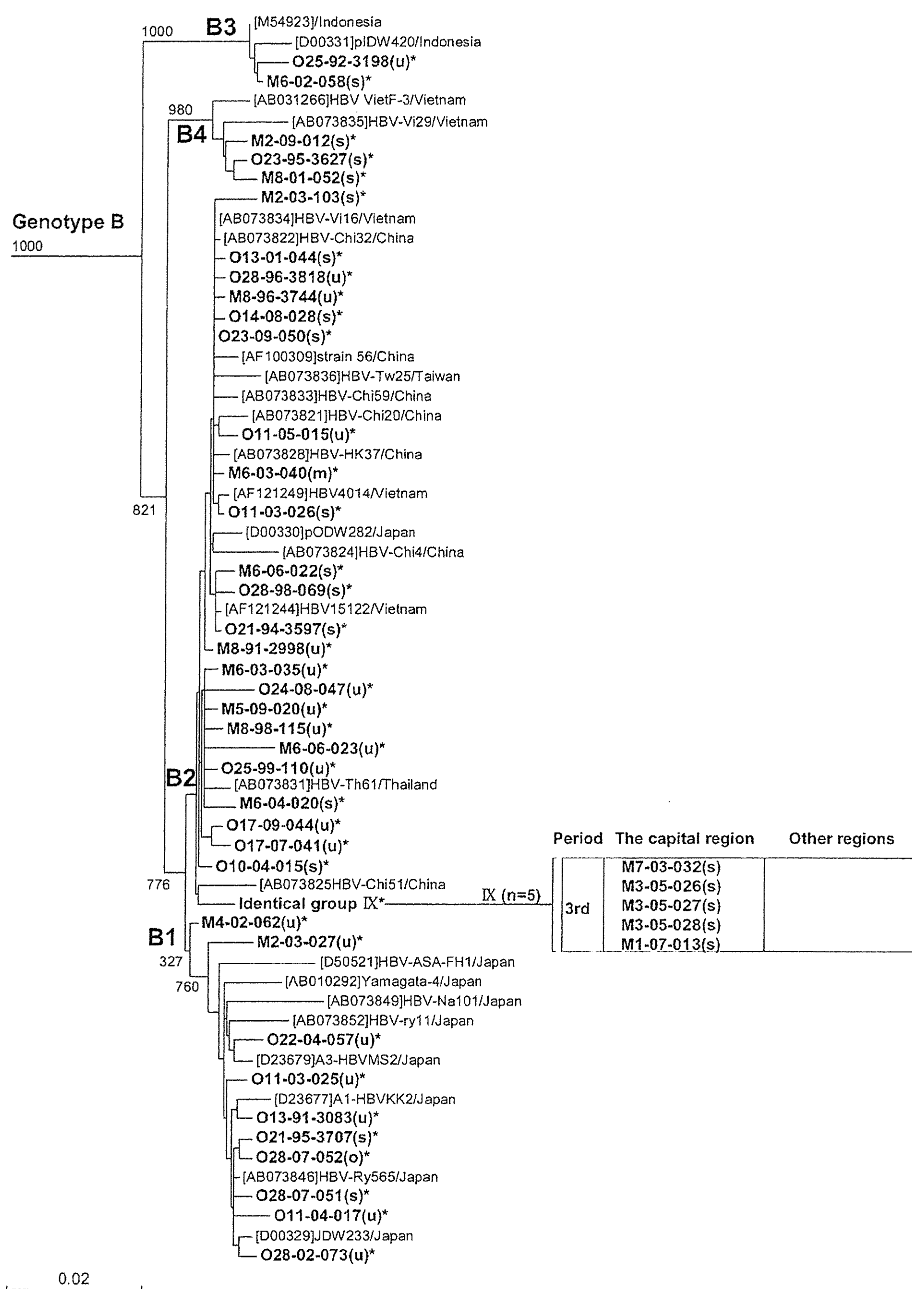

Figure 4 Phylogenetic analysis of genotype B strains by the neighbour-joining method. Hepatitis B virus (HBV) isolates obtained in the present study are specified in the same manner as in figure 3, and isolates with an identical sequence are bracketed in 'Identical group IX' on the tree. Of them, 10 reference isolates of subgenotype B1 and 13, two and two of those of B2, B3 and B4, respectively, were retrieved from the database; they are specified as in figure 3.

those with genotype C. Remarkably, all five patients with AHB who acquired chronic infection possessed HBV genotype A, either alone (four patients) or together with HBV genotype G (one). Increasing genotype A infections may have changed the genotype distribution in patients with AHB and those with chronic HBV infection. In Japanese patients with chronic hepatitis B, the proportion of genotype A has doubled, from 1.7% in 1999–2000 to 3.5% in 2005–2006.[37]

The genotype was A in 29 of the 32 (91%) homosexual men. Of the 29 homosexuals with genotype A, 10 gave consent to anti-HIV testing, and four of these (40%) were found to be positive. Of the five patients who acquired chronic HBV infection, anti-HIV was tested in four (three with genotype A and one with genotypes A and G), and one with genotype A was found to be positive. There is a possibility that co-infecting HIV in this patient with genotype A may have promoted chronic HBV infection; HIV is known to prolong and aggravate HBV infection by compromising immune responses.[38]

Patients with FH in this study were infected with either HBV genotype B (1/48 (2.1%)) or C (8/359 (2.2%)); no patients with genotype A developed FH. PreC and/or CP mutations were significantly less common in genotype A (1/109 (3.7%)) than B (6/39 (15.4%)) or C (279/310 (5.5%)) infection. The single patient with genotype A who had PreC mutation was simultaneously infected with HBV genotype G. There is a possibility that the PreC mutation in this patient was from HBV genotype G.[26] FH did not develop in any patients with genotype A, which may be attributable, at least in part, to the lack of PreC mutation in genotype A infections.[39]

Previous reports have shown that genotype A is common in patients with AHB in Metropolitan Tokyo,[20 21 40] as well as around Aichi located in the middle of Mainland Japan.[22]

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Yotsuyanagi *et al*[23] reported that genotype A is more common in patients with AHB in the metropolitan region than in other regions. Sugauchi *et al*[41] found that, in patients with AHB, the proportion with genotype A has increased over time. The present study indicates that the number of patients with AHB in Japan would not have decreased. We found that the proportion of patients with genotype A infection is increasing in the 28 national hospitals in Japan (6.0% in the 1st period, 15.4% in the 2nd, and 39.4% in the 3rd (figure 2)), with the prevalence much higher in the capital than other regions (35.5% vs 19.8% (table 2)).

In this study, there was a time lag in the increase in genotype A infection between the capital region and other regions of Japan (table 2). In the capital region, the prevalence of genotype A started to increase in the late 1990s, and kept increasing through the early 2000s (4.8% in the 1st period, 29.3% in the 2nd, 50.0% in the 3rd, and 56.3% in 2009). In other regions, by contrast, the frequency of genotype A did not change during the late 1990s, and increased significantly in the 2000s (6.5% in the 1st period, 8.5% in the 2nd, 33.1% in the 3rd, and 34.4% in 2009). Thus infiltration of genotype A infection into other regions occurred 5–6 years behind the epidemic in the capital region. This indicates that genotype A infection originated in the capital region and then spread to other areas of Japan.

Some genotypes are classified into several subgenotypes, and they have distinct geographical distributions.[42] Hence, subgenotypes are useful in tracing the route of HBV infection. By phylogenetic analysis (figures 3 and 4), 88.6% of genotype A isolates had the European–American type (A2), and the remaining 11.4% possessed the Asian–African type (A1). Likewise, 76.7% of genotype B isolates had Asian types (B2–B4), and the remaining 23.3% possessed the type endemic to Japan (B1). Of the 157 HBV isolates of genotype A or B, 147 (93.6%) had subgenotypes foreign to Japan. They are thought to have been transmitted from foreign sex workers, and spread among certain populations who share particular sexual behaviours in Japan.[41]

Of note, some HBV isolates of distinct subgenotypes possessed an identical sequence in the preS1/S2/S gene. The isolates of subgenotype A2 were prominent in this regard, and more often had the same sequence than those of other subgenotypes, such as A1, B1 and B2. The high prevalence of subgenotype A2 isolates with an identical sequence would not have been caused by cross-contamination. If cross-contamination had occurred, it would have affected isolates of all subgenotypes, and not influenced subgenotype A2 isolates preferentially. As many as 35% of subgenotype A2 isolates had an identical sequence, and those with the same sequence increased to 56.3% in the recent 2009 survey in Metropolitan Tokyo. Furthermore, some subgenotype A2 isolates in groups I, III and VII clustered locally within short periods, whereas others in groups II and VI were scattered widely over a long period of time. On the basis of these results, it is tempting to speculate that some subgenotype A2 strains would have been transmitted from person to person without undergoing mutations for many years.

In summary, the present study indicates the following. (1) AHB in the 28 national hospitals in Japan has not decreased, because genotype A infections are increasing. (2) Genotype A infections started to increase in the capital region, and then spread to local areas 5–6 years later. (3) Approximately 90% of genotype A in patients with AHB is subgenotype A2. (4) Subgenotype A2 strains with an identical sequence are spreading among younger generations with high sexual activity. (5) On the basis of the results obtained, AHB in Japan is not decreasing, because HBV of subgenotype A2 is prevailing in particular subpopulations at high risk. Finally, in order to prevent further increases in AHB in Japan, universal vaccination of young people deserves consideration.

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Competing interests None.

Ethics approval Approved by the ethics committee of each institution.

Contributors YT, HY and HI designed data collection tools, monitored data collection for the whole study, wrote the statistical analysis plan, cleaned and analysed the data. YT, HY and YM drafted and revised the paper. HY, NM, MN, EM, TK, YW, TM, MS, TH, TS, YM, TK, MT, HK, HO, SH and SA collaborated in data and sample collection.

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Cirrhosis

Development of a new *in situ* hybridization method for the detection of global bacterial DNA to provide early evidence of a bacterial infection in spontaneous bacterial peritonitis

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Background & Aims: Despite the importance of identifying the causative pathogen(s), ascitic fluid cultures are occasionally negative in patients with spontaneous bacterial peritonitis (SBP). A novel strategy using the *in situ* hybridization (ISH) method was introduced to detect the bacterial genomic DNA phagocytized in the blood of patients with sepsis. In the present study, we developed a new ISH probe to detect global bacterial DNA (named as GB probe) and evaluated its utility for detecting the phagocytized bacterial DNA in SBP ascites.

Methods: Hybridization of bacterial DNA with the GB probe was examined by dot-blot and ISH tests. In addition, the utility of the ISH method to detect the bacterial DNA in the leukocytes of SBP ascites was evaluated.

Results: The GB probe hybridized with the genomic DNA of all 59 bacterial strains tested (59 species of 36 genus). Eleven of 51 patients with ascites (out of total 542 cirrhotic inpatients) were categorized as SBP. The ISH tests showed positive results in 10 of 11 SBP cases. However, the ISH tests all showed negative results in the 40 non-SBP ascitic samples. Therefore, the ISH tests yielded highly sensitive and specific results for detecting the phagocytized bacterial DNA in the leukocytes of SBP ascites. Moreover, all of the ISH test results were obtained within only one day.

Conclusions: Our newly established ISH method was found to provide both a rapid and sensitive detection of bacterial DNA in SBP ascites, thus suggesting its utility for providing early and direct evidence of bacterial infection in SBP ascites.

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Abbreviations: SBP, spontaneous bacterial peritonitis; ISH, *in situ* hybridization; PMN, polymorphonuclear neutropils; PCR, polymerase chain reaction; rRNA, Ribosomal RNA.

Introduction

Spontaneous bacterial peritonitis (SBP) is a severe life-threatening complication in cirrhotic patients with ascites, and its prevalence in hospitalized patients ranges between 10% and 30% [1–3]. Although the identification of the pathogen(s) is important for the management of infectious diseases, it takes several days to detect the casual bacteria from ascitic fluid cultures. Furthermore, despite the use of sensitive methods, ascitic fluid cultures are negative in about 10–60% of patients with the clinical manifestations of SBP [4–7]. On the other hand, the elevated count of polymorphonuclear neutrophils (PMN) in the ascitic fluid has been proven to be a very useful and sensitive method for diagnosis of SBP. A PMN count in ascitic fluid equal to or over 250/μl is definitive for SBP [8]. Since early diagnosis and treatment play a key role in the treatment of SBP, antibiotic therapy must be started immediately after the diagnosis of SBP, without knowledge of the causative organisms and their *in vitro* susceptibility. Therefore, SBP is an infectious disease that is usually diagnosed only by the PMN count in the ascitic fluid without the identification of the pathogen. Although it is clinically very important to confirm the bacterial infection, a definitive method for early detection of the bacterial pathogen in ascitic fluid has not been well established.

In order to identify the causal bacteria in sepsis, we previously reported a novel strategy using an *in situ* hybridization (ISH) method to detect the genomic DNA of bacteria phagocytized in neutrophils and macrophages [9–11]. Our previous studies established the utility of the ISH method by demonstrating evidence supporting the presence of a bacterial infection. The ISH method was almost four times more sensitive than the blood culture (42% *vs.* 11%) in detecting the causal bacteria of sepsis [10]. Furthermore, the results of ISH tests can be obtained within 1 day, while several days to 2 weeks are required for the results of the culture method. The aim of the study was to assess the utility of the ISH method by representing the evidence of bacterial infection in SBP ascites. An original probe was generated to detect the genomic DNA of broad-spectrum bacteria, and its utility to demonstrate the causative bacteria in SBP ascites was evaluated. The ISH test succeeded in achieving a rapid and sensitive detection of

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intra-cellular bacteria in SBP ascites, thus suggesting this new method to be an effective tool to obtain direct evidence of a bacterial infection in SBP patients.

Patients and methods

The purpose and design of the study

SBP is one of the potentially fatal complications of cirrhotic patients with an infection of ascitic fluid. However, ascitic fluid cultures are often negative in patients with the clinical manifestations of SBP. In the present study, we developed a new ISH probe to detect global bacterial DNA and assessed its utility for detecting the phagocytized bacterial DNA in the leukocytes of SBP ascites.

The present study was an observational, cohort, and prospective study. The cohort/group included the consecutive cirrhotic patients who admitted to our department from January 2007 to March 2011. Ascitic fluid was obtained by paracentesis according to the usual clinical management of such patients. The condition evaluated in the study was SBP, and the intervention was ascitic liquid puncture. The purpose of the study was to evaluate the efficacy of the ISH test to offer direct evidence of a bacterial infection in SBP patients.

Study population

All cirrhotic patients with ascites admitted to our department from January 2007 to March 2011, were included in the present study. Cirrhosis was diagnosed by histological criteria and/or by clinical (laboratory, endoscopic and/or ultrasonographic) findings [12]. Exclusion criteria included the presence of any intra-abdominal, surgically treatable source of infection. Cases possibly affected by the antibiotic treatment, were also excluded from the analysis. Most of the 542 cirrhotic patients were admitted to receive the invasive treatment for hepatocellular carcinoma or esophageal varices, and their ascites was well controlled before admission. Only 58 cirrhotic patients could provide sufficient ascitic samples, and four patients were excluded due to receiving antibiotic treatment (one patient was treated due to the suggestive clinical manifestations of SBP in another hospital before admission to our hospital). In addition, three patients (one patient with hepatocellular carcinoma and two patients with pancreatic cancer) were excluded due to the complication of peritonitis carcinomatosa. Finally, 51 consecutive cirrhotic patients who met the inclusion criteria were analyzed in the present study (Fig. 1). All the patients had evidence of advanced liver disease, and Child–Pugh classification was grade B in 15 patients and grade C in 36 patients. Cirrhosis was diagnosed as non-viral hepatitis in 28 patients and chronic viral hepatitis in 23 patients (HBV 6, HCV 16, both 1). Non-viral cirrhotic patients were associated with alcoholic, autoimmune, and cryptogenic cirrhosis. The characteristics of the study population are summarized in Table 1. The study protocol confirmed the ethical guidelines of the 1975 Helsinki declaration, and patients were enrolled after providing their written informed consent to participate in this study. Blood samples were obtained to perform routine studies, including hematological, biochemical, and coagulation tests.

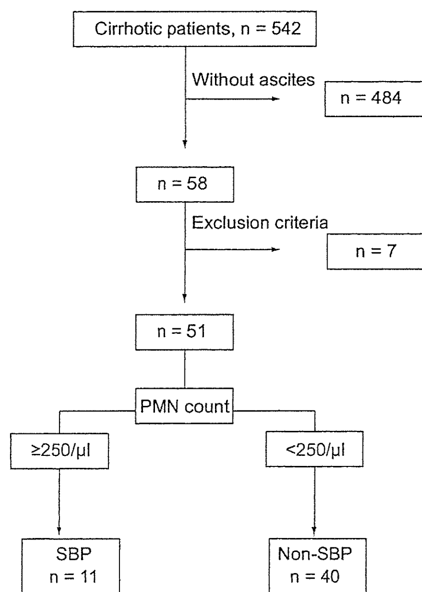

Fig. 1. Algorithm for the classification of cirrhotic patients.

Table 1. Characteristics of the study population.

Age (years)	62.5 ± 1.2
Gender (Male/Female)	35/16
Etiology (HBV/HCV/Both/Non-viral)	6/16/1/28
Child-Pugh classification (A/B/C)	0/15/36
Child-Pugh score	10.24 ± 0.20
Total bilirubin (mg/dl)	2.3 (0.4-28.3)
Albumin (g/dl)	2.68 ± 0.05
Prothrombin time (%)	61.2 (24.0-96.2)
Creatinine (mg/dl)	1.45 ± 0.15
MELD score	17 (7-40)
Hepatocellular carcinoma (present/absent)	13/38
Variceal hemorrhage (present/absent)	4/47
History of SBP or sepsis (present/absent)	3/48
Use of β-blocker (present/absent)	4/47
Use of PPI (present/absent)	40/11

HBV, hepatitis B virus; HCV, hepatitis C virus; PPI, proton-pump inhibitor.
Data are expressed as the mean ± standard deviation or median (range).

Paracentesis

All 51 cirrhotic patients underwent diagnostic paracentesis to search for SBP in aseptic conditions following the usual procedures, and a routine biochemical study was carried out and the PMN count of the ascitic fluid was determined. The diagnosis of SBP was based on the elevated PMN count (⩾250/µl) in the ascitic fluid. Distinction from secondary bacterial peritonitis was performed according to the criteria previously reported [3,4,13,14]. Ascitic fluid culturing was carried out using both aerobic and anaerobic blood-culture bottles.

Probes

A series of grouped probes (designated SA, SE, PA, EF, and EK) were previously established for the specific detection of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and groups of enterobacteria (*Escherichia coli*, *Enterobacter cloacae* and *Klebsiella pneumoniae*), respectively [9]. These probes were generated to detect the agents that most frequently cause bacteremia and specifically find the intended pathogen.

The present study attempted to develop a new probe which could detect all kinds of bacteria. A cDNA probe for the 23S ribosomal RNA (rRNA) gene was thought to serve this purpose, because 23S rRNA is common to all bacteria. Therefore, polymerase chain reaction (PCR) was performed as described previously [15], and several cDNA fragments that corresponded to the 23S rRNA genes of various bacteria were obtained. Although every generated cDNA probe for 23S rRNA gene could detect the genomic DNA of plural bacteria, no probe could cover all kinds of bacteria with sufficient sensitivity (data not shown). This suggested that each type of bacteria had own unique sequences in their 23S rRNA genes, and DNA sequences were partially different from one another. Therefore, it would be difficult to establish a single cDNA probe that could solely detect all kinds of bacteria universally. Finally, plural cDNA fragments were mixed and used as new probe cocktails. This strategy succeeded in detecting the genomic DNA of all bacteria examined. The new probe mixtures were designated as the "global bacteria (GB) probe". The GB probe consisted of cDNA fragments for 23S Ribosomal RNA genes of various bacteria, including *S. aureus*, *Bacteroides fragilis*,

Table 2. Probes used for the *in situ* hybridization.

Probe	Detective pathogen
SA	*Staphylococcus aureus*
SE	*Staphylococcus epidermidis*
PA	*Pseudomonas aeruginosa*
EF	*Enterococcus faecalis*
EK	*Escherichia coli* *Enterobacter cloacae* *Klebsiella pneumoniae*
GB	Global Bacteria

Pseudomonas aeruginosa, *Enterococcus faecalis*, and *E. coli* (Genbank Accession No.: X68425, CR626927, Y00432, AJ295306, EU146962, respectively). Therefore, the probe cocktails called "GB probe" have been developed to detect the genomic DNA of whole bacteria, but the probe mixture cannot specifically identify the kind of bacteria. In contrast, the previously established probes can detect only limited types of bacteria, but can specifically identify the targeted pathogens (Table 2).

Dot-blot hybridization of the GB probe

Genomic DNA was isolated from various bacterial strains, including both Gram-positive and Gram-negative bacteria, and dot-blot hybridization was performed as described previously [9] to examine the utility of the newly developed GB probe for the detection of global bacterial DNA. Genomic DNA samples extracted from the fungi were used as negative control samples.

In order to determine the lower limit of detection of bacterial DNA, samples containing serially diluted genomic DNA of either Gram-positive (*S. aureus*: ATCC12400) or Gram-negative (*E. coli*: JCM1649) bacterial strains were prepared (final concentration; 100,000, 10,000, 1000, 100, 10, and 1 pg/μl). In addition, samples containing serially diluted DNA fragments that corresponded to the 23S rRNA genes of either Gram-positive (*S. aureus*: ATCC12400) or Gram-negative (*E. coli*: JCM1649) bacterial strain were also prepared (final concentration; 100, 10, 1, 0.1, 0.01, and 0.001 pg/μl). Two microliters of each sample was spotted on the membrane, and these were used for the detection of bacterial DNA with the GB probe.

In situ hybridization of the GB probe in bacterial smear samples

In order to examine whether the ISH test could detect and visualize the genomic DNA of a single bacterial cell, smear samples of either Gram-positive (*S. aureus*: ATCC12400) or Gram-negative (*E. coli*: JCM1649) bacterial strains were prepared. In addition, a representative smear sample of a fungus (*C. albicans*: NBRC1594) was also prepared as a negative control sample. The slides were fixed for 20 min in Carnoy's solution, and ISH was performed according to the methods described previously [9–11].

In situ hybridization of the GB probe in an experimental infection model

A previous study [16] successfully detected intracellular bacteria in mouse phagocytic cells by the ISH method. The present study used human leukocytes extracted from blood samples derived from a member of our laboratory. First, various bacterial strains were phagocytized by leukocytes *in vitro*, according to the methods described in the published patent (US 7651837 B2: "Method for detecting and identifying organism causative of infection"), and the ability of the GB probe to detect intracellular bacteria was investigated. In addition, leukocytes which ingested fungus were also prepared and used as negative control samples. The procedures used for the ISH test were performed as previously described [9–11], and positive signals were detected in the cellular cytoplasm as purple-brown stains.

Next, in order to determine whether the ISH test can detect the phagocytized bacterial DNA in fluid samples with a lower concentration of leukocytes, we prepared samples with various leukocyte concentrations of approximately 1000, 300, and 100/μl. The samples containing these dilutions of leukocytes were spiked with either Gram-positive (*S. aureus*: ATCC12400) or Gram-negative (*E. coli*: JCM1649) bacteria. After the 1-h spiking procedure, leukocytes were collected and the ability of the GB probe to detect the bacterial DNA was investigated.

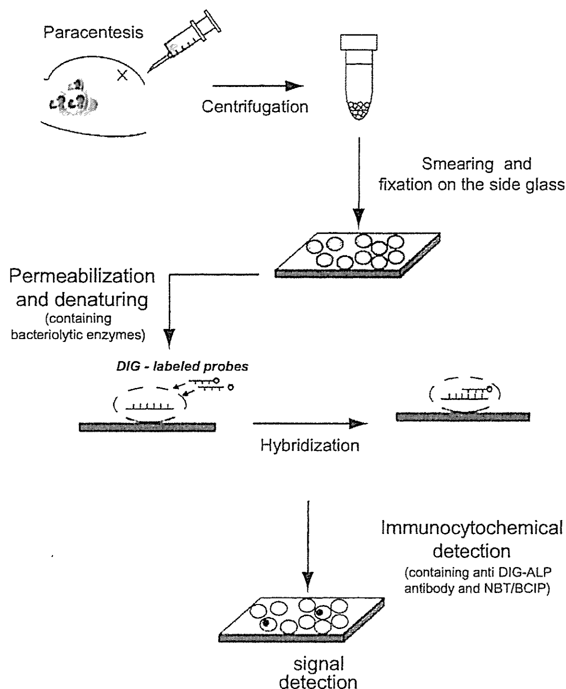

Fig. 2. Schematic representation of the *in situ* hybridization (ISH) for ascitic leukocytes. Floating leukocytes in ascitic fluid were collected by centrifugation and samples were spread on glass slides and prepared for ISH. ISH was performed according to the methods described in "Patients and methods".

Detection of the bacterial DNA in ascitic leukocytes

Ascitic samples were obtained by routine paracentesis as described above. Leukocytes were collected from ascitic fluid in the research laboratory, and ISH tests were performed to detect the bacterial genomic DNA. The procedure of ISH was based on the previously reported method with some modifications. In brief, migrated leukocytes in the ascitic fluid were collected by centrifugation (140–180*g* for 10 min). Bloody ascites underwent hemolysis with hypotonic buffer, and the leukocytes were re-collected by centrifugation and re-suspended in PBS (concentration: 5×10^4/μl). A 5–10 μl sample of the cellular suspension was spread onto a glass slide, and then allowed to air-dry. The slides were fixed for 20 min in Carnoy's solution, and the ISH was performed according to the methods as previously described [9–11]. A schematic illustration of the method is shown in Fig. 2. The ISH tests with all probes (GB, SA, SE, PA, EF, and EK probes) were performed simultaneously.

Statistical analysis

Baseline differences between SBP patients and non-SBP patients were statistically evaluated. Quantitative variables were expressed as the mean values ± SD, and were evaluated using Student's *t* test. Data with an abnormal distribution were expressed as the median values (range) and were evaluated using the Mann–Whitney *U*-test.

Results

Characteristics of patients and clinical data

Fifty-one cirrhotic patients with ascites (out of 542 cirrhotic inpatients) were consecutively included in the present study, based on the inclusion and exclusion criteria as described in

Table 3. Clinical data of the groups distributed according to the absence or presence of SBP.

	SBP (n = 11)	Non-SBP (n = 40)	*p value
Age (years)	63.5 ± 4.3	62.2 ± 1.0	0.63
Gender (Male/Female)	7/4	28/12	0.54
Etiology (HBV/HCV/Both/Non-viral)	0/5/0/6	6/11/1/22	
(Viral/Non-viral)	5/6	18/22	0.77
Child-Pugh classification (A/B/C)	0/3/8	0/12/28	0.78
Child-Pugh score	10.64 ± 0.51	10.13 ± 0.22	0.30
Total bilirubin (mg/dl)	2.4 (0.9-18.5)	2.2 (0.4-28.3)	0.32
Albumin (g/dl)	2.66 ± 0.11	2.69 ± 0.06	0.82
Prothrombin time (%)	52.7 (24.0-71.1)	63.0 (35.0-96.2)	0.09
Creatinine (mg/dl)	1.58 ± 0.37	1.42 ± 0.17	0.68
MELD score	19 (10-40)	16.5 (7-34)	0.12
HCC (present/absent)	2/9	11/29	0.53
Variceal hemorrhage (present/absent)	3/8	1/39	0.0068*
History of SBP or sepsis (present/absent)	2/9	1/39	0.050
Use of β-blocker (present/absent)	2/9	2/38	0.15
Use of PPI (present/absent)	9/2	31/9	0.76
PMN count of ascites (cells/μl)	1767 (281-20,593)	9.5 (0-168)	6.3×10^{-6}*

HBV, hepatitis B virus; HCV, hepatitis C virus; PMN, polymorphonuclear neutrophils; PPI, proton-pump inhibitor.
*A significant p value was a p value <0.05. Data are expressed as the mean ± standard deviation or median (range).

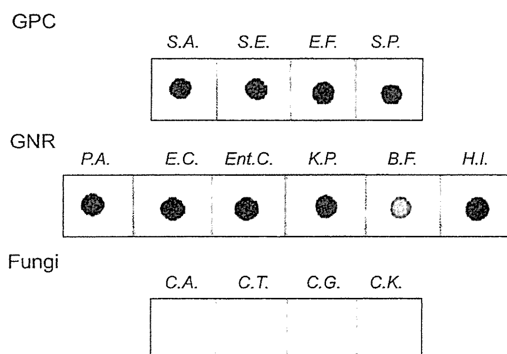

Fig. 3. Dot-blot hybridization of the GB (designed for Global Bacteria) probe with genomic DNA of various bacterial strains. Genomic DNA was isolated from various Gram-positive and Gram-negative bacterial strains, and dot-blot hybridization was performed as described previously [9]. Genomic DNA samples extracted from several fungi were used as negative control samples. Positive signals were observed in the DNA spots of various Gram-positive (GPC) and Gram-negative (GNR) bacterial strains. In contrast, the GB probe did react with the fungal DNA spots (Fungi). The names of bacterial and fungal strains shown are: S.A., *S. aureus* ATCC12600; S.E., *Staphylococcus epidermidis* ATCC14990; E.F., *Enterococcus faecalis* JCM5803; S.P., *Streptococcus pneumoniae* ATCC39938; P.A., *Pseudomonas aeruginosa JCM5962*; E.C., *Escherichia coli* JCM1649, Ent.C, *Enterobacter cloacae* JCM1232; K.P., *Klebsiella pneumoniae* JCM1662; B.F., *Bacteroides fragilis* SMUM2275; H.I., *Haemophilus influenzae* ATCC33391; C.A., *Candida albicans* NBRC1594; C.T., *Candida tropicalis* NBRC1400; C.G., *Candida glabrata* NBRC0622; C.K., *Candida krusei* NBRC0011. GPC, Gram-positive cocci; GNR, Gram-negative rods.

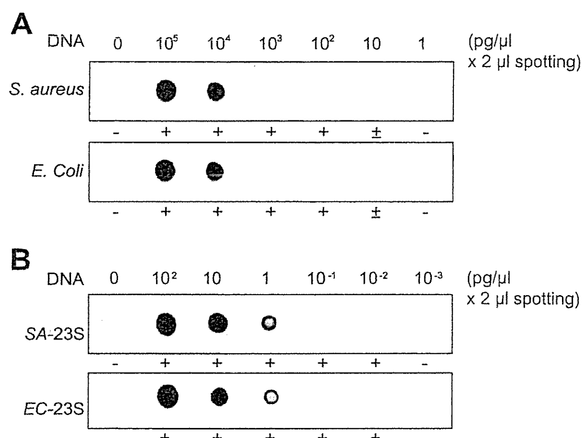

Fig. 4. Sensitivity of the ISH test for detecting bacterial DNA by the dot-blot hybridization with GB-probe. (A) The sensitivity analysis for the detection of bacterial genomic DNA with the GB probe. Samples containing serially diluted genomic DNA of either Gram-positive (*S. aureus*: ATCC12400) or Gram-negative (*E. coli*: JCM1649) bacterial strains were prepared (final concentration; 100,000, 10,000, 1000, 100, 10 and 1 pg/μl). Two microliters of each sample was spotted onto the membrane, and these were used for the hybridization with the GB probe. The ISH test showed positive signals on the spots which contained more than 200 pg of genomic DNA, whereas a positive signal was barely detected on the spot which contained 20 pg of genomic DNA. (B) The sensitivity analysis for the detection of target DNA (*S. aureus* [SA-23S] and *E. coli* [EC-23S]-DNA) with the GB probe. Samples containing serially diluted DNA fragments that corresponded to the 23S rRNA genes of either Gram-positive (*S. aureus*: ATCC12400) or Gram-negative (*E. coli*: JCM1649) bacterial strains were prepared (final concentration; 100, 10, 1, 0.1, 0.01, and 0.001 pg/μl). Two microliters of each sample was spotted onto the membrane, and these were used for the detection of bacterial DNA by the GB probe. The GB probe showed positive signals on the spots which contained more than 0.02 pg of the target DNA.

"Patients and methods". Eleven of the 51 patients had SBP defined by PMN counts equal to or over 250/μl (SBP patients), and the remaining 40 patients with the PMN counts below 250/μl were defined as non-SBP patients. The baseline clinical

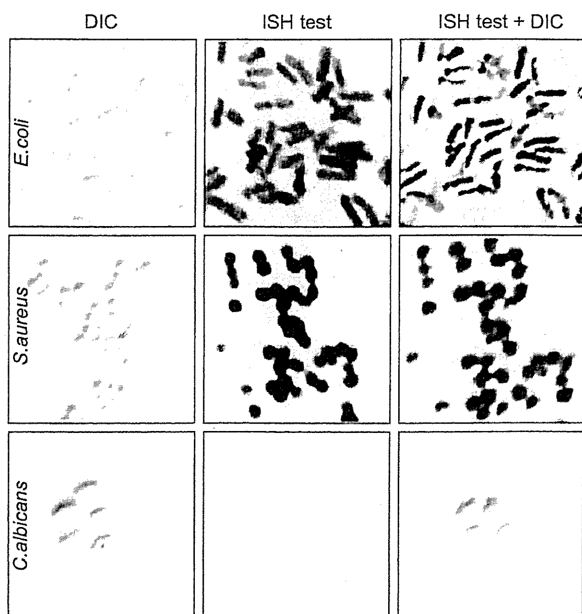

Fig. 5. ***In situ* hybridization of the GB probe for the bacterial smear samples.** In order to examine whether the ISH test can detect and visualize the genomic DNA of a single bacterial cell, smear samples of either Gram-positive (*S. aureus*: ATCC12400) or Gram-negative (*E. coli*: JCM1649) bacterial strains were prepared. In addition, smear samples of a fungus (*C. albicans*: NBRC1594) were also prepared as negative control samples. We assessed the results of the ISH tests using the differential interference contrast microscope (DIC). The ISH tests succeeded in providing visualization of all smeared bacterial cells, thus suggesting that the GB probe can detect the small amount of bacterial DNA which is derived from a single bacterial cell.

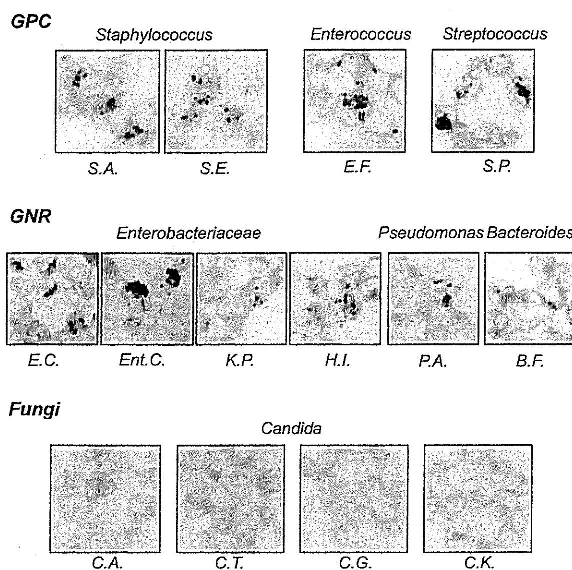

Fig. 6. Detection of the phagocytized bacterial DNA in leukocytes by the ISH method using the GB probe. Various bacterial strains were ingested by leukocytes *in vitro* according to the method described in "Patients and methods", and whether or not the GB probe could detect intracellular bacteria was determined. Both Gram-positive and Gram-negative strains were examined, and the ISH test was performed. Smear samples of leukocytes which had phagocytized fungi were used as negative control samples. Positive (purple brown) signals were observed in leukocytes (counterstained by fast green) which had phagocytized various Gram-positive (GPC) and Gram-negative (GNR) bacteria. However, no positive signal was observed in the leukocytes of intra-cellular fungal DNA (Fungi). The names of the bacterial or fungal strains are shown in the legend of Fig. 3. GPC, Gram-positive cocci; GNR, Gram-negative rods.

data of the groups distributed according to the absence or presence of SBP are shown in Table 3. The PMN counts of the SBP patients were significantly higher than those of the non-SBP patients, which is consistent with the diagnostic definition of SBP. The rate of variceal bleeding at the time of admission was significantly higher in the SBP patients than that in the non-SBP patients. The comparison of the other parameters analyzed did not show statistical significance, although there was a trend of more advanced liver disease (lower serum albumin level and prothrombin time; higher total bilirubin level, Child–Pugh score, and MELD score) in the patients of the SBP group. The SBP prevalence according to the etiology of cirrhosis was as follows: HBV: 0/6, HCV: 5/16, HBV + HCV: 0/1, non-viral: 6/28. Although there were no HBV-related cirrhotic patients who had SBP, the difference between the viral group (5/23) and the non-viral group (6/28) was not significant.

Hybridization of the new GB probe with bacterial genomic DNA

A previous study established five kinds of probes (designated SA, SE, PA, EF, and EK) and reported their specific detection of the intended bacterial genomic DNA. The present study attempted to generate a new probe, which was expected to hybridize with the genomic DNA sequences of 23S rRNA genes in global bacteria. At first, dot-blots were used to determine whether the newly developed GB probe could hybridize with the genomic DNA of various bacteria. As shown in Fig. 3, the GB probe detected all kinds of genomic DNA isolated from broad-spectrum bacterial strains, but did not react with the DNA of fungi, thus showing selective hybridization with the bacterial genomic sequences. In order to determine the lowest limit of the detectable bacterial DNA, serial dilutions of both whole genomic DNA and the target DNA for the genomic sequences of 23S rRNA were prepared and hybridized with the GB probe. As shown in Fig. 4A, the GB probe showed positive signals on the spots which contained over 200 pg of genomic DNA, and a weak signal was detected on the spot which contained 20 pg of genomic DNA. With regard to the detection of target DNA for 23S rRNA, the GB probe showed positive signals on the spots which contained over 0.02 pg of the target DNA (Fig. 4B). Therefore, the lowest amount of DNA detectable by the GB probe, was estimated to be about 20 pg of genomic DNA and about 0.02 pg of target DNA.

Furthermore, in order to determine the lowest limit of the detectable bacterial DNA, we examined whether the ISH test could detect and visualize the genomic DNA of a single bacterial cell. As shown in Fig. 5, the ISH test succeeded in the visualization of all smeared bacterial cells, thus suggesting that the GB probe can work in the detection of the small amount of bacterial DNA present in a single bacterial cell.

Next, the ISH method was used to determine whether the GB probe could detect intracellular bacterial DNA. As shown in Fig. 6,

Table 4. Bacterial strains detected by the GB probe.

Bacteria	genus	species	Strain
Actinobacteria	*Eggerthella*	*lenta*	JCM9979
	Corynebacterium	*diphtheriae*	JCM1310
		pseudodiphteritcum	JCM11665
		jeikeium	JCM9384
	Propionibacterium	*acnes*	JCM6425
	Micrococcus	*luteus*	JCM1464
Firmicutes	*Lactobacillus*	*fermentum*	JCM1173
		acidophilus	JCM1132
	Bacillus	*cereus*	IFO15305
	Staphylococcus	*aureus*	ATCC12600
		epidermidis	ATCC14990
	Enterococcus	*faecalis*	JCM5803
		faecium	JCM5804
		avium	JCM8722
	Streptococcus	*pneumoniae*	ATCC39938
		sanguinis	JCM5708
		pyogenes	JCM5674
		agalacticae	JCM5671
		salvarius	IFO13956
	Clostridium	*perfringens*	JCM1290
	Peptoniphilus	*asaccharolyticus*	JCM8143
Bacteroides	*Bacteroides*	*fragilis*	JCM11019
		ovatus	JCM5824
	Porphyromonas	*asaccharolytica*	JCM6326
Fusobacterium	*Fusobacterium*	*nucleatum*	JCM8532
		necrophorum	JCM3724
Proteobacteria α	*Brevundimonas*	*diminuta*	NBRC14213
Proteobacteria β	*Burkholderia*	*cepacia*	SMUM2242
	Achromobacter	*xylosoxidans*	JCM9659
Proteobacteria (γ)	*Pseudomonas*	*aeruginosa*	JCM5962
		fluorescens	JCM5963
		putida	JCM6157
	Acinetobacter	*calcoaceticus*	JCM6842
	Escherichia	*coli*	JCM1649
	Enterobactor	*cloacae*	JCM1232
		sakazakii	JCM1233
		aerogenes	JCM1235
		gergoviae	JCM1234
	Klebsiella	*pneumoniae*	JCM1662
		aerogenes	IFO13541
		oxytoca	SUMUM2071
	Raoultella	*terrigena*	IFO14941
	Haemophillus	*influenzae*	ATCC33391
	Serratia	*marcescens*	JCM1239
		liquefaciens	JCM1245
	Citrobacter	*koseri*	JCM1659
	Hafnia	*alvei*	JCM1666
	Edwerdsiella	*tarda*	JCM1656
	Proteus	*vulgaris*	IFO3851
		mirabilis	JCM1669
	Providencia	*rettgeri*	JCM1675
		alcalitaciens	JCM1673
		stuartii	ISTU683
	Morganella	*morganii*	JCM1672
	Salmonella	*enterica*	IFO12529
	Pantoea	*agglomerans*	JCM1236
	Kluyvera	*intermedia*	JCM1238
	Raoultella	*planticola*	IFO14939
	Stenotrophomonas	*maltophilia*	JCM1975

Data are expressed as the mean ± standard deviation or median (range).

positive signals were observed in cells which ingested various bacterial strains, but the GB probe did not react with cells that had phagocytized fungi. Therefore, positive signals detected by the GB probe were not due to non-specific hybridization, but were caused by the hybridization with genomic DNA of bacteria, suggesting the specific detection of intracellular bacterial genomic DNA. The GB probe was confirmed to hybridize with the genomic DNA of 59 bacterial strains (59 species of 36 genus) without exception (Table 4), thus suggesting that it was a new probe to detect global bacteria.

In order to examine whether the ISH test also work in fluid with lower concentration of leukocytes, serial dilutions (1000, 300, and 100/μl) of leukocytes were spiked with bacteria, and then leukocytes were collected to prepare smear samples. Using the experimental system of the *in vitro* phagocytization, the ISH test showed positive signals in all samples (Fig. 7), thus suggesting that the ISH test should work in the fluid with a lower concentration of leukocytes.

Preparation of ascitic leukocyte-smears and detection of bacterial DNA

The ISH tests against leukocytes samples isolated from blood samples used 5 ml of the peripheral blood samples for the tests. The current study used 100–200 ml of ascitic fluid samples, and leukocytes were collected by centrifugation, since the number of leukocytes in ascites is predicted to be less than one-tenth of that in blood samples. The average recovery rate of ascitic leukocytes was about 24.6%, and that was a sufficient number of cells for the investigation (data not shown). In addition, cellular shapes were well maintained after the centrifugation and re-suspension procedure, resulting in the successful preparation of smear samples.

Next, the ISH method was used to detect the bacterial DNA ingested in phagocytes. Smear samples of ascitic leukocytes were prepared according to the procedure described in "Patients and methods". As shown in Fig. 8, positive signals were detected in

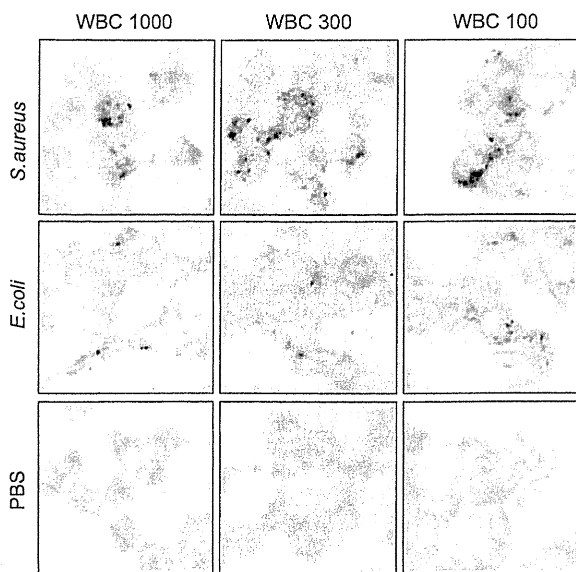

Fig. 7. *In vitro* phagocytosis of bacteria by low counts of WBCs, and the detection of bacterial DNA with the GB probe. In order to examine whether the ISH test works in ascitic fluid with a lower concentration of leukocytes, serial dilutions (1000, 300, and 100/µl) of leukocytes were spiked with either Gram-positive (*S. aureus*: ATCC12400) or Gram-negative (*E. coli*: JCM1649) bacterial strains, and then leukocytes were collected to prepare the smear samples. Using the same experimental system used for the *in vitro* phagocytization, the ISH test showed positive signals in all samples, thus suggesting that the ISH test can work in fluid samples with a lower concentration of leukocytes.

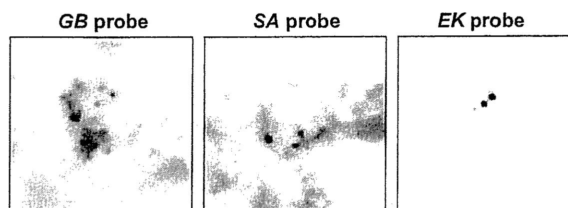

Fig. 8. Detection of the ingested bacterial DNA in ascitic leukocytes of SBP patients using the ISH method. ISH tests were performed to detect the bacterial genomic DNA in ascitic leukocytes obtained from cirrhotic patients. The procedure of ISH was based on the previously reported method with some modifications (see Fig. 2). Positive signals were detected in the ascites-derived leukocytes of SBP cases, as well as in the experimental studies shown in Fig. 6. Representative data of positive signals detected by the various ISH probes (GB probe, SA probe, and EK probe) are shown.

ascites-derived leukocytes of SBP cases, as well as they were observed in the peripheral leukocytes of septic patients in the previous reports [9,10].

The detailed results of the ISH tests and ascitic cultures in SBP patients are shown in Table 5. The previously developed probes for specific pathogens (SA, SE, PA, EF, and EK) detected the bacterial genomic DNA in 4 of the samples from SBP cases (Nos. 1, 2, 4, and 9). In addition, positive signals were detected in 10 of the 11 cases when all available probes were used (including GB probe). Both the ISH test and culture showed positive results in the three cases (No. 1, 6, and 7). ISH and culture methods detected *S. aureus* in case No. 1. An ascitic culture revealed an infection of bacteria in cases No. 6 and No. 7, which were not identified by the five previously developed probes, but an infection could be successfully detected by the GB probe. Therefore, the results of the ISH test were consistent with the results of ascitic cultures in these three cases, suggesting the validity of the positive results obtained with the ISH method. In contrast, neither ISH tests nor ascitic cultures showed positive results in the 40 cirrhotic patients without SBP (Table 6). Therefore, when SBP was diagnosed at the threshold of 250 PMN count/µl with the ascitic fluid, the ISH test detected intra-cellular bacterial DNA in the SBP ascites with a high sensitivity (91%) and specificity (100%), despite the fact that the number of patients in the present study was small. The ISH tests with all probes (GB, SA, SE, PA, EF, and EK

Table 5. Results of *in situ* hybridization test for SBP patients.

Case No.	WBC (PMN) (cells/µl)	Results of ISH tests						Results of ascitic culture
		New probe	Previously established probes for specific pathogens					
		GB	SA	SE	PA	EF	EK	
1	5430 (4832)	+	+	-	-	-	-	MRSA
2	1400 (1141)	+	-	-	-	-	+	n.d.
3	1030 (991)	+	-	-	-	-	-	n.d.
4	1880 (1767)	+	-	-	-	-	+	n.d.
5	310 (281)	-	-	-	-	-	-	n.d.
6	7230 (6652)	+	-	-	-	-	-	*Serratia liquefaciens*
7	24,370 (20,593)	+	-	-	-	-	-	NF-GNR
8	5710 (4653)	+	-	-	-	-	-	n.d.
9	810 (535)	+	-	-	-	-	+	n.d.
10	3130 (2612)	+	-	-	-	-	-	n.d.
11	1380 (994)	+	-	-	-	-	-	n.d.

ISH, *in situ* hybridization; SBP, spontaneous bacterial peritonitis; PMN, polymorphonuclear neutropils; MRSA, Methicillin-resistant *S. aureus*; n.d.: not detected; NF-GNR: non-fermenting Gram-negative rod (unidentified: other than *Achromobacter xylosoxidans*, *Acinetobacter baumannii*, *Burkholderia cepacia*, *Chryseobacterium meningosepticum*, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia*).

Table 6. ISH test and culture of ascitic fluid in the cirrhotic patients.

	SBP (%)	Non-SBP (%)
ISH test (+)	10/11 (91)	0/40 (0)
ISH test (-)	1/11 (9)	40/40 (100)
Culture (+)	3/11 (27)	0/40 (0)
Culture (-)	8/11 (73)	40/40 (100)

probes) were performed simultaneously. Therefore, consistent with our previous reports [9,10], all of the ISH test results were obtained within one day.

Discussion

In the present study, a new ISH method to detect broad-spectrum bacteria was developed, and its utility to provide direct evidence of a bacterial infection in clinically defined SBP patients was evaluated. The principle of the method is to detect bacterial genomic DNA in the phagocytic leukocytes using ISH within 8 h, and this method was originally developed for the purpose of diagnosing sepsis. In addition to septicemia, a case report using the ISH test for the detection of bacterium in the peritoneal dialysis fluid was recently reported [17]. The current study showed that the ISH test is considered to be useful for obtaining early and direct evidence of a bacterial infection in SBP ascites.

While a delayed diagnosis of SBP often causes a fatal outcome, early and effective antibiotic treatment can improve the prognosis. Therefore, the diagnostic criteria of the complication focus on simplicity and rapidity, and the elevated PMN count in ascites is the gold standard for the diagnosis [2–4,8]. Although several days to weeks are required to reveal the casual bacteria by the ascitic cultures, the results are occasionally negative in patients with clinical symptoms of SBP. The amount of bacteria in the ascitic fluid of SBP patients is thought to be low even at the onset, and phagocytosis and digestion of bacteria by leukocytes should reduce the amount of proliferative and suspending bacteria in the ascitic fluid, thus leading to difficulty in identifying the pathogen. Previous studies assessed the clinical utility of several reactions induced by the host-immune responses of patients in order to develop a new method of SBP diagnosis. For example, reagent strips measuring leukocyte esterase activity have been reported to show a possible diagnostic utility of SBP [18,19]. In addition, the levels of several inflammatory mediators in ascitic fluids, such as tumor necrosis factor (TNF) and interleukin-6 (IL-6), are suggested to be associated with the severity of SBP [20,21]. However, these cytokines are generated by the host-reactions against inflammatory stimulations, thus these targets cannot directly demonstrate the bacterial infection of SBP. Moreover, the diagnostic accuracy of these methods has not yet been confirmed [2,22], although a new diagnostic method using the leukocyte esterase method was reported [23].

Recently, a PCR method has been reported to have a high sensitivity to detect the bacterial DNA in ascites [24,25]. However, a small amount of bacteria is suggested to invade into the peritoneal cavity of cirrhotic patients (so-called "bacterial translocation"), and the PCR method detects bacterial genomic DNA in about 40% of non-SBP ascitic samples [24,25]. Therefore, the higher sensitivity of the PCR method in comparison to the ISH test might lead to a lower diagnostic specificity. The present study attempted to detect ingested bacterial DNA by the ISH method. Although the sensitivity of the ISH test was 91%, no positive signal was detected in the non-SBP samples, thus indicating the highly specific (100%) detection of the bacterial infection. The lower detective sensitivity of ISH than the PCR method may, in turn, result in a higher specificity, because a fair amount of bacterial organisms causing clinical symptom(s) are required to show positive results based on the findings of ISH.

Previous reports have shown some advantages of the ISH method in the diagnosis of sepsis. At first, ISH showed high diagnostic sensitivity and the results were not affected by contamination [9]. In addition, ISH could be used for the diagnosis of septic patients receiving antibacterial treatment [11]. Consistent with the former advantage, the present data showed no false positive results in 40 non-SBP samples, suggesting that positive signals in SBP samples are not affected by contamination. The present study excluded patients treated with antibiotics, and, therefore, it was not possible to determine whether the latter advantage of ISH was also observed in SBP cases. However, there was one patient, who was diagnosed as SBP by the clinical manifestations and transferred to our hospital after the administration of antibiotics (one excluded case described in "Patients and methods"). The ISH test showed a positive result despite the low leukocyte count in the ascitic fluid (170/μl), implying the usefulness of ISH even after the initiation of the antibiotic treatment (Supplementary Table 1; patient A). These results suggest that several diagnostic merits of ISH in septic cases could be also applied to SBP patients. Since it is hypothesized that bacterial inflammation in ascites may induce episodes of systemic bacterial circulation, the ISH tests which were performed using the blood of SBP patients could provide useful information about the patients' status.

On the other hand, the ISH test fails to determine the *in vitro* susceptibility of causative organisms and this could be a problem with this method. However, the early detection of enterobacteria by the EK probe in the three cases of SBP (Nos. 2, 4, and 9) allowed for appropriate empirical treatments to be continued and, as a result, the patients recovered. Case No. 1 had a past history of severe acute cholecystitis, and had been treated with antibiotics for an extended period (over 2 weeks) until one month before the onset of SBP. This clinical information, in addition to the positive signals of SA probe (*S. aureus*), allowed the assumption of a Methicillin-resistant *S. aureus* infection. In the present study, we performed the ISH test with all probes (GB, SA, SE, EF, PA, and EK) simultaneously, and obtained positive signals of the SA probe within one day. One major advantage of the ISH test would be its orientation toward non-Gram-negative bacteria (including MRSA) which third-generation cephalosporins do not cover. Despite the lack of information on drug susceptibility, these cases suggested some clinical advantages of the ISH test, in comparison to the cases diagnosed only by the PMN counts without the information about the pathogen(s).

The ISH test showed positive signals in about 40% of the septic bloody samples in our previous study, whereas a higher sensitivity (91%) was obtained in the ascitic samples of the SBP patients in the present study. Since positive signals were detected in only 37% (4/11) cases by the previously established five probes (Table 5), the GB probe positively contributed to an increased sensitivity by detecting broad-spectrum bacteria in the present study. However, the GB probe cannot identify the characteristics of the pathogen, irrespective of the high sensitivity for the detection of bacterial DNA. In two SBP cases (Nos. 6 and 7), the

ingested bacterial DNA was detected by the ISH method, but the types of bacteria were only identified by the culture method. Although the present study showed the probable usefulness of ISH to detect the causative organisms of SBP, new probes for more kinds of bacteria are required, because such information regarding the pathogen is clinically important.

Previous studies reported the culture-positive rate of SBP ascites to be high, namely ranging between 72% and 90% of cases [3–5]. On the other hand, the rate of culture-positive cases was obviously low in the present study despite the fact that ascitic fluid cultures were performed by the standard (culture-bottle) method. However, this low culture-positive rate of SBP ascites has been also observed in several reports, with a proportion between 39% and 59% [7,14,26,27]. In addition, a recent report mentioned that positive results of ascitic culture are obtained in about only 40% of SBP cases [28]. Fernandez *et al.* showed low culture-positive rate (39%: 54/138), and they suggested that the low rate probably depended on an earlier diagnosis of the infection [7]. The current results may also reflect the earlier diagnosis, although that does not fully explain the low positive rate of cultures. A clinical trial with a larger scale should be performed to evaluate this new method in detail.

In the present study, we did not determine whether the ISH test works in bacterascites (positive bacterial culture without an elevated PMN count), because there were no patients who met the criteria for this conditions. However, our experimental studies suggested that ingested bacterial DNA can be detected even in fluid samples with a low count of leukocytes. Indeed, as described above, the ISH test showed a positive result in one patient who was treated with antibiotics for clinical symptoms of SBP, although the count of ascitic leukocytes was low. In contrast, when we investigated three cirrhotic patients with peritonitis carcinomatosa who showed high counts of ascitic leukocytes, no positive signal was observed (Supplementary Table 1; patients B, C, and D). Therefore, we think that the results of the ISH test should provide some clinical advantages in comparison to the cases diagnosed by the PMN counts alone. Since the ISH test detects phagocytized bacteria in SBP ascites, the results may, therefore, be independent of the leukocyte count. Although our preliminary results suggest that the ISH method is sensitive in cases with a low number of PMN, it remains to be demonstrated, in another study, that our technique is highly sensitive in SPB cases with PMN ranging from 250 to 1000/μl.

In summary, SBP is an infectious disease that is often only diagnosed by the PMN count without detecting the causal bacteria. The present study demonstrated the efficacy of a new probe to detect the DNA of global bacteria and showed the utility of the ISH test for providing early evidence of a bacterial infection in SBP ascites. The ISH test may, therefore, be a novel and effective approach for the management of SBP.

Conflict of interest

S.I. and A.M. are employees of Fuso Pharmaceutical Industries. The remaining authors have no conflicts to disclose.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2011.06.025.

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ORIGINAL ARTICLE

Long-term interferon therapy after radiofrequency ablation is effective in treating patients with HCV-associated hepatocellular carcinoma

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Abstract
Purpose This study investigates the usefulness of long-term interferon (IFN) therapy following radiofrequency ablation (RFA) for HCV-associated hepatocellular carcinoma (HCC).
Methods This is a retrospective observational study. Patients underwent pegylated IFN-α/ribavirin combination therapy for 48 weeks and then were maintained on IFN-α administration on average for 68 weeks (mean total duration 116 weeks). Patients who underwent IFN monotherapy were maintained on IFN administration on average for 78 weeks.
Results There were biases in the background factors between the IFN and non-IFN groups. Therefore, a covariate adjustment was performed using the propensity score. An analysis of 20-matched patients from each group showed the 5-year cumulative survival rate was higher in the IFN group than in the non-IFN group (100 and 76%, respectively), and the 3-year cumulative recurrence rate was significantly lower in the IFN group than in the non-IFN group (38.0 and 64.2%, respectively). In 14 patients (i.e., IFN responders), the serum alanine aminotransferase (ALT) level remained normalized at 30 IU/mL or lower, regardless of disappearance of serum HCV RNA. In these patients, the cumulative recurrence rate was low, the hazard ratio was 0.158 (95% confidence interval = 0.045–0.561, $P = 0.004$), and the serum albumin level was retained.
Conclusion These results show the importance of maintaining the liver function and suggest that long-term IFN administration after RFA inhibits recurrence and contributes to an improved outcome in patients (in particular, IFN responders) who initially develop HCC.

Keywords Hepatitis C virus · Interferon · Hepatocellular carcinoma · Prevention · Radiofrequency ablation

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Introduction

More than 500 million people in the world are infected with hepatitis B and C viruses (HBV and HCV, respectively). Persistent infection with these hepatitis viruses is strongly associated with the development of hepatocellular carcinoma (HCC). The HCC patient is ranked fifth among cancer patients throughout the world and the number of deaths from HCC is ranked at third [1]. Its pathogenesis is being progressively elucidated; in most instances, HCC develops in patients who have a background of HBV- and HCV-induced chronic hepatitis and hepatic cirrhosis [2]. Advances in the early detection of and therapy for HCC have increasingly led to curative treatment. However, HCC is likely to recur and the incidences of intrahepatic metastasis and multicentric recurrences are high and are a

major problem in improving the treatment outcomes [3, 4]. There have been marked advancements recently in interferon (IFN) therapy for chronic hepatitis C. The incidence of HCC is, according to reports, significantly reduced in patients who achieved a sustained viral response (SVR) with IFN therapy and in patients for whom IFN therapy normalizes liver function. This indicates the importance of IFN therapy as a primary prevention against developing HCC [5–9]. IFN therapy after curative treatment of HCV-associated HCC is reportedly useful as secondary prevention against carcinogenesis [10–14]. There are reports that long-term IFN administration inhibits recurrence [15], but the evaluation of this has not been fully established [16]. In this study, after administering radical radiofrequency ablation (RFA), we performed long-term IFN therapy in patients with the initial development of HCV-associated HCC. We retrospectively investigated the recurrence-inhibitory effect of the treatment.

Patients and methods

Patients

Between April 2001 and March 2008, there were 226 patients who underwent RFA at the Division of Hepatobiliary and Pancreatic Diseases, Hyogo College of Medicine. Of these, 135 patients were negative for the HBs antigen and positive for HCV RNA, with tumor diameter $\leq$3 cm, with three or fewer tumors, and underwent RFA alone for the initial development of HCC. These 135 patients were selected for subjects. There were 71 male and 64 female patients who ranged from 39 to 85 years old (the median age was 68 years). The background liver was clinically and histologically evaluated. HCC was diagnosed using abdominal ultrasonography, dynamic computed tomography (CT), and magnetic resonance imaging (MRI). Markedly enhanced tumors were noted in the early phase of contrast imaging on CT or MRI in all patients and washout was observed in the portal phase or late phase 3 min after injection of the contrast medium. RFA was performed with ultrasonographic guiding using the Cool-tip Radiofrequency Ablation system (Tyco Healthcare Group LP, Burlington, MA). The lesions were evaluated by dynamic CT or MRI 1 and 8 weeks after RFA. The treatment was considered markedly effective when the intensely stained tumors disappeared and a treated area was sufficiently maintained.

IFN therapy was indicated for patients meeting the following conditions: (1) platelet count $\geq$ 70,000 μL^{-1}, (2) white blood cell count $\geq$ 1,500 μL^{-1}, (3) hemoglobin $\geq$ 10 g/dL, (4) Child-Pugh classification stage A, and (5) age $\leq$ 80 years. IFN therapy was performed in 20 patients for whom RFA was markedly effective. Nineteen of 20 patients requested IFN therapy and met the criteria. One patient with Child-Pugh classification stage B underwent IFN therapy because of the patient's strong desire for this therapy. One hundred and fifteen patients did not undergo IFN therapy. Fifty-one of 115 patients did not meet the criteria. IFN therapy was not performed in 64 patients because of their unwillingness to undergo this therapy (despite meeting the indications for treatment) or because ursodeoxycholic acid or strong neominophagen C were being administered instead of IFN because the patients had complications such as hypertension and diabetes (Table 1).

Since this was a retrospective study, covariate adjustment using the propensity score was performed to adjust for between-group confounding factors [17, 18]. The following ten factors were adopted as matching covariates: age, gender, platelet count, serum alanine aminotransferase (ALT) level, α-fetoprotein (AFP) level, protein induced by vitamin K absence or antagonist-II (PIVKA-II) level, Child-Pugh classification, tumor diameter, number of tumors, and the Japanese tumor, node, and metastasis (TNM) stage [19]. Based on the results of the adjustments, 20 patients with IFN therapy and 20 patients without IFN therapy (i.e., IFN and non-IFN groups, respectively) were assessed. Recurrence of HCC was classified as a local tumor progression or as an ectopic recurrence. A local tumor progression was defined as recurrence in an area previously treated with RFA and an ectopic recurrence was defined as a recurrence outside this area.

IFN therapy

IFN therapy that was initiated 1 month after RFA was markedly effective in treating the initial HCC. In the IFN group, 19 patients had the genotype 1b and a high viral load. In Japan, pegylated IFN-α/ribavirin (PEG-IFN/RBV) combination therapy was approved at the end of 2004, and we have planned to give PEG-IFN/RBV combination therapy since 2005. IFN therapy was administered after requests from individual patients who gave informed consent. Twelve patients underwent PEG-IFN/RBV combination therapy. The therapy regimen consisted of PEG-IFN-α-2b (Peg-Intron, Schering Plough Corporation, Kenilworth, NJ) administered at a dose of 1.5 μg/kg/week; PEG-IFN-α-2a (Pegasys, Hoffman La Roche, Nutley, NJ) administered at a dose of 180 μg/week; and RBV administered for 48 weeks at a dose based on body weight (600 mg for $\leq$60 kg, 800 mg for 60–80 kg, and 1,000 mg for >80 kg). Two of the 12 patients achieved SVR, which was defined as the persistent negative conversion of serum HCV RNA after the 24th week of IFN therapy. After the combination therapy, nine patients underwent IFN-α (Sumiferon, Dainippon Sumitomo Pharma Co., Ltd.,

Table 1 Demographic and baseline characteristics of the patients

Variable	IFN group	Non-1 FN group	*P*
Patients (*n*)	20	115	
Age (range), years	65 (52–76)	67 (39–85)	0.06
Gender (*n*)			0.04
Male	15	56	
Female	5	59	
White blood count ($\times 10^2$ μL^{-1})	40 (38, 50)	37 (28, 46)	0.08
Hemoglobin (g/dL)	12.9 (11.7, 13.9)	12.8 (11.3, 13.5)	0.35
Platelet count ($\times 10^4$ μL^{-1})	9.7 (8.7, 11.9)	9.7 (8.0, 12.2)	0.36
Prothrombin activity (%)	80.9 (79.5, 88.9)	81.9 (77.5, 84.2)	0.80
Total bilirubin (mg/dL)	0.8 (0.6, 1.2)	0.9 (0.5, 1.2)	0.51
Albumin level (g/dL)	3.9 (3.5, 4.1)	3.7 (3.3, 3.9)	0.006
AST (IU/L)	60 (33, 82)	57 (45, 86)	0.42
ALT (IU/L)	50 (37, 83)	53 (39, 72)	0.71
AFP (ng/mL)	11 (6, 41)	27 (6, 79)	0.03
PIVKA-II (mAU/mL)	25 (17, 60)	37 (21, 137)	0.95
Child-Pugh classification (*n*)			0.41
A	19	96	
B	1	18	
C	0	1	
Tumor size (mm)	21 (17, 24)	20 (19, 29)	0.75
Number of tumors (*n*)			0.17
Solitary	18	88	
Multiple	2	27	
Japanese TNM stage (*n*)			0.044
I	6	43	
II	13	45	
III	1	27	
Follow-up period (month)	37 (19, 57)	31 (17, 69)	0.13

Except where indicated, data are expressed as the median (25th, 75th percentile)

Osaka, Japan) monotherapy (300 MU, 3 times/week) for a mean duration of 68 weeks (mean total duration 116 weeks).

Eight patients underwent IFN monotherapy. One patient had the genotype 2a, one patient had the hemoglobin of 11.1 g/dL and six patients had low levels of the platelet count (<100,000 μL^{-1}). In accordance with Japan package insert of PEG-IFN-α-2b, we planned to undertake IFN monotherapy in eight patients on enough informed consent. Treatment was initiated with PEG-IFN-α-2a monotherapy (90 μg/week or 90 μg/2 weeks) in five patients, and IFN-α monotherapy (300–600 MU, 3 times/week) was initiated in three patients. The therapies were continued in these patients, except for one patient who ultimately achieved SVR. The mean duration of PEG-IFN-α-2a or IFN-α monotherapy was 78 weeks.

Follow-up procedure

After RFA for HCC, liver function testing and tumor marker measurement (AFP, PIVKA-II) were performed every month in all patients. Abdominal ultrasonography was performed every 3 months and dynamic CT or MRI was performed every 6 months. Histological examination was performed on suspected hypovascular HCC using fine-needle aspiration biopsy. When a recurrence occurred, the patient was admitted for appropriate therapy.

Statistical analysis

For between-group comparisons, the Mann–Whitney *U* test was used for continuous variables and the Chi-square and Fisher exact tests were used for categorical variables. The survival time and HCC recurrence were evaluated using the Kaplan–Meier method and between-group comparisons were performed using the log-rank test. The influence of IFN administration on recurrence after RFA was investigated using the Cox proportional hazards model. Covariate adjustment was performed using the propensity score, as previously reported [17, 18]. The *C*-statistic (ROCKIT software, Kurt Rossmann Laboratories, University of Chicago, Chicago, IL) [20] was used for the goodness-of-fit

index of patients matched using the propensity score. Analysis was performed using statistical software SPSS ver. 16.0 (SPSS, Inc., Chicago, IL) and SAS ver. 9.13 (SAS Institute, Inc., Cary, NC). A two-sided P value of less than 5% was regarded as significant.

Results

The virological and adverse effects of IFN therapy

Two of the 19 patients with the genotype 1b who had a high viral load and one patient with the genotype 2a achieved SVR. HCV RNA did not become negative in 14 patients. HCV RNA disappeared in the remaining three patients, but assessing SVR was difficult since the patients were undergoing IFN therapy. Normalization of the serum ALT level to 30 IU/mL or lower occurred in 11 of the 17 non-SVR patients. Since there were no severe adverse events, IFN therapy was continued in 12 patients who underwent PEG-IFN/RBV combination therapy and in eight patients who underwent IFN monotherapy. IFN therapy was suspended due to recurrence of HCC in two patients who underwent IFN monotherapy, but the treatment was resumed after a second RFA treatment in the patients. In the non-IFN group, normalization of the serum ALT level to 30 IU/mL or lower did not occur in any patient.

Recurrence of HCC

After radical RFA in 135 patients, the 1- and 3-year cumulative recurrence rates were 5.0 and 38.0%, respectively, in the IFN group, and 25.0 and 68.6%, respectively, in the non-IFN group. The lower rate in the IFN group was statistically significant ($P = 0.007$). A comparison of the

Table 2 Demographic and baseline characteristics of the matched patients

Variable	IFN group	Non-IFN group	*P*
Patients (*n*)	20	20	
Age (range), years	66 (52–76)	67 (56–73)	0.30
Gender (*n*)			1.00
Male	15	15	
Female	5	5	
White blood count ($\times 10^2\ \mu L^{-1}$)	40 (38, 50)	39 (29, 46)	0.14
Hemoglobin (g/dL)	12.9 (11.7, 13.9)	13.3 (11.9, 14.2)	0.67
Platelet count ($\times 10^4\ \mu L^{-1}$)	9.7 (8.7, 11.9)	10.9 (7.8, 14.6)	0.49
Prothrombin activity (%)	80.9 (79.4, 88.9)	83.8 (74.3, 90.1)	0.89
Total bilirubin (mg/dL)	0.8 (0.6, 1.2)	1.0 (0.7,1.2)	0.30
Albumin level (g/dL)	3.9 (3.5, 4.1)	3.7 (3.3,4.0)	0.08
AST (IU/L)	60 (33, 82)	62 (41, 87)	0.19
ALT (IU/L)	50 (37, 83)	52 (35, 67)	0.69
AFP (ng/mL)	11 (6, 41)	18 (8, 69)	0.29
PIVKA-II (mAU/mL)	25 (17, 61)	33 (18, 47)	0.75
Child-Pugh classification (*n*)			1.00
A	19	20	
B	1	0	
Tumor size (mm)	21 (17, 24)	21 (18, 26)	0.67
Number of tumors (*n*)			1.00
Solitary	18	18	
Multiple	2	2	
Japanese TNM stage (*n*)			0.46
I	6	9	
II	13	9	
III	1	2	
HCV genotype			1.00
1 b	19	18	
2a	1	2	
Viral load (kIU/mL)	900 (370, 1,600)	850 (270, 1,250)	0.76
Follow-up period (month)	37 (19, 57)	28 (17, 39)	0.24

Except where indicated, data are expressed as the median (25th, 75th percentile)