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

Feature	Total (n=547)	HBV genotypes			
		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).

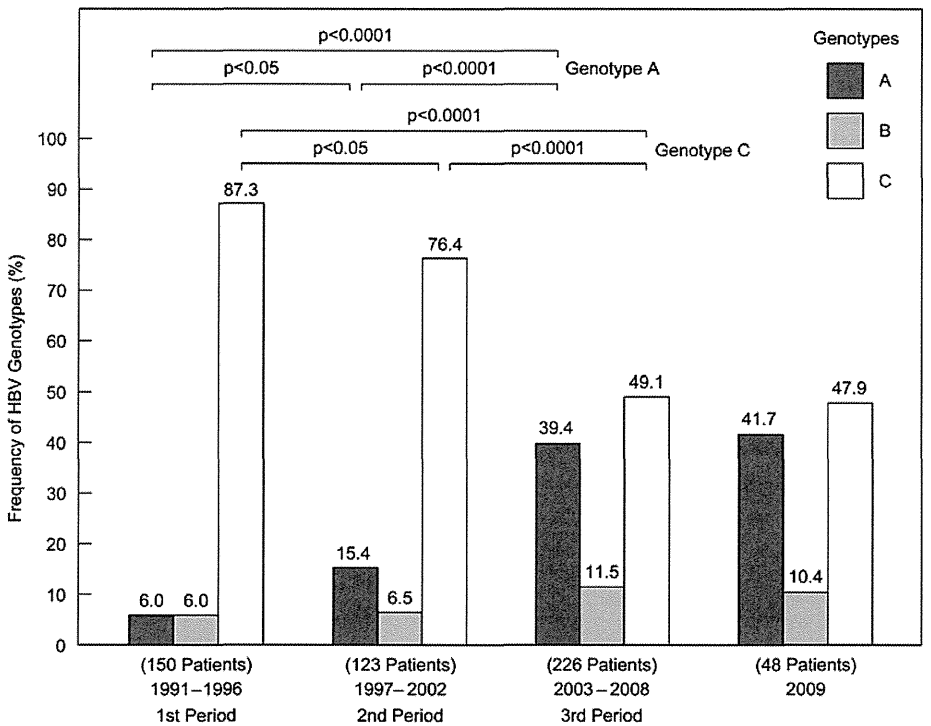

Figure 2 Distribution of hepatitis B virus (HBV) genotypes in three periods.

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.
*$p<0.05$, ***$p<0.0001$.

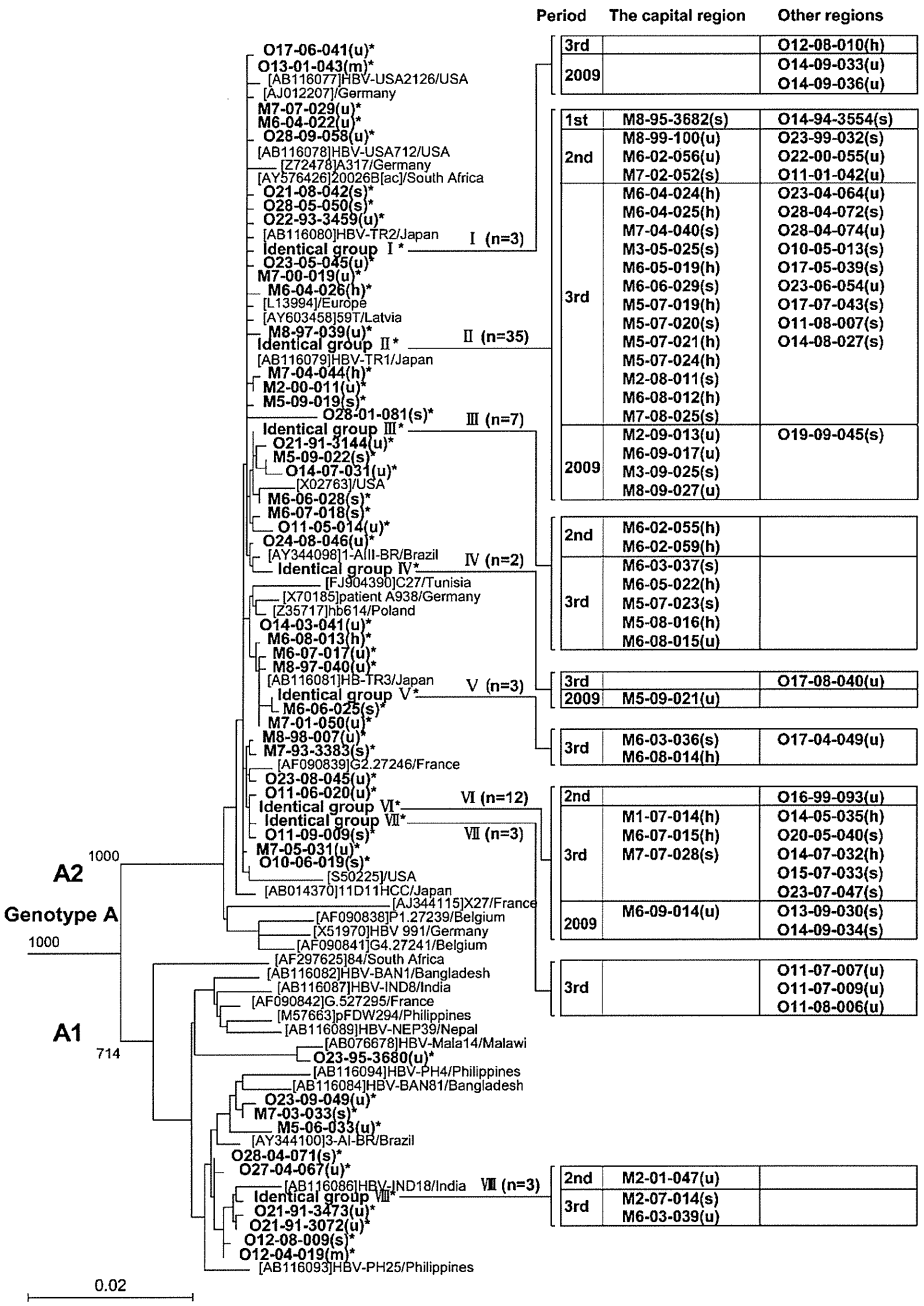

Figure 3 Phylogenetic analysis of genotype A strains by the neighbour-joining method. Isolates obtained in this study are shown in bold with asterisks. Hospitals in the capital region are labelled M1–M8 and those in other regions O9–O28 (corresponding to those in figure 1). Year of onset is indicated 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

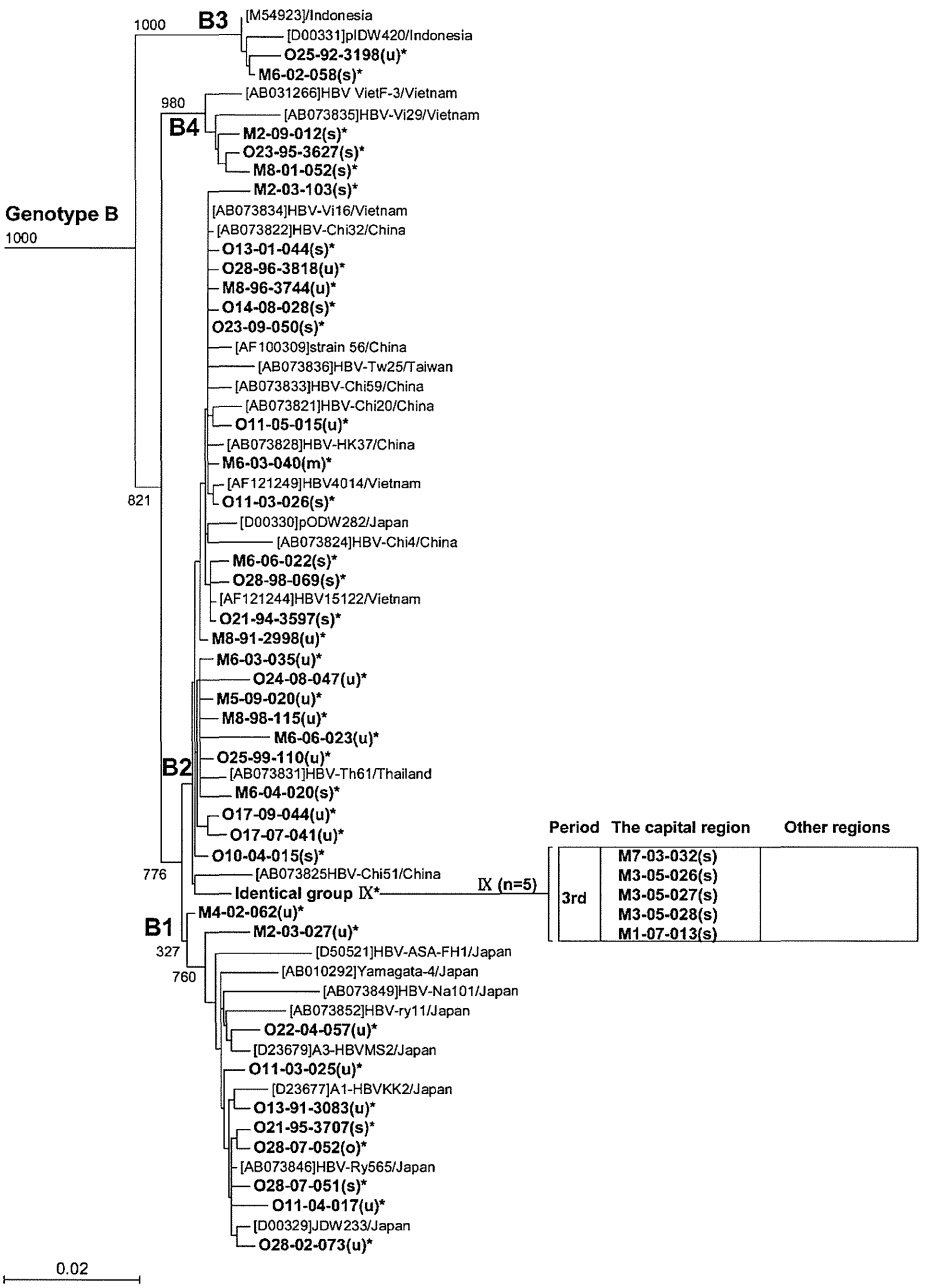

Figure 4 Phylogenetic analysis of genotype B strains by the neighbour-joining method. Hepatitis B virus (HBV) isolates obtained in the present study are specified in the same manner as in figure 3, and isolates with an identical sequence are bracketed in 'Identical group IX' on the tree. Of them, 10 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]

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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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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REFERENCES

1. **Arauz-Ruiz P,** Norder H, Robertson BH, *et al*. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 2002;**83**:2059–73.
2. **Norder H,** Courouce AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology* 1994;**198**:489–503.
3. **Okamoto H,** Tsuda F, Sakugawa H, *et al*. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* 1988;**69**:2575–83.
4. **Olinger CM,** Jutavijittum P, Hubschen JM, *et al*. Possible new hepatitis B virus genotype, southeast Asia. *Emerg Infect Dis* 2008;**14**:1777–80.
5. **Stuyver L,** De Gendt S, Van Geyt C, *et al*. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* 2000;**81**:67–74.
6. **Tran TT,** Trinh TN, Abe K. New complex recombinant genotype of hepatitis B virus identified in Vietnam. *J Virol* 2008;**82**:5657–63.
7. **Tatematsu K,** Tanaka Y, Kurbanov F, *et al*. A genetic variant of hepatitis B virus divergent from known human and ape genotypes isolated from a Japanese patient and provisionally assigned to new genotype J. *J Virol* 2009;**83**:10538–47.

8. **Chu CJ,** Lok AS. Clinical significance of hepatitis B virus genotypes. *Hepatology* 2002;**35**:1274–6.
9. **Kao JH.** Hepatitis B viral genotypes: clinical relevance and molecular characteristics. *J Gastroenterol Hepatol* 2002;**17**:643–50.
10. **Miyakawa Y,** Mizokami M. Classifying hepatitis B virus genotypes. *Intervirology* 2003;**46**:329–38.
11. **Kimbi GC,** Kramvis A, Kew MC. Distinctive sequence characteristics of subgenotype A1 isolates of hepatitis B virus from South Africa. *J Gen Virol* 2004;**85**:1211–20.
12. **Kramvis A,** Weitzmann L, Owiredu WK, *et al*. Analysis of the complete genome of subgroup A' hepatitis B virus isolates from South Africa. *J Gen Virol* 2002;**83**:835–9.
13. **Sugauchi F,** Kumada H, Acharya SA, *et al*. Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. *J Gen Virol* 2004;**85**:811–20.
14. **Sugauchi F,** Kumada H, Sakugawa H, *et al*. Two subtypes of genotype B (Ba and Bj) of hepatitis B virus in Japan. *Clin Infect Dis* 2004;**38**:1222–8.
15. **Sugauchi F,** Orito E, Ichida T, *et al*. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. *Gastroenterology* 2003;**124**:925–32.
16. **Tanaka Y,** Orito E, Yuen MF, *et al*. Two subtypes (subgenotypes) of hepatitis B virus genotype C: a novel subtyping assay based on restriction fragment length polymorphism. *Hepatol Res* 2005;**33**:216–24.
17. **Akuta N,** Suzuki F, Kobayashi M, *et al*. The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. *J Hepatol* 2003;**38**:315–21.
18. **Tanaka Y,** Hasegawa I, Kato T, *et al*. A case-control study for differences among hepatitis B virus infections of genotypes A (subtypes Aa and Ae) and D. *Hepatology* 2004;**40**:747–55.
19. **Orito E,** Ichida T, Sakugawa H, *et al*. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology* 2001;**34**:590–4.
20. **Kobayashi M,** Arase Y, Ikeda K, *et al*. Viral genotypes and response to interferon in patients with acute prolonged hepatitis B virus infection of adulthood in Japan. *J Med Virol* 2002;**68**:522–8.
21. **Ogawa M,** Hasegawa K, Naritomi T, *et al*. Clinical features and viral sequences of various genotypes of hepatitis B virus compared among patients with acute hepatitis B. *Hepatol Res* 2002;**23**:167–77.
22. **Takeda Y,** Katano Y, Hayashi K, *et al*. Difference of HBV genotype distribution between acute hepatitis and chronic hepatitis in Japan. *Infection* 2006;**34**:201–7.
23. **Yotsuyanagi H,** Okuse C, Yasuda K, *et al*. Distinct geographic distributions of hepatitis B virus genotypes in patients with acute infection in Japan. *J Med Virol* 2005;**77**:39–46.
24. **Kato H,** Orito E, Sugauchi F, *et al*. Frequent coinfection with hepatitis B virus strains of distinct genotypes detected by hybridization with type-specific probes immobilized on a solid-phase support. *J Virol Methods* 2003;**110**:29–35.
25. **Orito E,** Mizokami M, Sakugawa H, *et al*. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. *Hepatology* 2001;**33**:218–23.
26. **Kato H,** Orito E, Gish RG, *et al*. Hepatitis B e antigen in sera from individuals infected with hepatitis B virus of genotype G. *Hepatology* 2002;**35**:922–9.
27. **Sugiyama M,** Tanaka Y, Sakamoto T, *et al*. Early dynamics of hepatitis B virus in chimeric mice carrying human hepatocytes monoinfected or coinfected with genotype G. *Hepatology* 2007;**45**:929–37.
28. **Koyama T,** Matsuda I, Sato S, *et al*. Prevention of perinatal hepatitis B virus transmission by combined passive-active immunoprophylaxis in Iwate, Japan (1981–1992) and epidemiological evidence for its efficacy. *Hepatol Res* 2003;**26**:287–92.
29. **Noto H,** Terao T, Ryou S, *et al*. Combined passive and active immunoprophylaxis for preventing perinatal transmission of the hepatitis B virus carrier state in Shizuoka, Japan during 1980–1994. *J Gastroenterol Hepatol* 2003;**18**:943–9.
30. **Tanaka J,** Kumagai J, Katayama K, *et al*. Sex- and age-specific carriers of hepatitis B and C viruses in Japan estimated by the prevalence in the 3,485,648 first-time blood donors during 1995–2000. *Intervirology* 2004;**47**:32–40.
31. **Daniels D,** Grytdal S, Wasley A. Surveillance for acute viral hepatitis—United States, 2007. *MMWR Surveill Summ* 2009;**58**:1–27.
32. **Goldstein ST,** Alter MJ, Williams IT, *et al*. Incidence and risk factors for acute hepatitis B in the United States, 1982–1998: implications for vaccination programs. *J Infect Dis* 2002;**185**:713–19.
33. **Sherlock S.** The natural history of hepatitis B. *Postgrad Med J* 1987;63 Suppl 2:7–11.
34. **Mayerat C,** Mantegani A, Frei PC. Does hepatitis B virus (HBV) genotype influence the clinical outcome of HBV infection? *J Viral Hepat* 1999;**6**:299–304.
35. **Hayashi K,** Katano Y, Takeda Y, *et al*. Association of hepatitis B virus subgenotypes and basal core promoter/precore region variants with the clinical features of patients with acute hepatitis. *J Gastroenterol* 2008;**43**:558–64.
36. **Ozasa A,** Tanaka Y, Orito E, *et al*. Influence of genotypes and precore mutations on fulminant or chronic outcome of acute hepatitis B virus infection. *Hepatology* 2006;**44**:326–34.
37. **Matsuura K,** Tanaka Y, Hige S, *et al*. Distribution of hepatitis B virus genotypes among patients with chronic infection in Japan shifting toward an increase of genotype A. *J Clin Microbiol* 2009;**47**:1476–83.
38. **Bodsworth NJ,** Cooper DA, Donovan B. The influence of human immunodeficiency virus type 1 infection on the development of the hepatitis B virus carrier state. *J Infect Dis* 1991;**163**:1138–40.
39. **Li JS,** Tong SP, Wen YM, *et al*. Hepatitis B virus genotype A rarely circulates as an HBe-minus mutant: possible contribution of a single nucleotide in the precore region. *J Virol* 1993;**67**:5402–10.
40. **Suzuki Y,** Kobayashi M, Ikeda K, *et al*. Persistence of acute infection with hepatitis B virus genotype A and treatment in Japan. *J Med Virol* 2005;**76**:33–9.
41. **Sugauchi F,** Orito E, Ohno T, *et al*. Spatial and chronological differences in hepatitis B virus genotypes from patients with acute hepatitis B in Japan. *Hepatol Res* 2006;**36**:107–14.
42. **Norder H,** Courouce AM, Coursaget P, *et al*. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology* 2004;**47**:289–309.

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HEPATOLOGY

Long-term follow-up of patients with hepatitis B e antigen negative chronic hepatitis B

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HBe antibody, hepatitis B virus, long-term follow-up.

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Abstract

Background and Aim: After hepatitis B virus (HBV) e antigen (HBeAg) seroconversion, HBV-DNA continues to replicate, and HBeAg-negative patients still face the risk of liver disease progression. We investigated the predictive factors for alanine aminotransferase (ALT) elevation, antiviral drug use, and hepatocellular carcinoma (HCC) occurrence in HBeAg-negative patients.

Methods: Age, sex, ALT, platelet counts, HBV-DNA levels, genotype, antidiabetic drug use, body mass index, smoking, and alcohol consumption were analyzed for a total of 244 HBV carriers who were HBeAg-negative.

Results: Of 244 HBeAg-negative patients, 158 (64.8%) showed normal ALT levels at baseline. Multivariate Cox hazard regression analysis identified high HBV-DNA levels and high ALT at baseline as independent risk factors for ALT elevation in the patients with normal ALT at baseline. The threshold ALT and HBV-DNA levels were determined to be 31 IU/L and 5.3 logcopies/mL, respectively. Seventeen (7.0%) patients used antiviral drugs. Multivariate Cox hazard regression analysis identified high HBV-DNA levels (threshold, 5.7 log copies/mL), the use of antidiabetic drugs, and daily alcohol consumption at baseline as an independent risk factor for the use of antiviral drugs in HBeAg-negative patients. In 10 patients (4.1%), HCC was detected, and a low platelet count (threshold, $10.0 \times 10^4/mm^3$) was associated with the occurrence of HCC.

Conclusion: This study identified predictors of future active liver disease in HBeAg-negative patients, i.e. ALT elevation, unavoidable use of antiviral drugs, and occurrence of HCC.

Introduction

Chronic hepatitis caused by hepatitis B virus (HBV) often follows a fluctuating course characterized by periods of active hepatitis interspersed with quiescence. Therefore, close follow-up is necessary to understand the natural history of HBV patients. On the other hand, patients in which HBV is truly inactive have persistently quiescent disease with an excellent prognosis. Determining an accurate prognosis for HBV carriers based on clinical presentation is important for clinical management of the disease. Various studies have been performed to distinguish the positive and negative prognostic factors among HBV carriers.[1–3]

Hepatitis e antigen (HBeAg) seroconversion is an important event in the natural history of HBV infection. HBV-infected patients usually have a very good prognosis after HBeAg seroconversion.[4] Therefore, HBeAg seroconversion has become an important treatment goal during follow-up of HBV carriers.[5] However, it has also been shown that HBV-DNA replication and hepatic inflammation in seroconverted patients continue despite the persistent loss of HBeAg; thus, HBeAg-negative patients are likely to develop liver cirrhosis or hepatocelluar carcinoma.[6] In this study, we focused on the natural history of patients with HBeAg-negative chronic hepatitis B, particularly with respect to alanine aminotransferase (ALT) elevation, antiviral drugs, and hepatocellular carcinoma (HCC).

Recently, prognostic factors for HBeAg-negative patients have been investigated in Taiwan and Canada.[7,8] We expect to identify a unique constellation of prognostic factors for HBeAg-negative chronic hepatitis B in the Japanese population, due to differences in race and HBV genotype.

Methods

Patients

Between January 1985 and April 2007, all patients visiting the Chiba University Hospital with HBV infection were approached for participation in the study. This study was carried out only at

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one institute, Chiba University Hospital and was approved by ethical the committee of Chiba University. Written informed consent was obtained from all of the patients in accordance with the Declaration of Helsinki. New patients since 1985 and those who were already being followed-up in 1985 were eligible for inclusion in the study. A total of 881 patients were enrolled; of which, 862 were HBsAg positive at enrollment, and 319 were hepatitis B e antibody (HBeAb) positive. Patients who were positive for hepatitis C virus antibody or hepatitis D virus antibody or who had other potential cause of chronic liver diseases (autoimmune hepatitis, primary biliary cirrhosis) were excluded. Patients followed for less than 12 months were also excluded from the analysis. In total, 244 patients were included in the analysis. Serum samples from patients were stored at –20°C and the oldest sample for each patient was used for defining the level of HBV-DNA. The date of evaluation of HBV DNA level by PCR was defined as the baseline. Patient consent was obtained for storage and analysis of serum samples.

Laboratory methods

Serum ALT level was measured using a routine automated method. HBeAg and HBeAb were measured by standard enzyme-linked immunosorbent assays. Patients were screened for hepatitis C virus, hepatitis delta virus, and human immunodeficiency virus antibodies by a third-generation enzyme-linked immunosorbent assay.

HBV-DNA quantitative assay and genotyping

To investigate the level of HBV-DNA in serum, we chose polymerase chain reaction (PCR) assay with an accurate range of 500–200 000 copies/mL (Amplicor HBV monitor test, Roche Diagnostic Systems, Basel, Switzerland). The six major genotypes of HBV (A–F) were determined by enzyme-linked immunosorbent assay (ELISA) (HBV Genotype EIA, Institute of Immunology, Co., Ltd, Tokyo, Japan).

Statistical analysis

ALT elevation was defined as a change from normal ALT (< 42 IU/L) to elevated ALT (≥ 42 IU/L), and normalization was defined as a change from elevated ALT to normal from one visit to the next. Baseline data are presented as mean ± standard deviation (SD). Differences in clinical parameters between groups were analyzed by unpaired *t*-test, Welch *t*-test, and χ^2 tests. The Cox proportional hazards model was used to identify predictive factors for future ALT elevation/normalization, use of antviral drugs, and HCC occurrence using SPSS version 16.1 software (SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics

To investigate the natural course of HBV carriers with HBeAb, 244 carriers (HBeAg-negative and HBeAb-positive) were enrolled in the study. Follow-up was terminated when the use of antiviral drugs was started or the occurrence of HCC. The baseline clinical and virological characteristics of the 244 HBeAg-negative carriers are shown in Table 1. Because liver biopsy was performed only in 44 (18.0%) out of 244 patients, liver biopsy results could not be analyzed further. Age, sex, ALT, platelet count (PLT), HBV-DNA level, genotype, antidiabetic drug use, body mass index, smoking, and alcohol consumption were analyzed. The average (± SD) period of follow-up was 103.6 ± 74.8 months. Seventeen (7.0%) patients used antiviral drugs (lamivudine in eight and entecavir in nine) and HCC was detected in 10 (4.1%) patients. Two (0.82%) patients died of HCC. In addition, one died of intrahepatic cholangiocarcinoma, one of liver failure due to gastrointestinal bleeding, and one of tongue cancer during the follow-up period. In Japan, the majority of HBV cases are genotype C and B and these genotypes do not cause HBV carrier by way of horizontal infection in adults; therefore, the HBV infection in our HBV carriers mainly occurred by vertical infection or infection during childhood.[9] Thus, the period of HBV infection roughly coincided with the age of HBV carriers in Japan.

ALT and HBV-DNA levels

One hundred and fifty-eight of 244 (64.8%) HBeAg-negative patients had normal ALT levels at baseline. Of these 158 subjects, 85 (53.8%) continued to have normal ALT levels during follow-up, whereas 73 (46.2%) showed fluctuation of ALT levels with intermittently elevated ALT (Fig. 1). A total of 34 (21.5%) patients had ALT ≥ 84 IU/L (more than double the normal limit). Of the 86 patients who had elevated ALT levels at baseline, ALT elevation persisted in 10 (11.6%) and 76 (88.4%) showed ALT fluctuations with intermittently elevated ALT. Although HBV-DNA levels were associated with higher ALT levels in general, correlation was weak ($r^2 = 0.13$).

Platelet count

Patients were sub classified based on PLT as follows: (I) < 100 000 (II) 100 000–149 000 (III) 150 000–199 000 (IV) 200 000–249 000 (V) > 250 000 and more (/mm^3). The numbers of patients in groups I, II, III, IV, and V were 17, 28, 73, 68, and 58, respectively. A total of 84 (34.4%) patients reached a lower platelet count at the end of follow-up.

Risk factors for future ALT elevation in patients with normal ALT levels

Although 158 (64.8%) out of 244 HBeAb-positive patients had normal ALT levels at baseline, 73 patients showed fluctuation of ALT levels with intermittently elevated ALT. We investigated the risk factors for future ALT elevation in these patients. The predictive factors of ALT elevation (ALT > 42 IU/L) in patients with normal ALT levels were HBV-DNA and ALT levels at baseline (Table 2). We carried out an additional univariate analysis changing the threshold of HBV DNA from 3.5 to 7.0 log copies/mL in 0.1 log increments and that of ALT from 15 to 41 IU/L in 1.0 increments. We determined the threshold when the value of probability was smallest; the thresholds for ALT and HBV-DNA levels were 31 IU/L and 5.3 logcopies/mL, respectively. The time

Table 1 Baseline characteristics of hepatitis B virus (HBV) e antigen (HBeAg)-negative patients

	Total	Normal ALT	Elevated ALT	*P*
Number	244	158	86	
Age(years) : (mean ± SD)	44.1 ± 12.5	44.1 ± 13.1	44.0 ± 11.4	NS*
<30	35 (14.3%)	24 (15.2%)	11 (12.8%)	
30–39	52 (21.3%)	32 (20.3%)	20 (23.2%)	
40–49	66 (27.0%)	44 (27.8%)	22 (25.6%)	
50–	91 (37.3%)	58 (36.7%)	33 (38.4%)	
Sex				<0.001**
Male	141 (57.8%)	76 (48.1%)	66 (75.9%)	
Female	103 (42.2%)	82 (51.9%)	21 (24.1%)	
Alanine aminotransferase (ALT) (IU/L) (mean ± SD)	58.9 ± 108.1	20.9 ± 8.7	127.9 ± 160	<0.001*
<20	84			
21–30	47			
31–40	27			
42–84	47			
85–	39			
Platelet count ($\times 10^4/mm^3$) (mean ± SD)	205.5 ± 69.6	211.4 ± 60	193.3 ± 81.8	NS*
HBV-DNA (log copies/mL) (mean ± SD)	4.3 ± 1.5	3.8 ± 1.1	5.1 ± 1.7	<0.001*
<4.0	116 (47.5%)	91 (57.6%)	25 (29.1%)	
4.0–4.9	54 (22.1%)	38 (24.1%)	16 (18.6%)	
5.0–5.9	27 (11.1%)	18 (11.4%)	9 (10.5%)	
6.0–6.9	26 (10.7%)	5 (3.2%)	21 (24.4%)	
7.0–	16 (6.6%)	3 (1.9%)	13 (15.1%)	
Genotype				NS**
A	3 (1.2%)	2 (1.3%)	1 (1.2%)	
B	30 (12.3%)	16 (10.1%)	14 (16.3%)	
C	87 (35.7%)	49 (31%)	38 (44.2%)	
Not detected	124 (50.8%)	91 (57.6%)	33 (38.4%)	
Liver Histology ($n = 44$)				
Fibrosis 4/3/2/1	7/8/9/20	0/1/4/13	7/7/5/7	NS**
Activity 3/2/1	7/16/21	1/4/13	6/12/8	NS**
Use of anti-Diabetes drug	20 (8.2%)	3 (1.9%)	6 (7.0%)	NS**
Body mass index (kg/m^2) (mean ± SD)	23.3 ± 3.3	23.1 ± 3.2	24.0 ± 3.5	NS**
Smoker/ ever smoker/ non-smoker	32/15/89	16/5/56	16/10/33	NS**
Daily alcohol consumption	46 (27.1%)	24 (23.1%)	22 (33.3%)	NS**
Follow-up (months) (mean ± SD)	103.6 ± 74.8	109.5 ± 76.1	101.8 ± 74.6	NS*

*Unpaired *t*-test and **χ^2 test. NS, not significant difference.

interval from a visit with a normal ALT to a visit with an elevated ALT was used for Kaplan–Meier and Cox regression analysis. Kaplan–Meier curves were constructed for ALT and HBV-DNA levels (Fig. 2).

Risk factors for future use of antiviral drugs for HBV in HBeAg-negative patients

Seventeen (7.0%) patients used an antiviral drug (lamivudine in 8 and entecavir in 9). We investigated the risk factors for future use of antiviral drugs for HBV. The time interval from baseline to the use of an antiviral drug for HBV was used for Cox regression analysis. HBV-DNA levels, use of antidiabetic drugs, and daily alcohol consumption were predictive of future antiviral drug use for HBV, according to the results of multivariate Cox hazard regression analysis. Hazard ratios for HBV-DNA levels, antidiabetic drug use, and daily alcohol consumption were 1.519 (1.130–2.042, 95% confidence interval [CI]), 3.769 (1.203–11.81), and 3.011 (1.086–8.348), respectively. We repeated the univariate analysis, changing the threshold for HBV DNA from 3.5 to 7.0 log copies/mL in 0.1 log increments. We determined the threshold when the probability value was lowest; the HBV-DNA threshold level was 5.7 log copies/mL. Kaplan–Meier curves were constructed for HBV-DNA levels, antidiabetic drug use, and daily alcohol consumption (Fig. 3).

Risk factors for hepatocellular carcinoma in HBeAg-negative patients

In 10 patients (4.1%), HCC was detected. We investigated the risk factors for HCC in HBeAg-negative patients. The time interval from baseline to occurrence of HCC was used for Cox regression analysis. According to the results of multivariate Cox regression analysis, PLT was predictive of the development of HCC. The hazard ratio for PLT was 0.807 (0.724–0.899, 95% CI). We performed univariate analyses, changing the PLT threshold from 8.0 to $30.0 \times 10^4/mm^3$ in $1.0 \times 10^4/mm^3$ increments. We determined the threshold when the value of probability was smallest; the

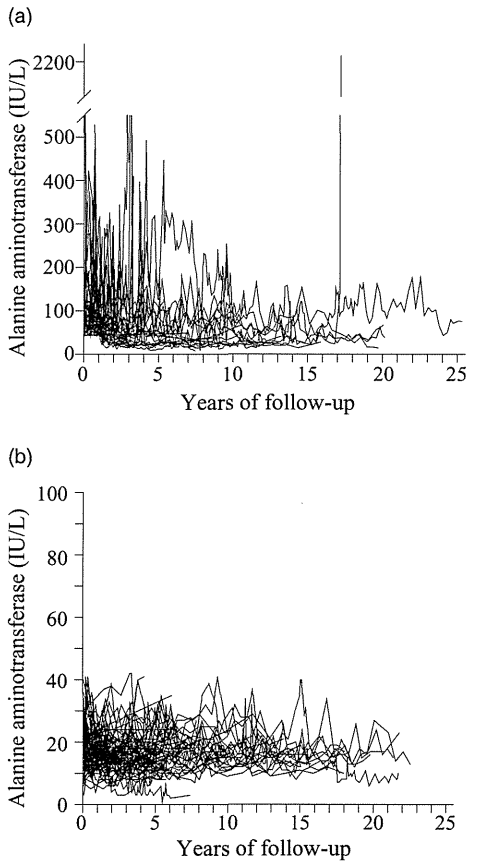

Figure 1 Level of alanine aminotransferase (ALT) in (a) patients with normal ALT at baseline and intermittently elevated ALT during follow-up ($n = 73$) and (b) patients with normal ALT at baseline and during follow-up ($n = 85$).

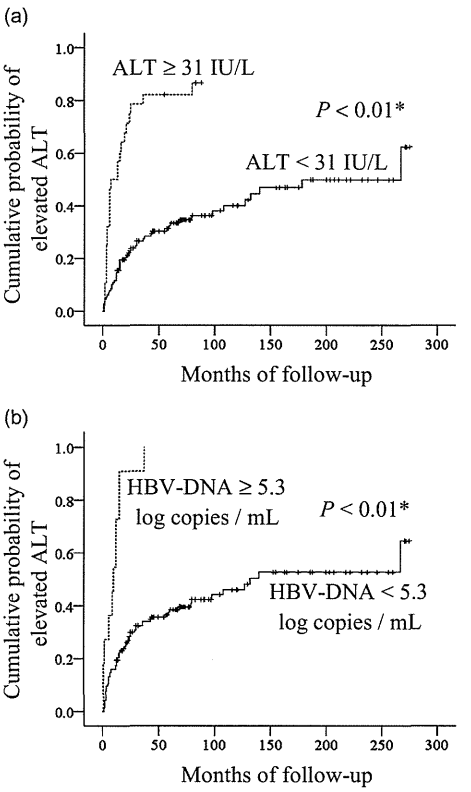

Figure 2 Cumulative occurrence of abnormal alanine aminotransferase (ALT) in HBeAg-negative patients with normal ALT based on (a) ALT and (b) HBV-DNA levels. We determined the threshold for ALT and HBV-DNA levels when the probability value was lowest in the univariate analysis. Kaplan–Meier curves show the time to ALT elevation. Solid lines indicated the control group. *A significant difference was determined by log-rank test.

Table 2 Univariate and multivariate analysis of factors associated with alanine aminotransferase (ALT) elevation in hepatitis B virus (HBV) e antigen (HBeAg)-negative patients with normal ALT levels

	Univariate analysis				Multivariate analysis			
	Standard error	Wald statistic	*P*-value	Hazard ratio (95% confidence interval)	Standard error	Wald statistic	*P*-value	Hazard ratio (95% confidence interval)
Sex (Male)	0.263	0.203	0.652	1.126 (0.673–1.885)				
Age (years)	0.011	5.704	0.017	1.027 (1.005–1.049)	0.252	0.068	0.794	1.015 (0.572–1.534)
HBV-DNA	0.109	17.773	<0.001	1.587 (1.280–1.966)	0.111	10.602	0.001	1.437 (1.155–1.788)
Genotype								
B	0.459	0.22	0.639	0.806 (0.328–1.982)				
C	0.435	0.055	0.815	1.107 (0.472–2.600)				
Alanine aminotransferase	0.014	42.440	<0.001	1.097 (1.067–1.128)	0.015	29.496	<0.001	1.086 (1.054–1.119)
Platelet count	0.019	5.928	0.015	0.955 (0.920–0.991)	0.021	0.754	0.385	0.982 (0.942–1.023)
Use of anti-diabetes drug	0.427	0.470	0.493	1.340 (0.581–3.091)				
Body mass index (kg/m^2)	0.042	0.033	0.855	0.992 (0.913–1.078)				
Smoker and ever smoker	0.374	0.111	0.739	1.133 (0.544–2.359)				
Daily alcohol consumption	0.333	0.512	0.474	1.269 (0.661–2.435)				

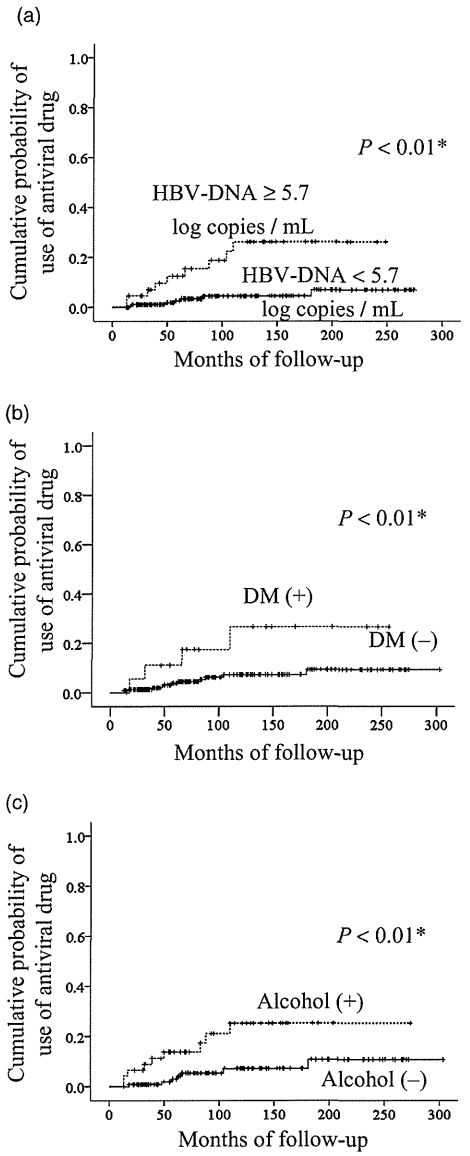

Figure 3 Cumulative occurrence of antiviral drug use for hepatitis B virus (HBV) in HBeAg-negative patients based on (a) HBV-DNA levels (b) use of antidiabetic drug, and (c) daily alcohol consumption. We determined the threshold for HBV-DNA levels when the probability value was lowest in the univariate analysis. Kaplan–Meier curves show the time to use of antiviral drugs for HBV. Solid lines indicated the control group. *A significant difference was determined by log-rank test.

PLT threshold was $10.0 \times 10^4/mm^3$. Kaplan–Meier curves were constructed for PLT (Fig. 4).

Stratification analyses of risk factors for clinical outcomes in HBeAg-negative patients by age, sex, and HBV genotype

The stratification analyses by age, sex, and HBV-genotype were performed to evaluate the risk factors for future ALT elevation in patients with normal ALT levels, future use of antiviral drugs for HBV, and HCC in HBeAg-negative patients (Table 3). The age threshold was 45 years, which was the average age of all the patients. We did not perform stratification analysis for patients infected with HBV genotype B because the number of such cases was very small.

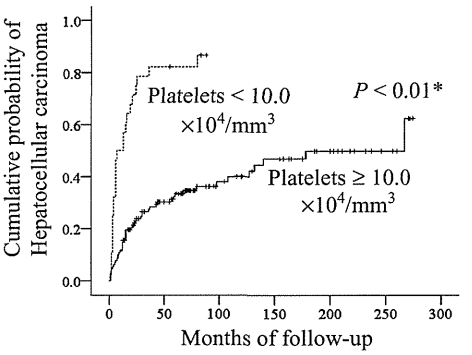

Figure 4 Cumulative occurrence of hepatocellular carcinoma (HCC) based on the platelet counts. We determined the threshold for HBV-DNA levels when the probability value was lowest in the univariate analysis. Kaplan–Meier curves show the time to HCC. Solid lines indicated the control group. *A significant difference was observed by log-rank test.

Discussion

Most patients who have undergone HBeAg seroconversion have normal serum ALT levels, which is indicative of a good clinical outcome.[10] Therefore, various therapies for early seroconversion have been used.[5] Recently, HBeAg-negative viral mutants have been shown to be responsible for continuous HBV-DNA replication.[7] That is, there exists the possibility that liver disease will get worse after HBeAg seroconversion. In fact, previous reports revealed that HBeAg status is not a predictive factor for HCC,[11,12] and fulminant hepatitis can occur by the infection of HBV with HBeAg-negative.[13] HBeAg-negative patients should be monitored closely, even though most of these patients show normal ALT levels and no progressive liver disease.[14] Therefore, predictive factors for active liver disease in HBeAg-negative patients need to be identified in order to facilitate optimal disease management. This study provides data regarding the prediction of future active liver disease, i.e. ALT elevation, unavoidable use of antiviral drugs, and occurrence of HCC.

Many previous reports have attempted to define a threshold HBV-DNA level that corresponds to the presence of active liver disease.[15] A National Institute of Health workshop demonstrated that an HBV-DNA level of 10^5 copies/mL could be used to distinguish active HBV infection from inactive HBV infection.[16] Other studies also suggested that the threshold HBV-DNA level lies somewhere between 10^4 and 10^6 copies/mL.[8] In this study, in order to clarify the natural course of HBeAg-negative patients with normal ALT levels, we used a HBV-DNA threshold of $10^{5.3}$ copies/mL. By log rank analysis, the ALT levels in patients with $>10^{5.3}$ copies/mL HBV-DNA level were significantly higher than in patients with HBV-DNA below this level. In HCV patients, ALT is

Table 3 Stratification analysis multivariate analysis of factors associated with alanine aminotransferase (ALT) elevation in hepatitis B virus (HBV) e antigen (HBeAg)-negative patients with normal ALT levels, future use of antiviral drugs for HBV, and occurrence of hepatocellular carcinoma

		Age (years)					Sex					Genotype				
		≥45 years			<45 years		Male			Female		C				
		$n = 126$			$n = 118$		$n = 141$			$n = 103$		$n = 87$				
	Factors	Hazard ratio (95% CI)	*P*-value	Factors	Hazard ratio (95% CI)	*P*-value	Factors	Hazard ratio (95% CI)	*P*-value	Factors	Hazard ratio (95% CI)	*P*-value	Factors	Hazard ratio (95% CI)	*P*-value
Future ALT elevation in the patients with normal ALT level	HBV-DNA	1.535 (1.146–2.057)	0.004	ALT	1.106 (1.059–1.156)	<0.001	ALT	1.194 (1.075–1.325)	<0.001	ALT	1.060 (1.015–1.108)	0.008	ALT	1.149 (1.075–1.228)	<0.001
	ALT	1.077 (1.035–1.122)	<0.001							HBV-DNA	1.739 (1.213–2.492)	0.003	HBV-DNA	1.902 (1.223–2.956)	0.004
Future use of anti-viral drugs for HBV	Alcohol	4.744 (1.362–16.52)	0.014	HBV-DNA	2.238 (1.107–4.526)	0.025	HBV-DNA	1.486 (1.053–2.098)	0.024	DM†	86.14 (1.842–4027.3)	0.023	Alcohol	5.617 (1.431–22.05)	0.013
										BMI†	0.408 (0.187–0.890)	0.024			
Occurrence of hepatocellular carcinoma	PLT	0.772 (0.659–0.905)	0.001	(–)†			PLT	0.832 (0.731–0.948)	0.006	PLT†	0.775 (0.635–0.947)	0.013	PLT	0.833 (0.732–0.948)	0.006

†Three patients were used in subgroup for HBV antiviral drugs and HCC occurrence.
Alcohol, Daily alcohol consumption; BMI, Body mass index; CI, confidence interval; DM, use of antidiabetic medication; PLT, platelet count.

a poor surrogate marker for inflammation and fibrosis.[17] Therefore, even if the patient's ALT level was within normal limits, they should still be monitored closely, and HCV eradication therapy is recommended under certain circumstances. Similarly, even if the ALT levels are within normal limits in HBV-infected patients who are HBeAg-negative, the higher their ALT levels were, the more frequently their ALT levels would be high in the future, which might cause progressive liver disease.[18]

Some of the patients with progressive liver disease caused by HBV infection were treated with the antiviral drugs lamivudine and entecavir. The use of lamivudine or entecavir might result in mutant HBV resistance to antiviral drugs[19,20] and the associated costs are not trivial. The baseline levels of HBeAg, ALT, and HBV-DNA, and the presence of either chronic hepatitis or cirrhosis have been established as determinants for eligibility for antiviral treatment.[21] According to treatment guidelines in the Unites States (National Guideline Clearinghouse, http://www.guideline.gov), patients with HBeAg-negative chronic hepatitis B should be considered for antiviral treatment based on their HBV-DNA and ALT levels (serum HBV-DNA >20 000 IU/mL and elevated ALT >2 times normal). In this study, only four out of 17 patients treated with an antiviral drug showed normal ALT levels at baseline, and all four patients showed elevated ALT levels in 8–57 months later. Therefore, this study revealed that patients with high HBV-DNA levels tended to have high ALT levels at baseline or in the future; as a result, such patients have a tendency for future treatment with and antiviral drug.

Hepatocellular carcinoma occurrence was noted in only 10 cases (4.1%). The only predictive factor for HCC occurrence was PLT, which meant that patients with advanced liver disease tended to develop HCC later, because the decrease in PLT corresponded to the extent of liver fibrosis. Four patients (1.6%) died of liver-related diseases and one (0.4%) died of cancer in another organ. The number of deaths was too small to determine the predictive factors for death of HBeAg-negative HBV carriers. Further analysis is needed to properly address this factor.

Stratification analyses of risk factors for clinical outcomes by age, sex, and HBV-genotype were performed. Because the numbers of female patients with future use of antiviral drugs for HBV ($n = 3$), HCC occurrence ($n = 3$), or who were under 45 years old with HCC occurrence ($n = 3$) were very small, it was not possible to properly evaluate these subgroups. The risk factors among subgroups for future ALT elevation in patients with normal ALT levels, and for HCC were almost equal to those of the entire patient population. However, daily alcohol consumption, not HBV-DNA level, was predictive of future use of antiviral drugs for HBV in patients ≥45 years old or in patients infected with HBV genotype C. In these subgroups, alcohol consumption was an important factor for predicting the clinical course of HBV carriers; i.e. advising patients to abstain from drinking might reduce the need for antiviral drugs in the future.

Coffee or caffeine consumption is reported to be strongly related to ALT levels and HCC occurrence.[22–24] In our study, we did not survey caffeine consumption; therefore, further analysis is needed to determine the importance of coffee or caffeine consumption as a predictive factor of the clinical course in HBeAg-negative HBV carriers.

In conclusion, we established that low HBV-DNA levels and ALT levels at baseline were good predictors for future ALT eleva-

tion in HBeAg-negative HBV carriers with normal ALT levels. In addition, this study provides data on the prediction of unavoidable antiviral drug use and HCC occurrence.

References

1 Fattovich G, Bortolotti F, Donato F. Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. *J. Hepatol.* 2008; **48**: 335–52.
2 Di Marco V, Lo Iacono O, Camma C *et al.* The long-term course of chronic hepatitis B. *Hepatology* 1999; **30**: 257–64.
3 Fattovich G, Olivari N, Pasino M *et al.* Long-term outcome of chronic hepatitis B in Caucasian patients: mortality after 25 years. *Gut* 2008; **57**: 84–90.
4 Hoofnagle JH, Dusheiko GM, Seeff LB *et al.* Seroconversion from hepatitis B e antigen to antibody in chronic type B hepatitis. *Ann. Intern. Med.* 1981; **94**: 744–8.
5 Korenman J, Baker B, Waggoner J *et al.* Long-term remission of chronic hepatitis B after alpha-interferon therapy. *Ann. Intern. Med.* 1991; **114**: 629–34.
6 Sumi H, Yokosuka O, Seki N *et al.* Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease. *Hepatology* 2003; **37**: 19–26.
7 Lin CL, Liao LY, Liu CJ *et al.* Hepatitis B viral factors in HBeAg-negative carriers with persistently normal serum alanine aminotransferase levels. *Hepatology* 2007; **45**: 1193–8.
8 Feld JJ, Ayers M, El-Ashry D *et al.* Hepatitis B virus DNA prediction rules for hepatitis B e antigen-negative chronic hepatitis B. *Hepatology* 2007; **46**: 1057–70.
9 Orito E, Ichida T, Sakugawa H *et al.* Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology* 2001; **34**: 590–4.
10 Lai CL, Yuen MF. The natural history of chronic hepatitis B. *J. Viral. Hepat.* 2007; **14** (Suppl. 1): 6–10.
11 Murata K, Sugimoto K, Shiraki K *et al.* Relative predictive factors for hepatocellular carcinoma after HBeAg seroconversion in HBV infection. *World J. Gastroenterol.* 2005; **11**: 6848–52.
12 Pokorski RJ, Ohlmer U. Long-term morbidity and mortality in Chinese insurance applicants infected with the hepatitis B virus. *J. Insur. Med.* 2001; **33**: 143–64.
13 Fujiwara K, Yokosuka O, Ehata T *et al.* The two different states of hepatitis B virus DNA in asymptomatic carriers: HBe-antigen-positive versus anti-HBe-positive asymptomatic carriers. *Dig. Dis. Sci.* 1998; **43**: 368–76.
14 Kumar M, Sarin SK, Hissar S *et al.* Virologic and histologic features of chronic hepatitis B virus-infected asymptomatic patients with persistently normal ALT. *Gastroenterology* 2008; **134**: 1376–84.
15 Mels GC, Bellati G, Leandro G *et al.* Fluctuations in viremia, aminotransferases and IgM antibody to hepatitis B core antigen in chronic hepatitis B patients with disease exacerbations. *Liver* 1994; **14**: 175–81.
16 Hoofnagle JH, Doo E, Liang TJ *et al.* Management of hepatitis B: summary of a clinical research workshop. *Hepatology* 2007; **45**: 1056–75.
17 Sanai FM, Benmousa A, Al-Hussaini H *et al.* Is serum alanine transaminase level a reliable marker of histological disease in chronic hepatitis C infection? *Liver Int.* 2008; **28**: 1011–18.
18 Lai M, Hyatt BJ, Nasser I *et al.* The clinical significance of persistently normal ALT in chronic hepatitis B infection. *J. Hepatol.* 2007; **47**: 760–7.
19 Villet S, Ollivet A, Pichoud C *et al.* Stepwise process for the development of entecavir resistance in a chronic hepatitis B virus infected patient. *J. Hepatol.* 2007; **46**: 531–8.
20 Sa-nguanmoo P, Tangkijvanich P, Payungporn S *et al.* Dynamics of HBV DNA levels, HBV mutations and biochemical parameters during antiviral therapy in a patient with HBeAg-negative chronic hepatitis B. *Asian Pac. J. Allergy Immunol.* 2007; **25**: 183–8.
21 Buster EH, van Erpecum KJ, Schalm SW *et al.* Treatment of chronic hepatitis B virus infection—Dutch national guidelines. *Neth. J. Med.* 2008; **66**: 292–306.
22 Ruhl CE, Everhart JE. Coffee and caffeine consumption reduce the risk of elevated serum alanine aminotransferase activity in the United States. *Gastroenterology* 2005; **128**: 24–32.
23 Ruhl CE, Everhart JE. Coffee and tea consumption are associated with a lower incidence of chronic liver disease in the United States. *Gastroenterology* 2005; **129**: 1928–36.
24 Larsson SC, Wolk A. Coffee consumption and risk of liver cancer: a metaanalysis. *Gastroenterology* 2007; **132**: 1740–5.

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MicroRNA122 is a key regulator of α-fetoprotein expression and influences the aggressiveness of hepatocellular carcinoma

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α-fetoprotein (AFP) is not only a widely used biomarker in hepatocellular carcinoma (HCC) surveillance, but is also clinically recognized as linked with aggressive tumour behaviour. Here we show that deregulation of microRNA122, a liver-specific microRNA, is a cause of both AFP elevation and a more biologically aggressive phenotype in HCC. We identify CUX1, a direct target of microRNA122, as a common central mediator of these two effects. Using liver tissues from transgenic mice in which microRNA122 is functionally silenced, an orthotopic xenograft tumour model, and human clinical samples, we further demonstrate that a microRNA122/CUX1/microRNA214/ZBTB20 pathway regulates AFP expression. We also show that the microRNA122/CUX1/RhoA pathway regulates the aggressive characteristics of tumours. We conclude that microRNA122 and associated signalling proteins may represent viable therapeutic targets, and that serum AFP levels in HCC patients may be a surrogate marker for deregulated intracellular microRNA122 signalling pathways in HCC tissues.

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The incidence of hepatocellular carcinoma (HCC), the third most common cause of cancer-related mortality worldwide[1], is currently increasing[2]. The recent discovery of the efficacy of sorafenib, a multikinase inhibitor, as a treatment for patients with advanced HCC, has represented a major breakthrough in the clinical management, although the survival benefit has been shown to be less than 3 months[3]. No other effective therapy is currently available for patients with advanced disease[4]. As such, there is a continuing need to develop novel therapeutics and approaches for treatment of advanced HCC[5].

To develop targeted cancer therapies, we must first identify aberrantly regulated molecular pathways specific to this cancer. Clinically, it is also important to discover useful and convenient surrogate serum biomarkers that reflect aberrations in molecular pathways due to the molecular mechanisms of their expression, to identify the deregulated intracellular signalling pathways and to spare the patients from invasive clinical tests.

Currently, α-fetoprotein (AFP) is the most widely used serum biomarker for HCC surveillance[6]. Although the regulation of AFP gene expression is not fully understood, p53 (ref. 7), β-catenin[8] and the recently identified zinc-finger protein, ZBTB20 (ref. 9), have been reported to be involved. Furthermore, whereas mounting clinical evidence indicates that AFP elevation is linked to a more aggressive tumour phenotype characterized by vascular invasion, metastasis and poor differentiation[10,11], it remains to be determined whether the two phenotypes represent anything more than coincidental epiphenomena[12].

MicroRNAs (miRNAs) are short, single-stranded, non-coding RNAs. Although first identified in *Caenorhabditis elegans*[13], miRNAs are now known to be expressed in most organisms, from plants to vertebrates[14]. Primary miRNAs, which possess stem-loop structures, are processed into mature miRNAs by Drosha and Dicer RNA polymerase III. These mature miRNAs then associate with the RNA-induced silencing complex, and the resulting complex directly binds to the 3′-untranslated regions of target messenger RNAs to act as suppressors of translation and gene expression. Thus, depending on the target mRNAs, miRNAs are responsible for the control of various biological functions including cell proliferation, apoptosis, differentiation, metabolism, oncogenesis and oncogenic suppression[15–17].

MiRNA122 (miR122) is a tissue-specific miRNA that is most abundant in the liver[18], wherein it is responsible for the maintenance of fatty acid metabolism[19,20] and circadian rhythms[21]. As shown for other tissue-specific miRNAs[22], expression of miR122 has been reported to be downregulated in carcinomas, particularly in more malignant tumours, although these results remain controversial because of conflicting reports[23–26]. The biological significance of the downregulation of miR122 expression in HCC at the molecular level has not yet been fully elucidated.

In the present study, we explored the role of microR122 in HCC by silencing it both in human HCC cells and in a transgenic mouse model. Our molecular analysis enabled us to define the complex regulatory cascades underlying the clinically recognized link between raised AFP levels and a more aggressive phenotype in HCC.

Results

Establishment of miR122-silenced HCC cell lines. To characterize the functional consequences of miR122 downregulation in HCC cells, Huh7 and PLC/PRF/5 cells were stably transduced with a lentivirus that expresses RNA hairpins that produce mature antisense RNA designed to silence miR122 function. These cells were selected on the basis of their relatively high levels of miR122 expression[24,27]. Several mismatches were intentionally inserted into the RNA hairpin sequences to produce more stable templates for miR122 binding and sequestration and to perturb the participation of miR122 in RNA-induced silencing complex-associated inhibition of translation (Fig. 1a).

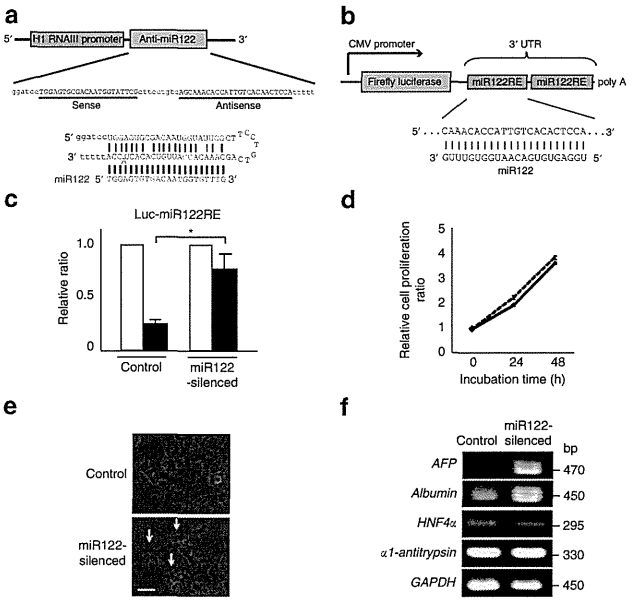

Figure 1 | Establishment of miR122-silenced HCC cell lines. (**a**), The miRZip122 construct, which yields a functional single-stranded full-length antisense miRNA complementary o miR122, processed from a stem-loop-stem molecule and transcribed from the constitutively active H1 RNA III promoter. Several mismatched nucleotides were included to efficiently produce a single-stranded antisense miR122 molecule. (**b**) Schematic representation of the luciferase reporter used to examine miR122 activity. The firefly luciferase gene, driven by a cytomegalovirus (CMV) promoter, contains two tandem miR122-binding sites (miR122-responsive elements; miR122-RE) in its 3′-UTR. (**c**) The suppressive effects of miR122 precursor expression (black bar) on luciferase activity in control and miR122-silenced cells. Test values were normalized to those obtained from the cells transfected with a miRNA precursor-non-expressing empty vector (white bar), which were set to 1. Data represent the mean±s.d. of three independent experiments using Huh7 cells. *$P<0.05$ (*t*-test). Similar results were obtained using PLC/PRF/5 cells. (**d**) Control (solid line) and miR122-silenced cells (dashed line) were plated at a density of 2×10^4 cells per well. After incubation for 24 and 48 h, numbers of cells were calculated as described in the Methods. Data represent the mean±s.d. of three independent experiments using Huh7 cells. Similar results were obtained using PLC/PRF/5 cells. (**e**) Changes in cellular morphology of Huh7 miR122-silenced cells are shown. Arrows indicate pseudopodia. Scale bar, 50 μm. Similar phenotypes were observed using PLC/PRF/5 cells. (**f**) The expression of several hepatocyte markers in control and Huh7 miR122-silenced cells was evaluated by semi-quantitative RT-PCR. Representative results from three independent experiments using Huh7 cells are shown. Similar results were obtained using PLC/PRF/5 cells.

To confirm effective miR122 silencing in transduced cells, we analysed the luciferase activity of reporters containing miR122-binding sites (the function of which is suppressed by miR122 overexpression) (Fig. 1b) in miR122-silenced and control cells. As expected, overexpression of the miR122 precursor greatly suppressed luciferase activity in control cells (Fig. 1c). In contrast, this suppressive effect was significantly reduced in miR122-silenced cells (Fig. 1c), indicating that miR122 was indeed functionally silenced.

To characterize the biological changes that result from the loss of miR122 function, we next analysed cell proliferation, morphology and differentiation in miR122-silenced cells. The rates of cell

proliferation were comparable between control and silenced cells (Fig. 1d); however, miR122-silenced cells exhibited a larger number of distinct pseudopodia (Fig. 1e). Next, as miR122 is specifically expressed in the liver, we hypothesized that it may have a role in hepatocyte differentiation and, therefore, we investigated the expression of several hepatocyte markers by semi-quantitative RT–PCR. We observed an increase in AFP expression and a slight elevation of albumin expression in miR122-silenced cells, but the expression levels of other hepatocyte markers, such as hepatocyte nuclear factor 4α (HNF4α) and α_1-antitrypsin, did not change (Fig. 1f).

MiR122-silenced HCC cells exhibit a more invasive phenotype. Because miR122-silenced cells exhibited an increased number of pseudopodia, we next characterized phenotypes associated with more biologically aggressive cell characteristics. We found that actin polymerization and pseudopod formation were significantly increased in miR122-silenced cells (Fig. 2a). The increase in the number of pseudopodia was confirmed by a quantitative pseudopodia assay (Supplementary Fig. S1). Although the expression levels of the mesenchymal marker α-smooth muscle actin were only slightly increased, we observed a significant decrease in the expression of the epithelial marker E-cadherin in miR122-silenced cells (Fig. 2b). Furthermore, the expression of other epithelial-to-mesenchymal transition markers such as fibronectin, N-cadherin, snail and Zeb1 was altered in miR122-silenced cells (Supplementary Fig. S1). These findings are consistent with the notion that loss of miR122 function leads to a more malignant phenotype.

We next performed scratch and invasion assays to characterize the invasive phenotype of miR122-silenced cells. Rates of cell migration and of cell invasion were significantly increased in miR122-silenced cells (Fig. 2c,d). As the proliferation rates of control and miR122-silenced cells were similar (Fig. 1d), altogether these results suggest that inhibition of miR122 function in HCC cells may lead to increases in malignancy-related cellular properties.

To investigate the molecular mechanisms underlying these cellular phenotypes, we assessed the activity of RhoA and Rac1, which are small GTPases that are closely associated with cell migration and invasion[28]. Although Rac1 activity did not significantly change, RhoA activity significantly increased in miR122-silenced cells (Fig. 2e), suggesting that the increase in cell migration and invasion in miR122-silenced cells may result from increased RhoA activity.

AFP expression is increased in miR122-silenced HCC cells. As we observed an increase in AFP expression in miR122-silenced cells (Fig. 1f), we next sought to quantify AFP concentrations in culture supernatants using an enzyme-linked immunosorbent assay (ELISA). AFP levels were approximately three times higher in the supernatant of miR122-silenced cells as compared with control cells (Fig. 3a). Consistent with this observation, immunofluorescence staining for AFP produced a stronger cytoplasmic signal and quantitative RT–PCR revealed a tenfold increase in AFP mRNA levels in miR122-silenced cells (Fig. 3b,c).

The 3′-UTR of the AFP mRNA did not contain predicted miR122 target sequences, based on sequence analyses performed using miRNA target search engines such as TargetScan (http://www.targetscan.org), suggesting that it is unlikely that miR122 directly regulates AFP expression. Therefore, to characterize the mechanisms underlying increased AFP expression in miR122-silenced cells, we first assessed the stability of the AFP mRNA in miR122-silenced cells. As expected, AFP mRNA stability was unaffected by silencing of miR122, as the amount of mRNA was comparable between control and miR122-silenced cells at 6, 12 and 24 h after inhibition of new transcription by treatment with actinomycin D (Fig. 3d). The increase in AFP mRNA levels in the absence of changes in mRNA stability suggested that transcription of AFP was increased

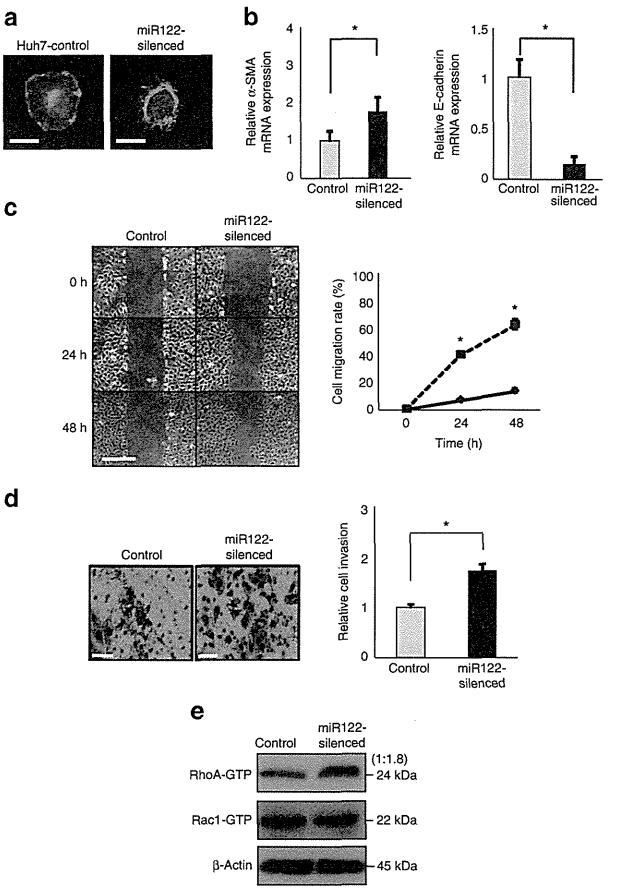

Figure 2 | HCC cells silenced for miR122 function exhibit a more invasive phenotype. (**a**) Cells were treated with 2 ng ml^{-1} TGF-β for 12 h, and actin filaments were stained with Alexa Fluor 488-conjugated phalloidin. Representative results from two independent experiments using Huh7 cells are shown. Similar results were obtained using PLC/PRF/5 cells. Scale bar, 50 μm. (**b**) Expression levels of α–smooth muscle actin and E-cadherin mRNAs were assessed by quantitative RT–PCR. Values shown represent mRNA expression levels in experimental cells relative to control cells. Data represent the mean±s.d. of three independent experiments using Huh7 cells. *$P<0.05$ (*t*-test). Similar results were obtained for PLC/PRF/5 cells. (**c**) The degree of cell migration was characterized using a scratch assay. The ratio of migrating cells was significantly increased in miR122-silenced cells at 24 and 48 h after scratching. Left panels show representative images. Right panel shows the results from cell counts for four randomly chosen fields per experiment. Data are represented as the mean±s.d. of three experiments using Huh7 control (solid line) and miR122-silenced cells (dashed line). *$P<0.001$ (*t*-test). Similar results were obtained for PLC/PRF/5 cells. (**d**) The degree of cell invasion was examined using cell invasion chambers. Representative images of stained invaded cells (left). The relative cell invasion ratio after normalization to control cell invasion levels (right). Data represent the mean±s.d. of three independent experiments using Huh7 cells. Scale bar, 100 μm. *$P<0.01$ (*t*-test). Similar results were obtained for PLC/PRF/5 cells. (**e**) Rho and Rac1 activity was determined by comparing the amounts of active GTP-bound RhoA (RhoA-GTP) and Rac1 (Rac1-GTP) between control and miR122-silenced cells. The indicated numbers represent the relative expression levels. Equal loading in pull-down assays was confirmed by analysis of β-actin levels in the cell lysates. Representative results of five independent experiments using Huh7 cells are shown. Similar results were obtained using PLC/PRF/5 cells.

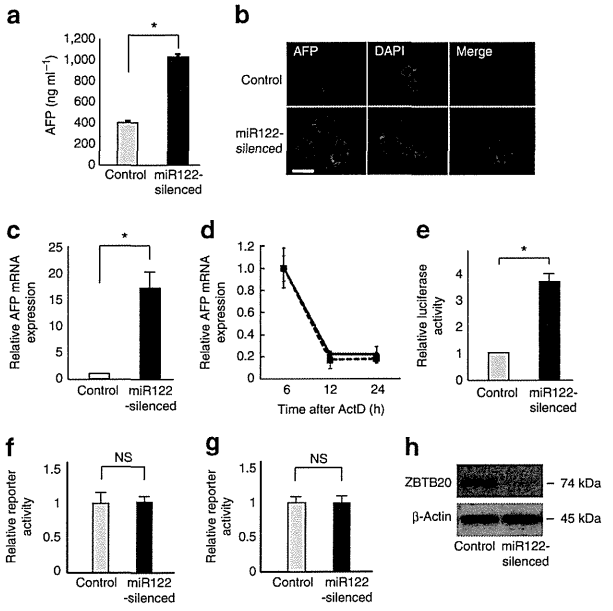

Figure 3 | Increased expression of AFP in HCC cells silenced for miR122 function. (**a**) The AFP concentration in the culture medium was determined by ELISA. Data represent the mean±s.d. of three independent experiments using Huh7 cells. *$P<0.01$ (*t*-test). Similar results were obtained for PLC/PRF/5 cells. (**b**) Immunofluorescent staining for AFP in the cytoplasm of control and miR122-silenced Huh7cells. Representative images of stained cells from three independent experiments are shown. Scale bar, 50 μm. (**c**) Amounts of AFP mRNA in Huh7 control and miR122-silenced cells were determined by quantitative RT-PCR. Data represent the mean±s.d. of three independent experiments. *$P<0.05$ (*t*-test). Similar results were obtained using PLC/PRF/5 cells. (**d**) AFP mRNA stability in Huh7 control (solid line) and miR122-silenced (dashed line) cells was determined by quantitative RT-PCR at 6, 12, and 24 h after treating cells with 10 μl ml^{-1} actinomycin D. Data represent the mean±s.d. of three independent experiments. Similar results were observed using PLC/PRF/5 cells. (**e**) AFP promoter activity was measured in reporter assays using Huh7 cells and AFP promoter-luciferase construct. Data represent the mean±s.d. of three independent experiments. *$P<0.05$ (*t*-test). Similar results were obtained using PLC/PRF/5 cells. (**f**, **g**) Luciferase assays were performed using reporter plasmids to measure p53 (**f**) and β-catenin (**g**) activities. Data represent the mean±s.d. of three independent experiments. Similar results were obtained using PLC/PRF/5 cells. (**h**) ZBTB20 protein levels in miR122-silenced cells. A representative result from three independent experiments using Huh7 cells is shown. Similar results were obtained using PLC/PRF/5 cells.

in miR122-silenced cells as a result of increased AFP promoter activity. Indeed, AFP promoter activity was almost four times higher in miR122-silenced cells than in control cells, as assessed by a reporter assay (Fig. 3e).

Because AFP promoter activity is in part regulated by p53 (ref. 7), we assessed p53 activity using reporter constructs. However, no changes in p53 activity were detected in miR122-silenced cells (Fig. 3f). As mutation of β-catenin has also been reported to be involved in upregulation of AFP expression[8], we next analysed β-catenin activity in miR122-silenced cells. Similar to p53, no change in β-catenin activity was evident in miR122-silenced cells compared with control cells (Fig. 3g).

Recently, it was reported that ZBTB20 acts as a repressor of AFP transcription[9]. This result led us to assess the expression of the ZBTB20 protein in miR122-silenced cells. Indeed, western blot analysis revealed that ZBTB20 expression was decreased in miR122-silenced cells (Fig. 3h). However, as ZBT20 lacks the presence of predicted miR122 target sequences based on computational searches of the 3′-UTR, it was also unlikely that miR122 directly regulates ZBTB20 expression. These observations suggest that other indirect mechanisms may lead to decreased ZBTB20 expression in miR122-silenced cells.

CUX1 is the regulator of phenotypes in miR122-silenced cells. To explore the mechanisms by which miR122 regulates cell motility, invasion and AFP expression, we used computational searches to identify potential miR122 target genes with known functions related to these processes. This analysis led to the identification of Cut homeobox 1 (CUX1, also known as CCAAT-displacement protein/cut homeobox, CDP/Cux/Cut) through the presence of a high probability miR122 target site located in the 3′-UTR and a perfect match in the seed sequences. CUX1 is a transcription factor that regulates multiple processes including cell cycle progression, chromosomal segregation and cell migration[29,30]. Consistent with the effects of miR122 silencing described above, CUX1 was reported to modulate cell motility and invasion through the control of RhoA activity[31–33]. We observed that whereas CUX1 mRNA levels remained unchanged (Fig. 4a), there was a significant increase in the steady-state level of the CUX1 p200 and p110 isoforms in miR122-silenced cells (Fig. 4b).

To investigate the contribution of CUX1 upregulation to the increase in AFP expression and invasive properties observed in miR122-silenced cells, we knocked down CUX1 protein expression using lentiviruses expressing CUX1 short hairpin RNAs (shRNAs) (Fig. 4c). In the resulting double-knockdown cells, AFP protein expression in cell-culture supernatant and cell invasion were both reduced to levels similar to that of the parental Huh7 cells (Fig. 4d–f).

CUX1 represses ZBTB20 expression via miR214. We next assessed whether miR122 directly targets CUX1 by constructing a luciferase reporter construct that possessed a portion of the CUX1 3′-UTR containing the putative miR122 target site (Fig. 5a). Co-transfection experiments revealed that luciferase activity was suppressed by overexpression of a miR122 precursor-expressing plasmid (Fig. 5b). This suppressive effect was prevented by introducing two point mutations into the seed sequences of the miR122 target site (Fig. 5a,b), demonstrating that miR122 directly targets these sequences.

To confirm these effects, we generated 293T cell lines that stably expressed the miR122-precursor construct by transducing cells with miR122 precursor-expressing lentiviruses tagged with green fluorescent protein (Supplementary Fig. S2a). As expected, the anti-miR122 construct did not affect control 293T cells, owing to the lack of miR122 expression. However, the anti-miR122 construct greatly enhanced luciferase activity in 293T cells stably expressing the miR122-precursor, confirming that miR122 was transduced into the 293T cells (Supplementary Fig. S2b). Consistent with the results described above, these cells exhibited decreased expression of CUX1, particularly the p200 isoform, and also showed a modest, but reproducible, increase in ZBTB20 expression (Fig. 5c). These results suggest that miR122 directly regulates CUX1 protein expression, which in turn may regulate ZBTB20 expression.

Because CUX1 can function as a transcriptional modulator[29], we initially hypothesized that CUX1 is a direct regulator of ZBTB20 transcription. However, quantitative RT–PCR analysis revealed that levels of the ZBTB20 mRNA were unchanged in miR122-silenced cells compared with controls (Fig. 5d). To explain the discrepancy between unchanged levels of ZBTB20 mRNA and decreases in protein expression levels in miR122-silenced cells, we searched for miRNAs that could potentially target the ZBTB20 3′-UTR. Based on computational searches, miR214 and miR375 were identified as candidate ZBTB20-regulatory miRNAs. Although levels of miR375 were unchanged in miR122-silenced cells (Fig. 5e), expression of miR214 was significantly increased (Fig. 5e).

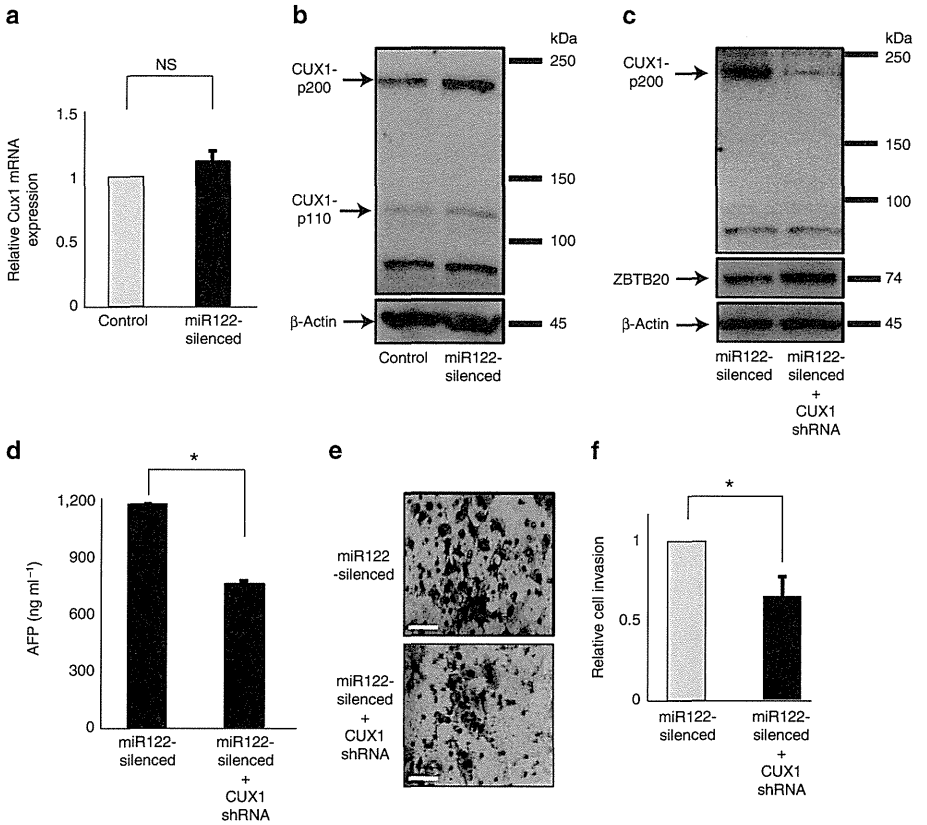

Figure 4 | CUX1-mediated regulation of AFP expression and phenotypic changes in miR122 functionally silenced cells. (**a**) CUX1 mRNA levels in control and miR122-silenced cells were analysed by quantitative RT-PCR. Data represent the mean±s.d. of three independent experiments using Huh7 cells. (**b**) p200 and p110 CUX1 protein levels were increased in miR122-silenced cells compared with control cells. A representative result from three independent experiments using Huh7 cells is shown. Similar results were obtained for PLC/PRF/5 cells. (**c**) CUX1 and ZBTB20 protein expression in double CUX1/miR122 knockdown Huh7 cells. Similar results were obtained using PLC/PRF/5 cells. (**d**) AFP concentrations in the culture medium supernatants were determined by ELISA. Data represent the mean±s.d. of three independent experiments. *$P<0.01$ (*t*-test). Similar results were observed using PLC/PRF/5 cells. (**e**, **f**) The change of cell invasion ratio by CUX1 knockdown in miR122-silenced Huh7 cells. Representative images of stained invading cells are shown (**e**). The relative cell invasion ratio after normalization to control invasion levels is shown (**f**). Data represent the mean±s.d. of three independent experiments. Scale bar, 100 μm. *$P<0.01$ (*t*-test). Similar results were obtained using PLC/PRF/5 cells.

To assess whether miR214 directly targeted the ZBTB20 3′-UTR, we constructed a luciferase reporter with the region of the ZBTB20 3′-UTR that contains the putative miR214 target site. Reporter assays revealed that luciferase activity was indeed suppressed by overexpression of the miR214 precursor, suggesting that miR214 directly targets the ZBTB20 3′-UTR and suppresses its expression (Fig. 5f). Consistent with these findings, cells that stably overexpressed the miR214 precursor exhibited decreased levels of ZBTB20 protein expression (Fig. 5g).

The putative promoter regions of miR214 contain multiple CUX1 binding sites as revealed by MATCH, a transcription factor binding site search engine (http://www.gene-regulation.com). A scanning chromatin immunoprecipitation (ChIP) experiment, followed by real-time PCR, using a series of primer pairs, confirmed that CUX1 binds to multiple genomic sites in the miR214 promoter region (Fig. 6a). We therefore hypothesized that CUX1 may regulate miR214 transcription. Consistent with this notion, we found that miR214 expression was decreased in CUX1 knockdown Huh7 cells (Fig. 6b). The role of CUX1 as an activator of miR214 transcription was further verified by knocking down or overexpressing CUX1 in another cell line. Levels of miR214 decreased following the constitutive knockdown of CUX1 with shRNA (Fig. 6c). In contrast, retroviral infection with a vector expressing p110 CUX1 led to an increase in miR214 (Fig. 6d). These findings were confirmed using doxycycline-inducible CUX1 shRNA. As previously observed for other transcriptional targets of CUX1 (refs 30,34), levels of miR214 were reduced in the presence of doxycycline, and then returned to levels higher than in untreated cells upon removal of the doxycycline inducer miR214 (Fig. 6e).

Next, to assess the contribution of miR214 to the control of ZBTB20 expression in miR122-silenced cells, we measured ZBTB20 expression after parallel silencing of miR214 in miR122-silenced cells. Although ZBTB20 protein expression was reduced by almost 50% by miR122 silencing, it was restored to >90% of control levels by miR214 silencing (Supplementary Fig. S3). Thus, CUX1-induced miR214 regulates, at least in part, ZBTB20 expression in miR122-silenced cells, leading to the upregulation of AFP expression.

Regulation of of CUX1 and AFP expression by miR122 was also confirmed in other HCC cell lines in which miR122 was overexpressed or silenced. Northern blotting showed that the expression of miR122 was relatively low in Hep3B and HepG2 cells, but was relatively high in Huh1, Huh7 and PLC/PRF/5 cells (Supplementary Fig. S4a). We therefore overexpressed the miR122 precursor in Hep3B and HepG2 cells and silenced miR122 in Huh1, Huh7 and PLC/PRF/5 cells (Supplementary Fig. S4b). CUX1 expression was respectively suppressed and enhanced by miR122 precursor overexpression and miR122 silencing (Supplementary Fig. S4c). In contrast, AFP expression was respectively enhanced and suppressed

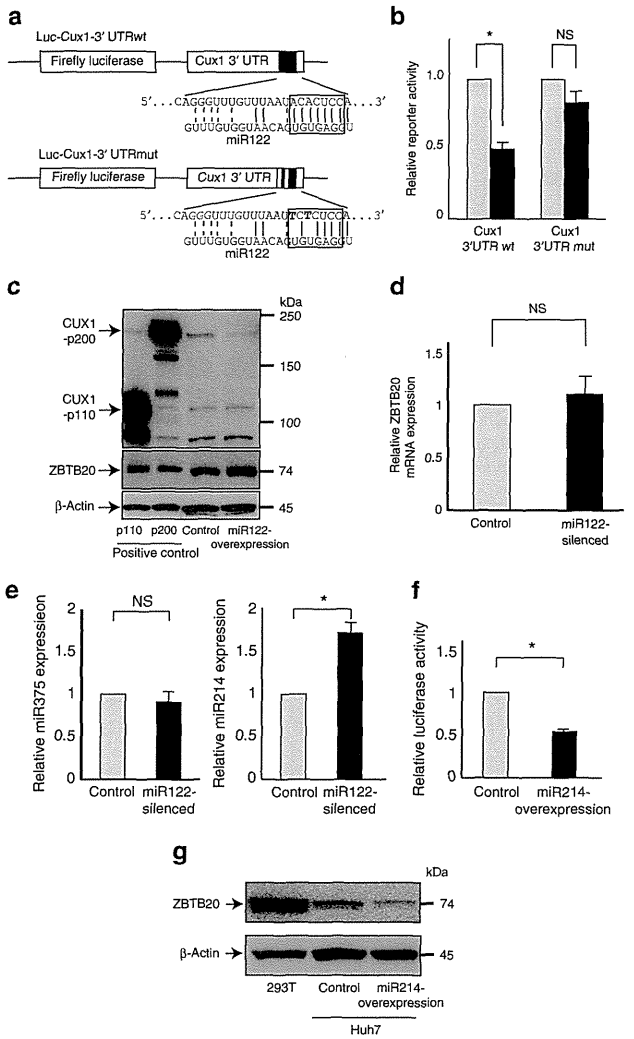

Figure 5 | MiR122 directly targets CUX1. (**a**) A luciferase reporter carrying a region of the wild type CUX1 3′UTR containing the putative miR122 target site (Luc-CUX1-3′UTRwt) was used to assess the effects of miR122 on expression of CUX1. A second luciferase reporter with two nucleotide mutations (indicated in bold) in the seed sequences (indicated by a rectangle) of the putative miR122 target sites (Luc-CUX1-3′UTRmut) was also utilized to assess specificity. (**b**) Huh7 cells were co-transfected with Luc-CUX1-3′UTRwt or Luc-CUX1-3′UTRmut and either an empty control vector (white bar) or a miR122 precursor expression plasmid (black bar). Data represent the mean±s.d. of three independent experiments. *$P<0.05$ (*t*-test). (**c**) CUX1 and ZBTB20 expression in 293T cells-expressing the miR122 precursor. Cell lysates transiently transfected with CUX1 p200 or p110 expression plasmids were used as positive controls. Representative results from four independent experiments are shown. (**d**) ZBTB20 mRNA levels in miR122-silenced Huh7 cells were determined by quantitative RT-PCR. Data represent the mean±s.d. of three independent experiments. *Similar results were obtained using PLC/PRF/5 cells.* (**e**) Levels of miR375 (left) and miR214 (right) in miR122-silenced Huh7 cells were analysed by quantitative RT-PCR. Data represent the mean±s.d. of three independent experiments. *$P<0.05$ (*t*-test). (**f**) Huh7 cells were co-transfected with Luc-ZBTB20-3′UTR and either an empty control vector or an miR214 precursor expression plasmid. Data represent the mean±s.d. of three independent experiments. *$P<0.05$ (*t*-test). (**g**) ZBTB20 expression was decreased in Huh7 miR214-overexpressing cells. 293T cell lysate was used as a positive control. Representative results from four independent experiments are shown.

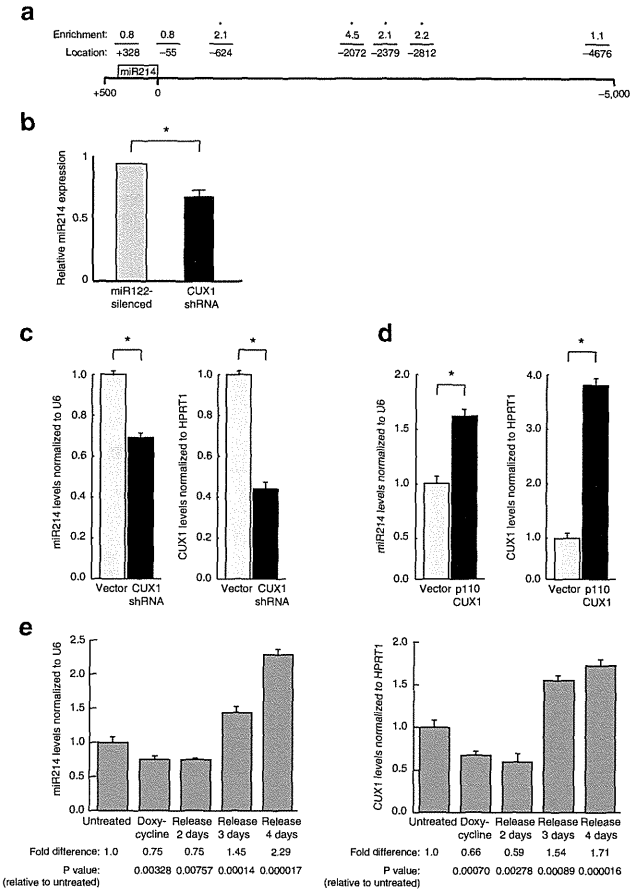

Figure 6 | CUX1 regulated miR-214 expression. (**a**), CUX1 enrichment at the miR214 locus. ChIP assays were performed using Hs578T cells. Fold enrichment and location of the center of each qPCR amplicon are shown. *$P<0.05$ (*t*-test). (**b**) MiR214 expression levels after CUX1 knockdown in miR122-silenced Huh7 cells were determined by quantitative RT-PCR. Data represent the mean±s.d. of three independent experiments. *$P<0.05$ (*t*-test). *Similar results were obtained using PLC/PRF/5 cells.* (**c**) Levels of miR214 and CUX1 RNA in Hs578T cells infected with an empty vector or a lentiviral vector constitutively expressing CUX1 shRNA. Data represent the mean±s.d. of three independent experiments. *$P<0.05$ (*t*-test). (**d**) Hs578T cells were infected with a retrovirus expressing p110 CUX1. Levels of miR214 and CUX1 mRNA were measured 1 day later. Data represent the mean±s.d. of three independent experiments. *$P<0.05$ (*t*-test). (**e**) Levels of miR214 and CUX1 mRNA in Hs578T cells expressing a doxycycline-inducible CUX1 shRNA. Levels are shown prior to treatment, after 5 days of doxycycline treatment, and 2, 3 and 4 days after withdrawal of doxycycline. Fold changes with the mean±s.d. of three independent experiments and *P*-values are shown (*t*-test).

(Supplementary Fig. S4d), confirming that AFP expression is regulated by an miR122-CUX1 pathway in multiple HCC cell lines.

These results indicate that functional silencing of miR122 leads to an increase in CUX1 protein expression, resulting in repression of ZBTB20 through an increase in miR214 expression. Repression of ZBTB20, in turn, leads to an increase in AFP expression. Because CUX1 is a modulator of cell motility and invasion[35–37], upregulation of this protein also enhances RhoA activity, increasing the malignant properties of cancer cells.

Expression of CUX1-related molecules in miR122-silenced mice. To explore the pathway delineated above in an *in vivo* model, we gen-

erated transgenic mice expressing antisense miR122 under the control of an H1 promoter (Fig. 7a) to inhibit the function of endogenous miR122 (ref. 38). *In situ* hybridization analysis in these mice revealed weak miR122 staining in liver tissue in comparison with control mice, likely due to binding of the anti-sense miR122 to endogenous miR122, which produces a double-stranded DNA and likely inhibits hybridization of the probe (Fig. 7b). Although structural development of the liver appeared normal based on haematoxylin and eosin staining (Supplementary Fig. S5), AFP mRNA expression (Fig. 7c) and p200 and p110 CUX1 protein expression were upregulated in the liver of anti-miR122 transgenic mice (Fig. 7d). Moreover, whereas levels of ZBTB20 mRNA were unchanged, ZBTB20 protein expression was decreased in the liver (Fig. 7d), in agreement with *in vitro* results demonstrating the regulation of ZBTB20 at the translational level (Fig. 5c,d). This was associated with a significant increase in the levels of miR214 in anti-miR122 transgenic mice (Fig. 7e). Thus, results from mouse liver tissue confirm that the miR122/CUX1/miR214/ZBTB20 regulatory pathway is also functional in an *in vivo* model.

Invasiveness of miR122-silenced cells in xenograft model. Next, we transplanted control and miR122-silenced PLC/PRF/5 cells under the liver capsule of nude mice (Fig. 7f) to determine whether miR122 silencing in HCC actually produces a more malignant phenotype *in vivo*. PLC/PRF/5 cells were chosen because of their transplantability in nude mice[39]. Neither intrahepatic metastases nor vascular invasion were detected in the livers of mice transplanted with control cells at 4 weeks post-transplantation. In contrast, vascular invasion was observed in the livers of mice transplanted with miR122-silenced HCC cells (Fig. 7g). These results suggest that miR122 silencing in HCC leads to a more aggressive phenotype.

HCC staging and the expression of miR122-related molecules. To assess the relevance of these results to human disease, we examined miR122 and AFP expression in several clinical-grade human HCC samples. We analysed miR122 expression by *in situ* hybridization (Fig. 8a) and AFP expression by immunohistochemistry (Fig. 8b). Both AFP expression and malignancy grading were inversely correlated with miR122 expression levels (Fig. 8c,d). In addition, CUX1, miR214 and ZBTB20 expression was also correlated with miR122 expression, as determined using serial sections (Supplementary Fig. S6a, b and c). These results, together with our studies in tissue culture systems and a transgenic mouse model, suggest that a reduction in the expression of miR122 increases AFP expression via a miRNA122-CUX1-miRNA214-ZBTB20 pathway and that the development of more biologically aggressive forms of HCC occurs via a miRNA122-CUX1-RhoA pathway (Supplementary Fig. S7). The miRNA-mediated mechanism described in this report may explain the clinically known link between increased AFP levels and more biologically aggressive cell characteristics in HCC.

Discussion

High AFP levels have been clinically shown to be an unfavourable prognostic factor in HCC patients[40]. In this study, we demonstrate that reduced expression of miR122 in HCC cells contributes to elevated AFP expression and, subsequently, a more aggressive phenotype. These results provide a molecular framework that explains the reported link between elevated AFP levels and a poor clinical outcome in HCC patients.

Clinically, high AFP expression is correlated with more biologically aggressive properties of HCC, as patients with high AFP levels have a significantly higher frequency of portal vein invasion and intrahepatic metastases. Additionally, these patients display significantly lower rates of recurrence-free survival and a trend towards lower overall survival[41]. In the present study, we have presented several lines of evidence indicating that decreased expression of miR122 in HCC leads to the two phenomena that

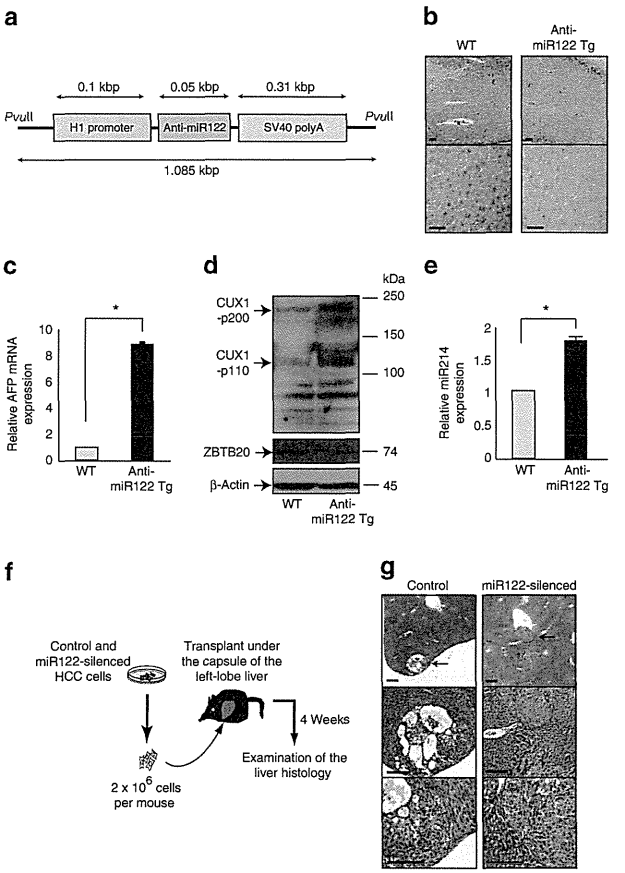

Figure 7 | AFP expression is increased in the liver of anti-miR122 transgenic mice. (**a**) DNA construct used to establishment transgenic mice in which miR122 is functionally silenced (anti-miR122 transgenic mice). This construct (like the construct used in the *in vitro* studies) generates a functional single-stranded full-length antisense miRNA complementary to miR122. (**b**) Confirmation of the expression of an antisense RNA directed against miR122 in anti-miR122 transgenic (anti-miR122 Tg) mice. Amounts of miR122 detected by *in situ* hybridization (blue/purple staining) in the liver tissues of anti-miR122 transgenic mice and WT mice. Results shown are representative of three independent experiments performed using littermates from four different mouse lines. Scale bar, 100 μm. No specific staining was observed when a negative control probe (LNA-scramble) was used. (**c**) AFP mRNA expression in mouse liver tissues was analysed by quantitative RT-PCR. Data represent the mean±s.d. of results for five mice in each group. *$P<0.05$ (*t*-test). (**d**) CUX1 and ZBTB20 expression in mouse liver tissues was assessed by Western blotting. A representative result from three independent experiments is shown. (**e**) Levels of miR214 in liver tissues were determined by quantitative RT-PCR. Data represent the mean±s.d. of three independent experiments. *$P<0.05$ (*t*-test). (**f**) Protocols of the orthotopic xenograft models of HCC cells. Control and miR122-silenced PLC/PRF/5 cells were prepared and injected under the capsule of the left-lobe of the nude mouse liver. Six mice were included in each group. At 4 weeks after transplantation, liver tissues were collected and sliced in series. Histological examination by H&E staining was performed to examine the tumour cell invasion status. (**g**) Representative liver histology images at 4 weeks after transplantation of tumour cells are shown. Whereas only the transplanted HCC cells beneath the capsule of the liver edge could be detected (arrow, upper left panel), neither intrahepatic metastasis nor vascular invasion was observed in the livers of mice transplanted with control cells. In contrast, vascular invasion by multiple tumour cells (arrow, upper right panel) was observed in mice transplanted with miR122-silenced cells. Scale bar, 500 μm. WT, wild type.