Table 2. Comparison between patients with chronicity following acute hepatitis B and those with self-limited acute infections determined by the persistence of HBsAg for more than 6 or 12 months

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

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

[a]Transmission routes of 41 patients were unknown

[b]Transmission routes of 8 patients were unknown

[c]Transmission routes of 46 patients were unknown

[d] Transmission routes of 3 patients were unknown

Table 3. Multivariate analysis of factors independently associated with persistence of HBsAg positivity following acute hepatitis B

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

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

Table 4. Characteristics of patients who progressed to chronicity following acute hepatitis B

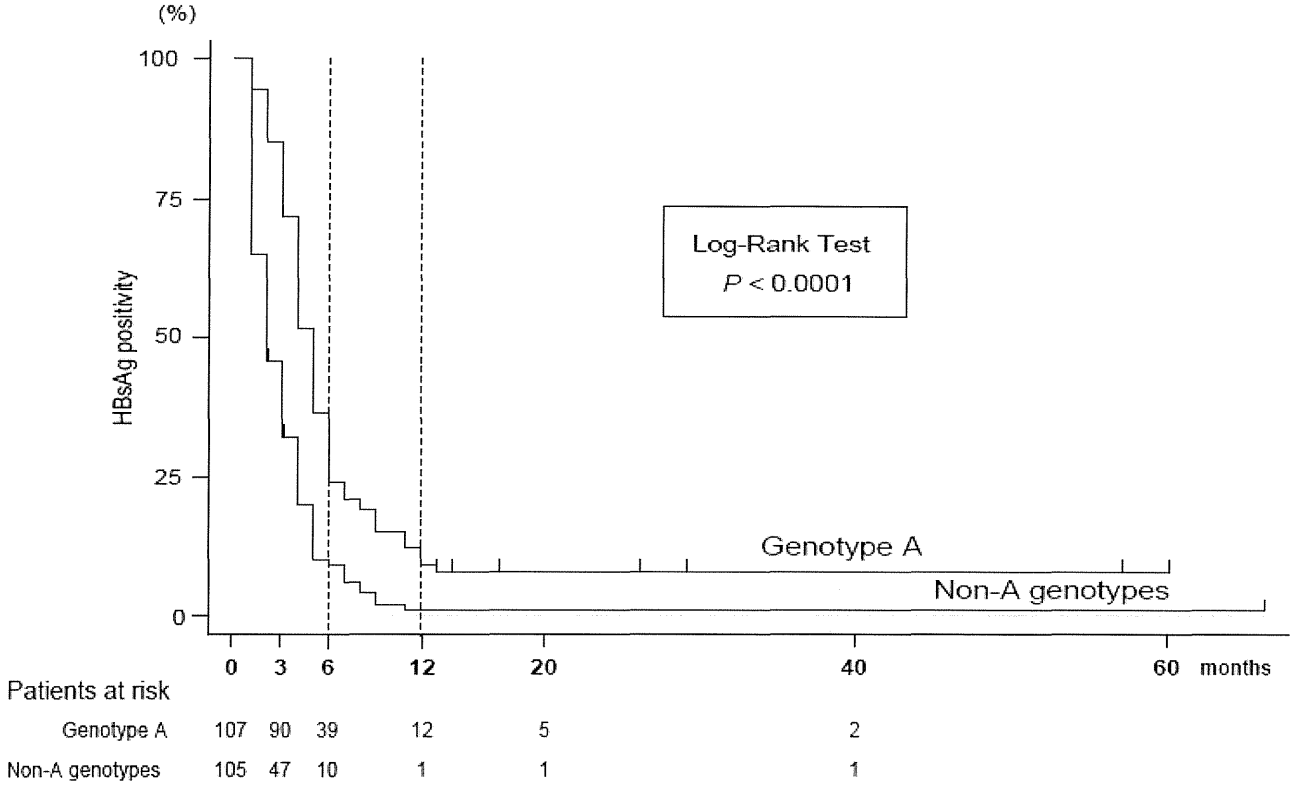
Case	Age	Gender	HIV	HBeAg	HBV DNA (log copies/mL)	Total bilirubin (mg/dL)	ALT (IU/L)	Observation period (months)	NAs treatment	Duration until NAs treatment (days)	Transmission routes	Genotype
1	23	Male	(-)	(+)	7.6	1.7	1271	26	ETV	570	Heterosexual	A
2	40	Male	(-)	(-)	8.8	1.4	568	13	ETV	240	Heterosexual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

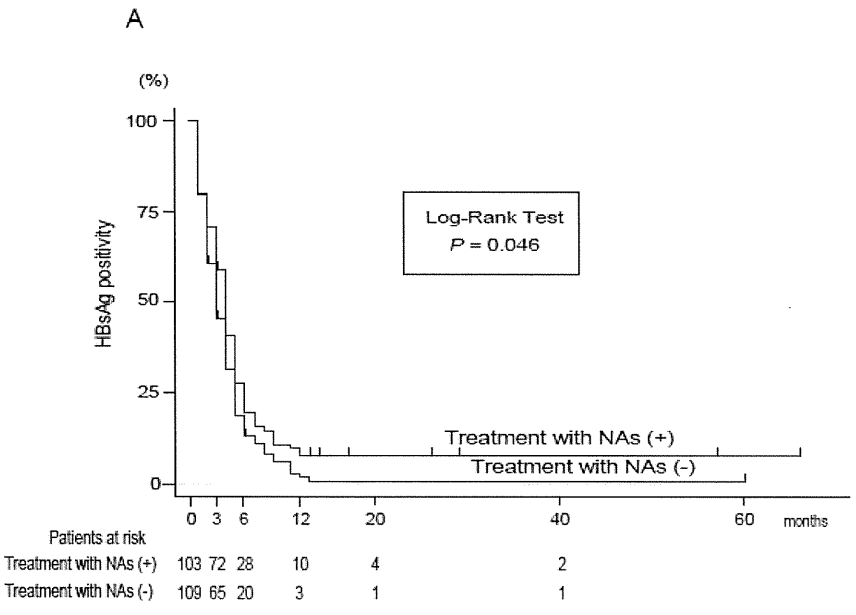
HIV, human immunodeficiency virus; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; NAs, nucleotide analogues, ETV; entecavir.

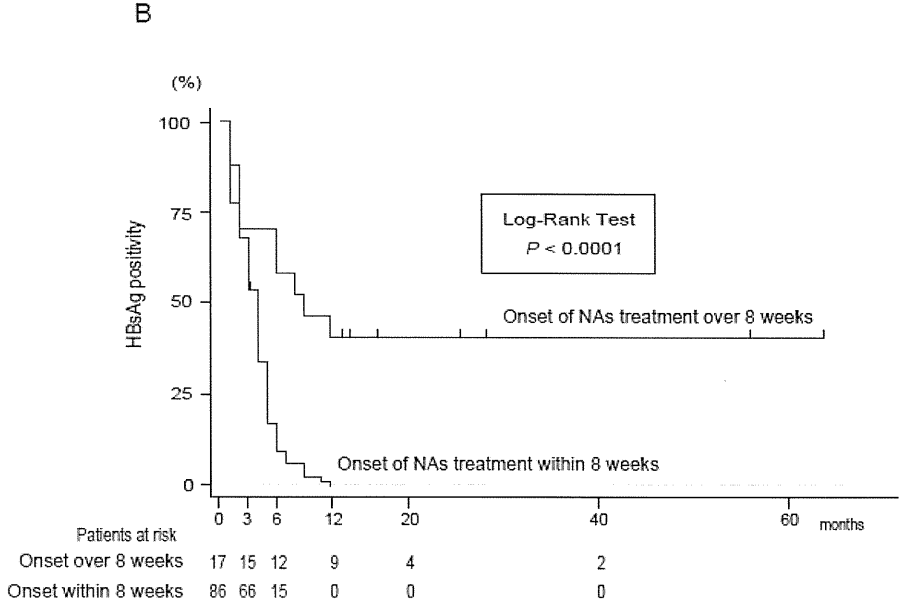
Table 5. Proportion of patients in whom HBsAg persisted for more than 6 or 12 months among patients categorized based on the number of weeks until the onset of NAs treatment

Duration until onset of NAs treatment (weeks)	Persistence of HBsAg for more than 6 months	Persistence of HBsAg for more than 12 months	Total patients
<4 weeks (n, %)	9 (12.7)	0 (0)	71
5–8 weeks (n, %)	6 (37.5)	0 (0)	16
9–12 weeks (n, %)	1 (33.3)	1 (33.3)	3
13–16 weeks (n, %)	4 (100)	1 (25.0)	4
>17 weeks (n, %)	9 (100)	6 (66.7)	9
Total	29	8	103

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


(%)
100
75
50
25
0
HBsAg positivity
Log-Rank Test
P < 0.0001
Genotype A
Non-A genotypes
0 3 6 12 20 40 60 months
Patients at risk
Genotype A 107 90 39 12 5 2
Non-A genotypes 105 47 10 1 1 1


A
(%)
100
75
50
25
0
HBsAg positivity
Log-Rank Test
P = 0.046
Treatment with NAs (+)
Treatment with NAs (-)
0 3 6 12 20 40 60 months
Patients at risk
Treatment with NAs (+) 103 72 28 10 4 2
Treatment with NAs (-) 109 65 20 3 1 1


B
(%)
100
75
50
25
0
HBsAg positivity
Log-Rank Test
P < 0.0001
Onset of NAs treatment over 8 weeks
Onset of NAs treatment within 8 weeks
0 3 6 12 20 40 60 months
Patients at risk
Onset over 8 weeks 17 15 12 9 4 2
Onset within 8 weeks 86 66 15 0 0 0

Supplementary Table 1. Characteristics of patients with NAs or without NAs treatment when acutely infected with hepatitis B virus

Features	NAs treatment (+) (n = 103)	NAs treatment (-) (n = 109)	*P* Value
Age (years)	40.9 ± 14.2	36.1 ± 12.0	0.019
Male sex	92 (89.3)	85 (77.9)	0.020
HBeAg positive	92 (89.3)	90 (82.6)	0.159
ALT (IU/L)	2014 ± 2773	1484 ± 1033	0.688
Total bilirubin (mg/dL)	9.2 ± 9.4	7.9 ± 6.8	0.837
HBV DNA (log copies/mL)	7.0 ± 1.5	5.9 ± 1.6	<0.0001
Duration until disappearance of HBsAg (month)	6.2 ± 9.4	4.2 ± 6.0	<0.0001
HBV genotype			
Non-A	42 (40.8)	63 (57.8)	
A	61 (59.2)	46 (42.2)	0.013
Sexual transmission	70/74 (94.6)[a]	82/89 (92.1)[b]	0.533

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

[a]Transmission routes were unknown for 29 patients.

[b]Transmission routes were unknown for 20 patients.

Supplementary Table 2. Characteristics of patients on NAs treatment 8 weeks before or 8 weeks after been acutely infected with hepatitis B virus

Features	Onset within 8 weeks (n = 87)	Onset over 8 weeks (n = 16)	*P* Value
Age (years)	39.8 ± 13.6	45.5 ± 15.8	0.161
Male sex	78 (89.7)	14 (87.5)	0.798
HBeAg positive	76 (87.4)	15 (93.8)	0.524
ALT (IU/L)	2256 ± 2946	727 ± 543	0.0003
Total bilirubin (mg/dL)	10.3 ± 9.5	3.9 ± 6.5	0.0003
HBV DNA (log copies/mL)	6.9 ± 1.6	7.7 ± 1.3	0.035
Duration until disappearance of HBsAg (month)	3.8 ± 2.5	18.9 ± 19.0	<0.0001
HBV genotype			
Non-A	37 (42.5)	5 (31.3)	
A	50 (57.5)	11 (68.7)	0.399
Sexual transmission	60/64 (93.8)[a]	10/10 (100)[b]	0.416

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

[a]Transmission routes were unknown for 23 patients.

[b]Transmission routes were unknown for 6 patients.

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Short Communication

In vitro replication competence of a hepatitis B genotype D/A recombinant virus: dissimilar biological behaviour regarding its parental genotypes

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Hepatitis B virus (HBV) DNA recombinants contribute to ~30 % of the overall full-length sequences already deposited in GenBank. However, their biological behaviour has not been analysed so far. In this study, the *in vitro* replication kinetics of the first D/A recombinant from the American continent differed from its parental genotypes, exhibiting higher extracellular levels of HBV DNA and hepatitis B e antigen. Southern blots of intracellular core-associated HBV DNA were in agreement with such results. Because this recombinant was obtained from an Argentinian injecting drug user belonging to a vulnerable community, these results are of singular relevance for regional public health. Further *in vivo* studies are urgently needed to determine the pathogenicity of these replicative competent clones.

Hepatitis B virus (HBV) infection is one of the most prevalent chronic viral infections among human beings. It often leads to cirrhosis and/or hepatocellular carcinoma, which is annually responsible for 1 million deaths worldwide. As a result, it is considered one of the major world health concerns.

Eight HBV genotypes (HBV/A–HBV/H) have been reported based on a sequence divergence greater than 8 % over the entire genome. Another two genotypes referred to as HBV/I and HBV/J have also been proposed. Genotypes are further subdivided into subgenotypes, which have been recognized in HBV/A–D and F, if the divergence in the whole genome reaches between 4 and 8 % (Lin & Kao, 2011). The global impact of HBV recombinants has also been described recently (Shi *et al.*, 2012).

Evidence for the influence of HBV genotypes and/or subgenotypes on the progression of liver diseases in acute, fulminant and chronic infection, the clinical outcome and the response to antiviral treatment have been reported by several researchers (Kramvis & Kew, 2005; Lin & Kao, 2011; Liu *et al.*, 2005). However, information about the effects of recombinant genomes on the clinical, prognostic and therapeutic aspects of the HBV infection is still lacking. Therefore, the aim of this study was to preliminarily analyse the very early replication dynamics of the infection of a HBV D/A recombinant and compare them with those

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Two supplementary figures are available with the online version of this paper.

of its parental genotypes (HBV/D and HBV/A) and of a highly replicative genotype (HBV/C) in an *in vitro* experimental system.

Serum samples were obtained from two previously recruited subjects (Trinks *et al.*, 2008): (i) H-IDU6 who was chronically infected with an HBV/D3 genome, as determined by partial S and pre-C/C phylogenetic analysis (PHYLIP package version 3.5c: Joseph Felsenstein, University of Washington, Seattle, WA, USA); and (ii) H-IDU7 who showed a HBV 'false' occult infection (Raimondo *et al.*, 2008) by a D3/A2 recombinant genome (breakpoints at nt 147 and 636, according to *Eco*RI restriction site numbering), as characterized by full-length phylogenetic and Simplot version 3.5.1 (Stuart Ray, John Hopkins University, Baltimore, MD, USA) analysis. Accordingly, this strain exhibited a recombinant HBV/A2 DNA region which corresponded to nt 147–636 of the S gene inserted in a backbone corresponding to HBV/D3.

HBV DNA was extracted from serum using QIAamp DNA blood kits (Qiagen). First, in order to fully characterize H-IDU6 HBV DNA, the complete genome was amplified and analysed by a reported method (Trinks *et al.*, 2008). Then, pUC19 plasmids deprived of promoters (Invitrogen) carrying a 1.24-fold HBV genome of each sample were constructed as described previously (Sugiyama *et al.*, 2006). Plasmids for HBV/A2 and HBV/C (Sugiyama *et al.*, 2006) were also included in this study.

After 24 h of culture, Huh7 cells were transfected with plasmids equivalent to 24 μg HBV DNA constructs using Lipofectamine 2000 transfection reagent (Invitrogen). Transfection efficiency was monitored by GFP expression using flow cytometry (BD FACSCanto; BD Biosciences) after cell transfection with a pTARGET (Promega)–GFP expression vector. Except for Southern blotting, all experiments were conducted twice for each clone.

At 24 and 72 h post-transfection (p.t.), hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) were determined from the supernatant by ARCHITECT (Abbott). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were also measured to determine cell viability. Supernatants from cells treated solely with Lipofectamine were included as negative controls.

At 72 h p.t., in order to measure HBV DNA viral load in supernatants (ruling out extracellular free HBV DNA or HBV RNA), such fluids were ultracentrifuged at 22 000 ***g*** for 5 min to enrich HBV and HBV core particles; pellets were resuspended and jointly treated with DNase I and RNase A at 37 °C for 3 h (Sugiyama *et al.*, 2006). To confirm the validity of the DNA extraction method (only from virions and capsids, but not from free recombinant plasmid DNA) in the supernatants, Huh7 cells mock transfected with HBV/C plasmid in the absence of Lipofectamine were considered as a further (expected) negative control due to DNase and RNase treatment. HBV load was measured subsequently (COBAS TaqMan HBV Test; Roche).

At 72 h p.t., in order to confirm the HBV replication among all the studied clones, cells were lysed and the density of core-associated HBV DNA was compared by Southern blot hybridization with a mix of full-length probes of each genotype involved in the experiment (A2, C, D3 and D3/A2; Sugiyama *et al.*, 2006).

Student's *t*-test was used to compare the means and SD between any pair of samples: $P<0.05$ was considered statistically significant.

Biochemical and virological features of both patients from whom sera were obtained are shown in Table 1. The full-length genome was amplified from sample H-IDU6 and subjected to phylogenetic analysis. This sample was ascribed to HBV/D3 and the presence of recombination was ruled out by Simplot (Figs S1 and S2b, available in JGV Online). None of the isolates possessed the mutation G1896A, A1762T or G1764A, which could have interfered with the expression of HBeAg and the efficiency of pre-genome encapsidation for replication. As expected for HBV/D, T1858 was observed in both isolates.

For construction of HBV D3/A2 recombinant and HBV/D3 vectors (Sugiyama *et al.*, 2006), at least 25 clones for each PCR-amplified HBV hemigenome [fragments A (nt 17–1799) and B (nt 1595–239); Sugiyama *et al.*, 2006] from each sample were sequenced and phylogenetically analysed. All clones from the HBV/D3 sample were ascribed to the D3 subgenotype. With regard to the D3/A2 recombinant sample, all 25 clones derived from fragment B were ascribed to the D3 subgenotype; in contrast, 40 % of the analysed clones from fragment A were D3/A2 recombinants with breakpoints at nt 147 and 636, 32 % belonged to the A2 subgenotype, 16 % to recombinant clones with breakpoints at nt 505 and 630, 8 % to recombinant clones

Table 1. Biochemical and virological features of patients from whom HBV isolates were recovered

Feature	H-IDU6	H-IDU7
Gender	Male	Male
Age	35	26
HBsAg	+	–*
HBeAg	+	+
Anti-HBc Ab	+	+
Anti-HCV Ab	+	+
Anti-HIV Ab	+	+
HBV viral load	$>110\times10^6$ IU ml^{-1}	$>110\times10^6$ IU ml^{-1}
HBV genotype	D3	D3/A2 recombinant

*T113S and T131N mutants were detected within the major hydrophilic region of the deduced S amino acid sequence. A negative result for HBsAg had been originally obtained with the serum collected in 1995 and then studied with AxSYM (Abbott) (Trinks *et al.*, 2008). These mutants became detectable when supernatants collected from Huh7 transfected cells were tested by means of the ARCHITECT assay (Abbott) in this study.

with breakpoints at nt 519 and 630, and 4% to D3 subgenotype (Fig. 1a). Because the most abundant recombinant clones were those exhibiting breakpoints at nt 147 and 636 (Fig. 1a and Fig. S2a), they were considered representative of the whole viral population and thus selected for D/A replicon construction.

Transfection efficiency ranged from 24.1 to 24.4% and cell viability was similar in all groups ($P>0.05$; data not shown, available upon request).

At 24 h p.t., the D3/A2 clone produced the highest levels of both antigens ($P<0.0001$; Fig. 1b). Moreover, at 72 h p.t., the HBsAg levels from HBV/A2 and the recombinant clone were the highest ($P>0.05$), followed by HBV/C and HBV/D3 ($P<0.0001$; Fig. 1b). At this time point, the recombinant clone produced the highest levels of HBeAg compared with its parental genotypes and also the HBV/C clone ($P<0.0001$; Fig. 1b).

At 72 h p.t., the HBV/C clone showed the highest viral load in the supernatant, closely followed by the recombinant clone, whose extracellular HBV DNA level was, in turn, higher than those from its parental genotypes ($P>0.05$; Fig. 1c).

Southern blotting undoubtedly confirmed previously published results regarding the HBV/C clone, which exhibited the highest intracellular replication level (Sugiyama *et al.*, 2006). Interestingly, the level of the recombinant was higher than those from its parental genotypes (Fig. 1d).Negative controls processed in parallel confirmed the specificity of the above-mentioned results.

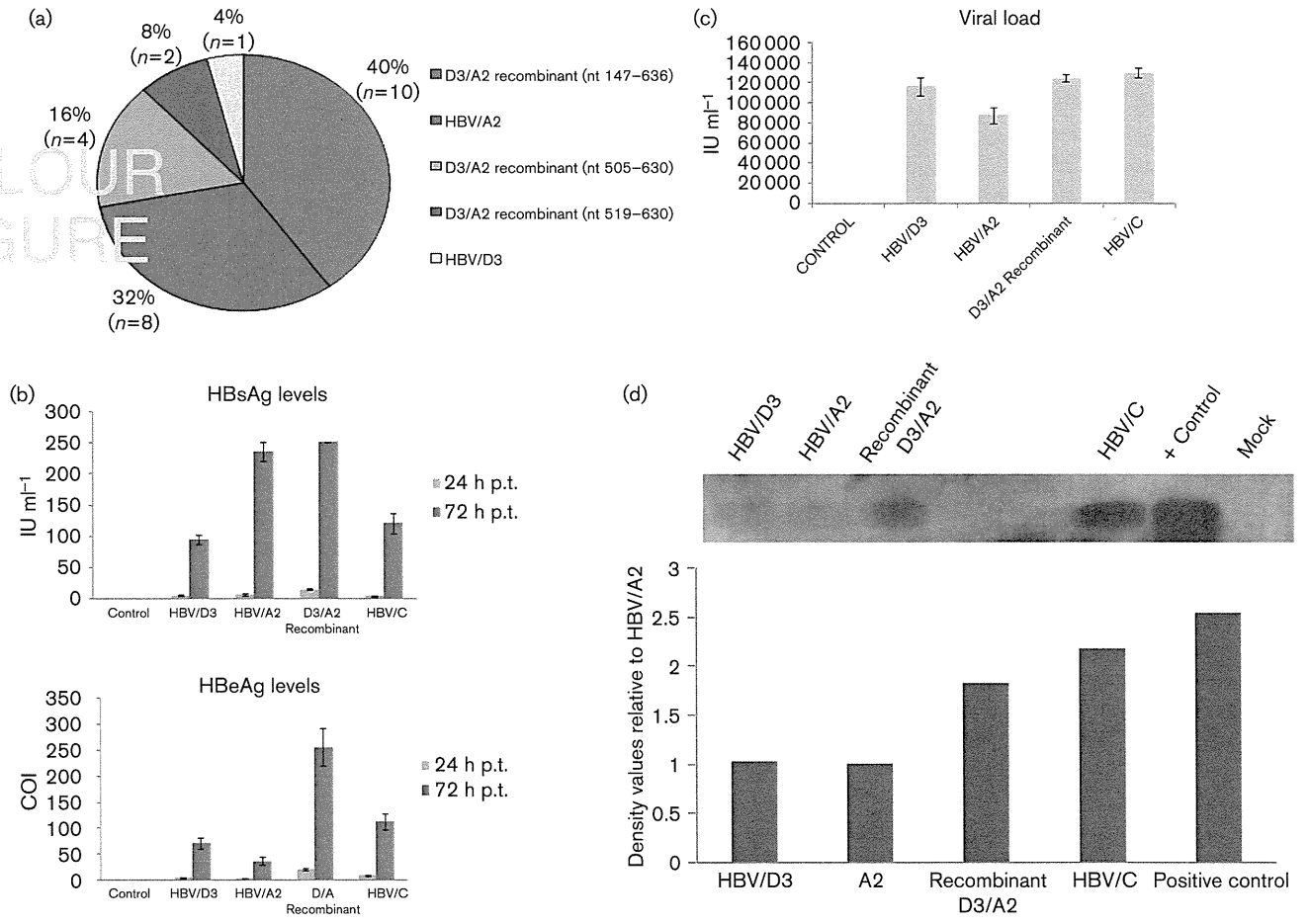

Fig. 1. (a) Analysis of clones derived from fragment A obtained from the recombinant strain H-IDU7. (b) HBsAg and HBeAg extracellular levels. COI, cut-off index. (c) HBV viral load in supernatant. (d) Core-associated HBV DNA in Southern blot analysis of Huh7 cell lysates transfected with plasmid constructs of genotype HBV/D3, HBV/A2, D3/A2 recombinant and HBV/C. An aliquot of non-transfected unlabelled full-length HBV/A2 probe (3.2 kb; positive control) and Huh7 cell lysates treated solely with transfection reagent (mock) were also included. The density of the bands corresponding to a hybridization signal was normalized to that obtained with the A2 clone, which exhibited the lowest density value (density=1). An asterisk represents a statistical difference of $P<0.0001$ when compared with all the remaining genotypes. Double asterisks indicate a statistical difference of $P<0.0001$ in comparison with genotypes HBV/D3 and HBV/C. The absence of asterisks represents no statistical difference.

The influence of genotypes and/or subgenotypes on disease progression and clinical outcome of HBV infection is well documented. However, information regarding HBV recombinant behaviour is unknown.

In a previous study (Trinks *et al.*, 2008), our group isolated a novel intergenotypic D/A recombinant strain from a patient (H-IDU7) co-infected with HIV/HCV. This strain, which was the first full-length D/A recombinant genome characterized from the American continent, exhibits a HBV/D3 backbone genome with an inserted segment of HBV/A2 within the Pol gene. After cloning this sample, we documented the co-circulation of HBV/D3, HBV/A2 genomes together with three different types of D/A recombinants of which one was dominant. In future studies, it will be interesting to compare the biological behaviour of these three dissimilar recombinants in an attempt to elucidate the reason(s) for the observed dominance of the D3/A2 recombinant clones with nt 147–636 breakpoints.

The observation of pure HBV/D3 and HBV/A2 clones confirms that co-infection with different HBV genotype strains is a prerequisite for recombination (Zhou *et al.*, 2012). However, the mechanism of selection of a given strain in mixed infections, i.e. DNA exchange or (less likely throughout the lifespan of a given individual) DNA mutation evolution, still remains unknown.

In this study, the replication kinetics of this recombinant differed from those of its parental genotypes, exhibiting higher extracellular levels of HBV DNA, similar (to A2) or higher (than D3) HBsAg, and higher (than both) HBeAg values. The significance of these findings should be explored by using the primary hepatocyte infection and also *in vivo* uPA-SCID mice models.

Taking into account that one of the HBV DNA-binding sites for CREB transcription factor is placed at nt 143–154 and that it enhances HBsAg expression levels, as previously shown for an A2 replicon (Tacke *et al.*, 2005), it seems plausible that those genomes showing the CCTGTG-ACGAAC binding site would exhibit similarly high HBsAg expression. This sequence was observed in the recombinant clone, as the 5′ breakpoint for the A2 insert is placed at nt 147. Interestingly, such a binding site is mutated in the HBV/D3 replicon (CCTGCGCTGAAC, mutations underlined), which could account for a lower level of pre-S/S transcription efficiency for such a genotype (in contrast to HBV/A2) and consequently for a lower level of HBsAg expression, as reported previously (Sugiyama *et al.*, 2006), the latter result also being observed in our study. Although HBsAg levels frequently reflect intrahepatic HBV replication in WT genomes (Chan *et al.*, 2011), they do not necessarily mirror HBV DNA levels in some mutated pre-S/S genomes (Pollicino *et al.*, 2012). Moreover, it has been shown that HBV/A2 is associated with higher HBsAg secretion and lower DNA replication compared with other genotypes (Sugiyama *et al.*, 2006). Interestingly, our recombinant clone produced high levels of HBsAg, HBeAg and DNA viral load, whose highest titres are usually associated with HBeAg secretion. These results might be explained by the presence of an A2 insert in the PreS2/S region and a D3 backbone in the pre-C/C region.

However, the recombinant exhibited even higher HBeAg values and intracellular HBV DNA levels than the parental D3 clone, which could be explained by the presence of mutations T1766 and A1770 in the D3 backbone of the recombinant D3/A2 clone, which form putative hepatocyte nuclear factors 1 (HNF1)- and HNF3-binding sites related to enhanced viral replication (Baumert *et al.*, 1996; Günther *et al.*, 1996; Fig. 2). Moreover, the single mutation T1664C observed within the core upstream regulatory sequence (CURS; nt 1636–1742) in the recombinant, but which was absent in the D3 parental genotype, might also account for such a difference in HBeAg secretion. As the CURS region exerts a strong stimulating effect on the basal core promoter (Yuh *et al.*, 1992), it is tempting to speculate that such a mutation might produce the higher HBeAg levels observed with the recombinant compared with the D3 parental clone.

Because this recombinant strain was obtained from an intravenous drug user belonging to a highly vulnerable

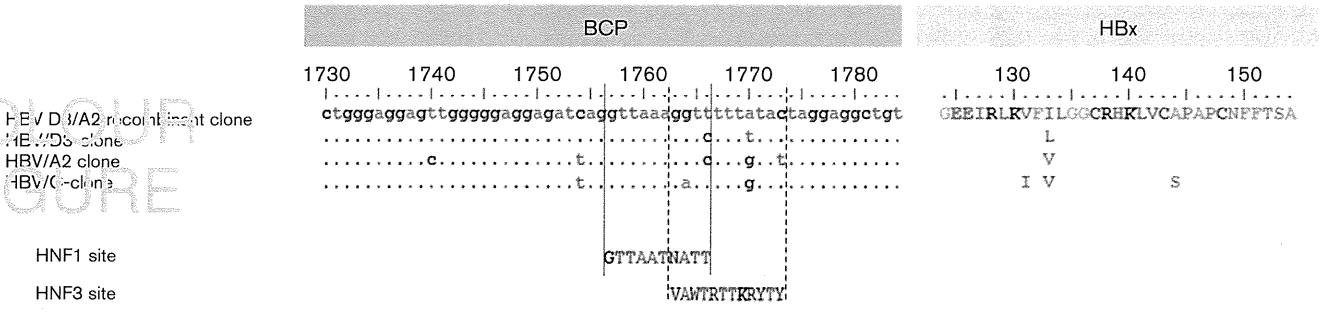

Fig. 2. Sequences of the basal core promoter (BCP) nt 1730–1785 and the overlapping region of the X protein of the HBV clones. The binding sites for HNF1 and HNF3 are aligned with the corresponding region of the HBV genome. The symbols for nucleotide ambiguities are as follows: V, A/C/G; W, A/T; R, A/G; K, G/T; Y, C/T.

group in Argentina, these results are of singular relevance for regional public health. Further *in vivo* studies are needed to determine the pathogenicity of these replicative competent clones.

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References

Baumert, T. F., Rogers, S. A., Hasegawa, K. & Liang, T. J. (1996). Two core promotor mutations identified in a hepatitis B virus strain associated with fulminant hepatitis result in enhanced viral replication. *J Clin Invest* **98**, 2268–2276.

Chan, H. L., Thompson, A., Martinot-Peignoux, M., Piratvisuth, T., Cornberg, M., Brunetto, M. R., Tillmann, H. L., Kao, J. H., Jia, J. D. & other authors (2011). Hepatitis B surface antigen quantification: why and how to use it in 2011: a core group report. *J Hepatol* **55**, 1121–1131.

Günther, S., Piwon, N., Iwanska, A., Schilling, R., Meisel, H. & Will, H. (1996). Type, prevalence, and significance of core promoter/enhancer II mutations in hepatitis B viruses from immunosuppressed patients with severe liver disease. *J Virol* **70**, 8318–8331.

Kramvis, A. & Kew, M. C. (2005). Relationship of genotypes of hepatitis B virus to mutations, disease progression and response to antiviral therapy. *J Viral Hepat* **12**, 456–464.

Lin, C. L. & Kao, J. H. (2011). The clinical implications of hepatitis B virus genotype: recent advances. *J Gastroenterol Hepatol* **26** (Suppl 1), 123–130.

Liu, C. J., Kao, J. H. & Chen, D. S. (2005). Therapeutic implications of hepatitis B virus genotypes. *Liver Int* **25**, 1097–1107.

Pollicino, T., Amaddeo, G., Restuccia, A., Raffa, G., Alibrandi, A., Cutroneo, G., Favaloro, A., Maimone, S., Squadrito, G. & Raimondo, G. (2012). Impact of hepatitis B virus (HBV) preS/S genomic variability on HBV surface antigen and HBV DNA serum levels. *Hepatology* **56**, 434–443.

Raimondo, G., Allain, J. P., Brunetto, M. R., Buendia, M. A., Chen, D. S., Colombo, M., Craxì, A., Donato, F., Ferrari, C. & other authors (2008). Statements from the Taormina expert meeting on occult hepatitis B virus infection. *J Hepatol* **49**, 652–657.

Shi, W., Carr, M. J., Dunford, L., Zhu, C., Hall, W. W. & Higgins, D. G. (2012). Identification of novel inter-genotypic recombinants of human hepatitis B viruses by large-scale phylogenetic analysis. *Virology* **427**, 51–59.

Sugiyama, M., Tanaka, Y., Kato, T., Orito, E., Ito, K., Acharya, S. K., Gish, R. G., Kramvis, A., Shimada, T. & other authors (2006). Influence of hepatitis B virus genotypes on the intra- and extracellular expression of viral DNA and antigens. *Hepatology* **44**, 915–924.

Tacke, F., Liedtke, C., Bocklage, S., Manns, M. P. & Trautwein, C. (2005). CREB/PKA sensitive signalling pathways activate and maintain expression levels of the hepatitis B virus pre-S2/S promoter. *Gut* **54**, 1309–1317.

Trinks, J., Cuestas, M. L., Tanaka, Y., Mathet, V. L., Minassian, M. L., Rivero, C. W., Benetucci, J. A., Gímenez, E. D., Segura, M. & other authors (2008). Two simultaneous hepatitis B virus epidemics among injecting drug users and men who have sex with men in Buenos Aires, Argentina: characterization of the first D/A recombinant from the American continent. *J Viral Hepat* **15**, 827–838.

Yuh, C. H., Chang, Y. L. & Ting, L. P. (1992). Transcriptional regulation of precore and pregenomic RNAs of hepatitis B virus. *J Virol* **66**, 4073–4084.

Zhou, B., Wang, Z., Yang, J., Sun, J., Li, H., Tanaka, Y., Mizokami, M. & Hou, J. (2012). Novel evidence of HBV recombination in family cluster infections in western China. *PLoS ONE* **7**, e38241.

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ORIGINAL ARTICLE

Specific mutations of basal core promoter are associated with chronic liver disease in hepatitis B virus subgenotype D1 prevalent in Turkey

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ABSTRACT

The role of hepatitis B virus (HBV) genetics in the clinical manifestations of infection is being increasingly recognized. Genotype D is one of eight currently recognized major HBV genotypes. The virus is ubiquitous worldwide, but shows different features in different regions. One hundred and ninety-eight patients with chronic HBV infection were enrolled in this study, 38 of whom had been diagnosed with cirrhosis of the liver and/or hepatocellular carcinoma. HBV DNA was isolated from the patients' blood samples and the entire genome and/or the basal core promoter/core promoter region sequenced. Phylogenetic analysis of the complete genomes revealed that subgenotype D1 is the most prevalent subgenotype in Turkey, but there was no definite phylogenetic grouping according to geography for isolates from different regions within Turkey, or for isolates in Turkey relative to other parts of the world. Turkish isolates tended to be genetically similar to European and central Asian isolates. Overall, HBV-infection in Turkey appears to be characterized by early HBeAg seroconversion, a high incidence of the A1896 core promoter mutation and a small viral load. Genotype D characteristic mutations A1757 and T1764/G1766 were found in the BCP region. T1773 was associated with T1764/G1766 and a larger viral load. In conclusion, infection with HBV genotype D in Turkey has a similar clinical outcome to that of Europe and central Asia. Genotypic mutations in genotype D may be linked with disease prognosis in Turkey, but further studies with higher sample numbers and balanced clinical groups are needed to confirm this.

Key words basal core promoter, genotype D, hepatitis B virus, Turkey.

Hepatitis B virus infection is a global public health problem, affecting more than 350 million people worldwide. The clinical manifestations of this infection vary greatly and include acute self-limiting disease, an inactive carrier state and CH with progression to LC and HCC (1, 2). An accumulating body of evidence indicates that the viral genotype (3, 4) and specific mutations in the viral genome (5, 6) are important viral factors contributing to the development of HCC. The main eight genotypes of HBV (A–H) have been identified based

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List of Abbreviations: γ-GTP, γ-glutamyl transpeptidase; AFP, alpha-fetoprotein; Alb, albumin; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate aminotransferase; BCP/CP, basal core promoter/core promoter; CH, chronic hepatitis; DB, direct bilirubin; EIA, enzyme-linked immunoassay; Glob, globulin; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinomas; HCV, hepatitis C virus; Kozak, Kozak sequence used in translation initiation site of eukaryotic mRNA; LC, liver cirrhosis; PLT, platelet; PT-INR, Prothrombin time-international normalized ratio; TB, total bililubin; TP, total protein.

on comparison of complete genomes, most genotypes having a distinct geographic distribution (7). There are some indications of correlations between HBV genotypes and clinical manifestations of this infection; one study showing that HBV genotype D is more strongly associated with severe liver disease and HCC than is genotype A (8). However, other studies found no association between genotype and clinical manifestations of this infection (9, 10). Specific mutations in the HBV genome reportedly affect both translation of the HBeAg and replication of HBV, thereby influencing the clinical manifestations of HBV infection and contributing to development of HCC (11, 12).

The aim of the current study was to investigate the distribution of HBV genotypes and subgenotypes in chronic hepatitis B patients in different regions of Turkey and to compare these distributions with those of HBV genotypes from other parts of the world. Our aim was to make it possible to draw inferences about disease transmission within Turkey, and between Turkey and other countries. This topic is particularly interesting, given Turkey's location at the crossroads of Europe and Asia. We also investigated the prevalence of BCP/CP mutations in patients with and without LC and/or HCC.

MATERIALS AND METHODS

Patients

In all, 198 patients with CHB were enrolled in the study. All were attendees at four clinical centers in geographically distinct parts of Turkey, namely Samsun (north), Ankara (center), Gaziantep (south) and Istanbul (west). The patients' ages ranged from 16 to 73 years. In 38 of the patients, LC or HCC had been diagnosed before enrollment (Table 1).

Diagnoses based on HBsAg seropositivity for longer than 6 months, clinical findings and liver biopsies were used to classify the patients into two clinical groups: (i) CH patients with persistently high serum ALT concentrations but no evidence of LC or HCC; and (ii) LC and/or HCC patients (hereafter referred to as LC/HCC patients) with clinical evidence of cirrhosis (e.g., coarse liver architecture, nodular liver surface and blunt liver edge) based on evidence of hypersplenism (e.g., splenomegaly demonstrated by ultrasonography or computed tomography and platelet counts of < 100,000 platelets mm^3) and complementary clinical information (e.g., ascites, jaundice, encephalopathy or esophageal varices), and/or HCC diagnosed on the basis of results of imaging studies together with high serum AFP concentrations (≥ 400 ng/mL). Sera were collected from each individual and stored immediately at −70°C until use. The serological and biochemical tests were performed at Ondokuz Mayis University (Kurupelit, Turkey). Molecular analyses were performed at the Department of Virology, Liver Unit, Nagoya City, University Graduate School of Medical Science, Nagoya, Japan. The study was approved by the Ethics Committee of the School of Medicine, Ondokuz Mayis University. Informed consent was obtained from all subjects and the study was conducted in accordance with the declaration of Helsinki (as revised in Tokyo 2004).

Table 1. Summary of samples collected

City (location)	N (%)
Samsun (north)	63 (31.8)
Ankara (center)	76 (38.4)
Gaziantep (south)	20 (10.1)
Istanbul (west)	39 (19.7)
Total	198 (100)

Serological analysis

Hepatitis B surface antigen, anti-HBs, HBeAg, anti-HBe, anti-HBc IgG, anti-Delta, and anti-HCV in patient serum samples were detected by ARCHITECT (Abbott Diagnostics, Lake Forest, IL, USA). Biochemical markers, including concentrations of anti-HCV, HBeAg, TP, Alb, Glob, PT-INR, AST, ALT, γ-GTP, ALP, TB, DB, and HBV DNA and PLT counts in all samples were measured at the local hospitals.

Genotyping of hepatitis B virus

Hepatitis B surface antigen-positive samples were subjected to HBV genotyping using commercially available EIA kits (Institute of Immunology, Tokyo, Japan). This method allows discrimination among the seven major HBV genotypes (A–G) by monoclonal antibodies targeted to the pre-S2 epitopes (2). HBV genotype H was not determined in this study because the EIA kit is unable to identify it.

Sequencing and phylogenetic analysis

Nucleic acids were extracted from 100 μL of serum using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Complete genomes were amplified using primer sets as described previously (13). Partial HBV genomes were also amplified in enhancer II/core promoter and precore regions as described previously (13).

PCR products were directly sequenced with the ABI PRISM BigDye v3.1 kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3100 DNA automated sequencer. All sequences were analyzed in both forward and reverse directions. Complete and partial genomes were assembled

using GENETYX Version 11.0 (GENETYX Corporation, Tokyo, Japan) Additional sequences were retrieved from the DNA Data Bank of Japan, EMBL Nucleotide Sequence Submissions and GenBank nucleic acid sequence databases for phylogenetic analysis. Phylogenetic relationships between sequences were determined using the neighbor-joining method using MEGA 4 software (14).

Quantification of serum hepatitis B virus DNA

Hepatitis B virus DNA was quantified using real-time detection PCR as previously described (15), with modifications as previously described (16). The detection limit of this assay was 100 copies/mL.

Statistical analysis

Statistical differences were evaluated by Fisher's exact test and the X^2 test, with Yates' correction for continuity where appropriate. Differences were considered significant for P-values < 0.05. All statistical analyses were performed using version 8.0 of the Stata Software package (StrataCorp LP, College Station, TX, USA).

RESULTS

Clinical characteristics of chronic hepatitis B patients in Turkey

Sera were collected from a wide area of Turkey. Figure 1 and Table 1 illustrate the locations of the four cities and the number of collected samples, respectively. The clinical characteristics of the 198 HBsAg-positive patients are summarized in Table 2. The LC/HCC patients were significantly older than the CH patients ($P < 0.0001$). Most (89.5%, 34 of 38 patients) were male. The concentrations of ALT, AST, and HBV DNA were not significantly different in LC/HCC patients compared to CH patients.

Hepatitis B virus genotypes

Hepatitis B virus genotypes were successfully determined in 185/198 HBsAg-positive patients by the EIA genotyping method. Genotyping was not possible for the remaining 13 patients because no HBV PCR products were detected in their samples.

Phylogenetic analysis of hepatitis B virus isolates based on complete genome sequencing

Whole HBV genomes were obtained from 36 of the HBsAg samples from four different geographical regions of Turkey, including 9 strains from Ankara, 15 from Samsun, 4 from Istanbul and 8 from Gaziantep (Fig. 2). All but one of these strains clustered with database reference strains representing genotype D, subgenotype D1. One strain isolated from a patient in Samsun clustered with subgenotype D3 references.

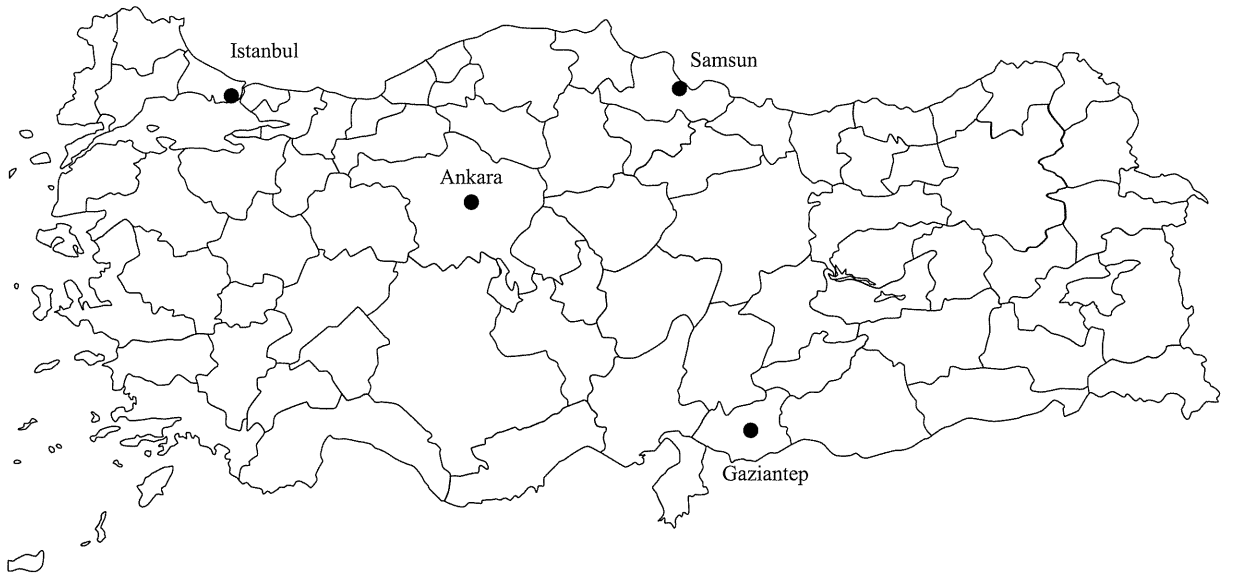

Fig. 1. Geographical locations from which samples were collected. All samples were collected at four clinical centers in geographically distinct parts of Turkey: Samsun (north), Ankara (center), Gaziantep (south), and Istanbul (west).

Table 2. Clinical characteristics of chronic viral hepatitis patients from Turkey with and without liver cirrhosis and/or hepatocellular carcinoma

Characteristic	Total ($n = 198$)	CH ($n = 160$)	LC/HCC ($n = 8$)	*P*-values
Age (years)†	41.4 ± 15	37.1 ± 13	58.9 ± 9	< 0.001
Male‡	132 (66.7)	98 (61.2)	34 (89.5)	< 0.001
Anti-HCV‡	1 (0.5)	1 (6%)	0	NS
Genotyped (HBV/D)‡	185 (93.4)	151 (94.4)	34 (89.5)	NS
HBeAg‡	68 (34.3)	59 (36.9)	9 (23.7)	NS
TP (g/dL)†	7.4 ± 0.8	7.6 ± 0.6	6.5 ± 1	< 0.001
Alb (g/dL)†	3.9 ± 0.7	4.1 ± 0.5	3.0 ± 0.7	0.001
Glob (g/dL)†	3.5 ± 0.6	3.5 ± 0.6	3.4 ± 0.6	NS
PLT count ($\times 10^3$ μL)†	150 ± 107	164 ± 112	94 ± 65	< 0.001
PT-INR†	1.1 ± 0.3	1.0 ± 0.2	1.5 ± 0.2	0.01
AST (IU/mL)†	93 ± 194	90 ± 208	107 ± 119	NS
ALT (IU/mL)†	123 ± 226	133 ± 247	80 ± 75	NS
	55 ± 47	52 ± 48	69 ± 37	NS
ALP (IU/L)†	133.1 ± 83.4	134 ± 84	130 ± 82	NS
TB (mg/dL)†	1.9 ± 5.5	1.1 ± 2.4	5.4 ± 10.9	< 0.001
DB (mg/dL)†	1.2 ± 4.7	0.5 ± 1.5	4.1 ± 9.9	< 0.001
HBV DNA ($\log_{10}$ copies/mL)†	5.4 ± 24	5.9 ± 25	0.2 ± 0.8	NS

NS, not significant.
†mean ± SD ‡number (%) of patients (percentage).

Basal core promoter and core region sequence analysis

To investigate genetic differences between LC/HCC and CH patients, the BCP/CP regions of HBV were successfully sequenced in samples from 22 LC/HCC patients and 52 age-, sex- and HBeAg-status-matched non-LC/HCC patients. Matching control subjects for these characteristics is important because HBV mutation rates are dependent on them. A summary of mutations observed in the BCP/CP region is presented in Table 3. There was a tendency toward a difference in the prevalence of the T1764G1766 double mutation ($P = 0.065$) and a statistically significant difference in prevalence of the A1896 mutation ($P = 0.03$) between LC/HCC and non-LC/HCC patients, a higher prevalence being found in LC/HCC patients. There was also a significant difference in the prevalence of the C1773 mutation, which was more frequently present in CH patients than in controls ($P = 0.05$).

Further, viral and host characteristics of the HBeAg-positive and HBeAg-negative patients were compared by using the samples from which the BCP/CP sequence were obtained (Table 4). Significant differences between the groups were observed in terms of the prevalence of V1753, A1757, and A1896 mutations ($P = 0.011$, 0.024, and 0.0001, respectively).

The T1773 mutation is associated with HBeAg-negative patients and is less often found in patients with advancing liver disease and infection with HBV genotypes B and C (17). However, in the present study, an excess of the T1773 mutation was not observed in patients with mild liver damage (Table 3) or HBeAg-negative patients (Table 4). On the other hand, specific mutation patterns were observed in HBeAg-negative patients infected with HBV/D. All the HBeAg-negative samples shown in Table 4 were allocated to two groups based on their 1773 mutation patterns (T1773 or C1773) and analyzed to determine any correlations with other mutations in the core promoter region. As shown in Table 5, the T1773 mutation coupled with the double mutation, T1764/G1766. In addition, the prevalence of A1757/T1764/G1766 mutations in the T1773 mutation group was statistically significant. The T1773 group had a larger viral load than did the C1773 group without the T1764/G1766 double mutation.

DISCUSSION

Viral hepatitis is one of the most prevalent and serious infectious diseases in the world and presents a serious public health problem. HBV infection follows different routes of inter- and intra-community transmission, various geographical, social and cultural factors playing important roles. The epidemiology of HBV genotypes provides useful information about population-specific behaviors, which may have direct or indirect roles in HBV transmission (18).

In this study, we investigated the genetic characteristics of HBV in a cohort of patients with CH with and without LC/HCC in Turkey. Phylogenetic analysis of complete genomes was carried out on HBV isolates from patients in different regions of Turkey to determine the distribution and transmission of different HBV genotypes within different areas of the country, and between Turkey and other parts of the world. In the present study in Turkey, all HBV genotypes (subgenotypes) were D1 type except for one isolate. There were no specific phylogenetic groupings of HBV isolates according to geography within Turkey. Previous studies have reported a high prevalence of genotype D1 (approximately 89%) in Mongolia (19, 20). They showed that HCV and Delta virus co-infections with HBV infection confer a high risk of HCC. These studies differ from the present study in that they investigated cases of co-infection with HBV and HCV or with HBV and Delta virus to assess association with HCC whereas we assessed cases of monoinfection. However, in Turkey genotype D1 infection without HCV co-infection is characterized by early HBeAg seroconversion, a small viral load upon seroconversion

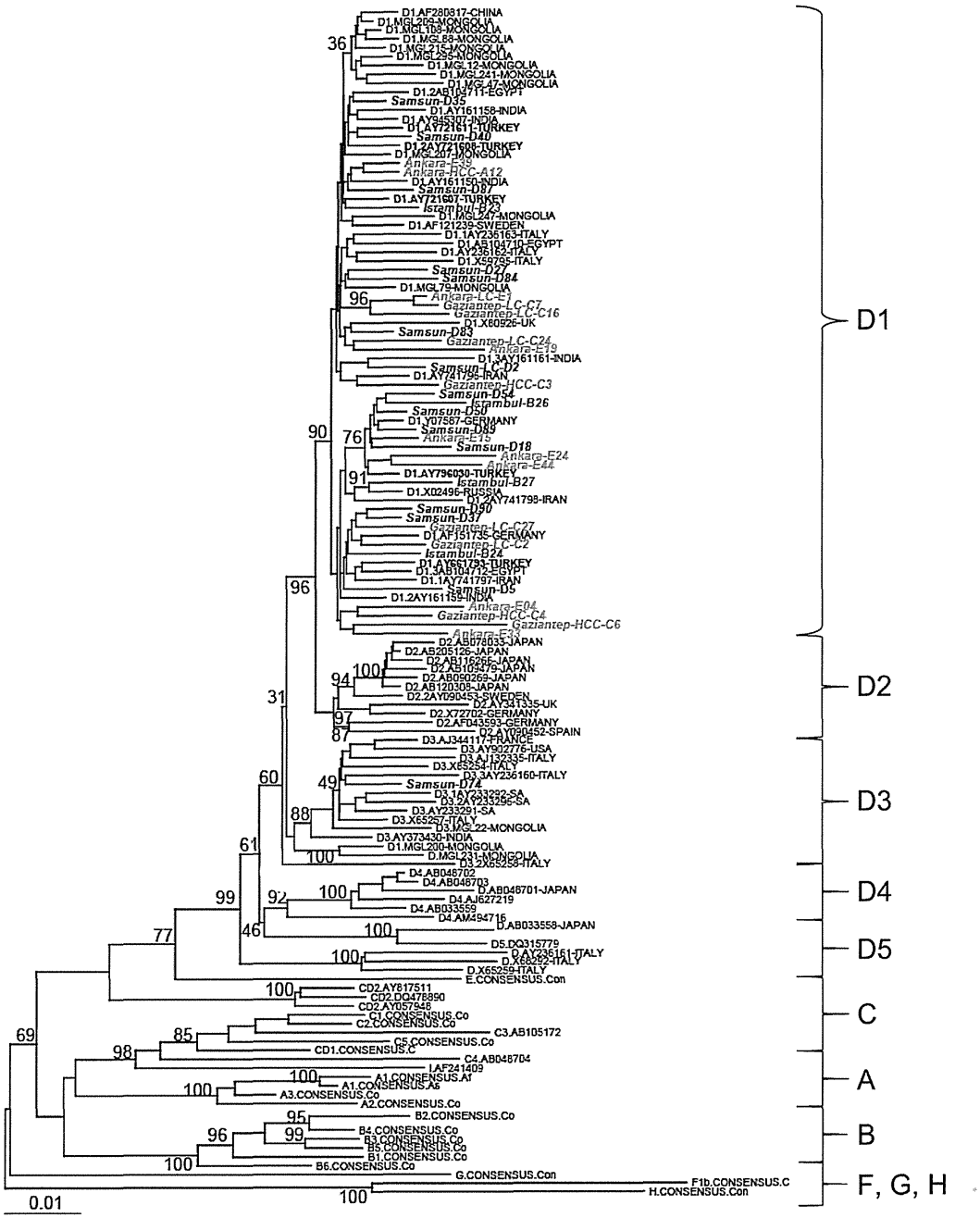

Fig. 2. Neighbor-joining phylogram based on complete hepatitis B virus genomes from Turkey and other countries. Sequences from the current study are color-coded according to the region of Turkey from which they were isolated (blue, Samsun; green, Ankara; orange, Gaziantep; red, Istanbul), and other Turkish sequences are labeled in bold. The study isolates were subjected to bootstrap re-sampling with all available complete genome sequences obtained from the EMBL, DDBJ, and GenBank nucleic acid sequence databases. Sequences used for the phylogenetic tree are indicated under the corresponding accession numbers from sequence databases and country of origin.

and a relatively low incidence of LC/HCC in those infected, which might indicate that HCV and Delta virus co-infection change the pathogenesis of HBV genotype D1.

Two previous studies on Iranian and Mongolian genotype D isolates reported a genotype-specific pattern of the functionally important BCP/CP region, with A1757 and T1764/G1766 (21, 22). Our *in vitro*