demonstrated significant associations between the HBV genotypes and the severity of liver disease, clinical outcomes and the response to antiviral therapies (Kramvis & Kew, 2005). Moreover, it was also demonstrated that the clinical and virological characteristics may also differ among patients infected with the same genotype (Miyakawa & Mizokami, 2003). The existence of different subtypes (subgenotypes) within same genotype helps to explain this for HBV/B, where one of the subtypes (subgenotypes) (widespread in Asia; Ba) possesses a recombination with genotype HBV/C, while another (indigenous to Japan; Bj) does not (Sugauchi *et al.*, 2003). Similarly, two subtypes (subgenotypes) have been reported for HBV/A: one of them, Aa (A′/A1) prevails in sub-Saharan Africa and South Asia, while the other, Ae (A2), is widely distributed in Europe and the USA (Bowyer *et al.*, 1997; Kramvis *et al.*, 2002; Sugauchi *et al.*, 2004). The subtypes (subgenotypes) of HBV/A show no evidence of distinguishing recombination; nevertheless, they are associated with differences in replicative activity, and in the mechanisms of HBeAg seroconversion as a result of specific nucleotide substitutions in the core promoter and precore regions (Kimbi *et al.*, 2004; Sugauchi *et al.*, 2004; Tanaka *et al.*, 2004).

The characterization of isolates from indigenous populations, especially in Africa where HBV is hyperendemic, may assist in revealing the origin of HBV and clarify the many questions about its evolutionary history (Kramvis *et al.*, 2005). The genetic diversity and distribution of HBV genotypes in Central West Africa, particularly in Cameroon, are poorly documented. No data were available for the HBV strains from Pygmies in this region. The objectives of the present study were to assess the prevalence of HBV and hepatitis C virus (HCV) markers among Bantus and Pygmies, to compare the distribution of HBV genotypes and to analyse the genomic characteristics of the HBV/A strain in Cameroon. Six full genome sequences, including four representing a new subtype (subgenotype) of HBV/A and two HBV/E strains from the Cameroonian Pygmies, were analysed.

METHODS

Blood serum samples. Blood serum samples were collected in 1994 from 544 voluntary donors, including representatives of two relatively isolated populations (Bantu and Pygmies) in Cameroon, Central West Africa. The Pygmies enrolled were from two forest encampments in the East province, and the Bantu were enrolled from five provinces across the country (Central, South, North, West and East). None of the donors had clinical symptoms of liver disease. Written informed consent was obtained from all subjects enrolled. After isolation of the serum fraction from whole blood, the samples were stored at −40 °C until use. The number of subjects studied in each group, their ages and sexes are summarized in Table 1.

Serological assays for hepatitis virus markers and HBV genotyping. HCV (anti-HCV) and HBV serological markers (HBsAg, HBeAg, anti-HBs and anti-HBc) were examined using a chemiluminescent immunoassay (Ortho Clinical Diagnostics).

HBsAg-positive samples were subjected to HBV genotyping using an enzyme-linked immunoassay (EIA) with monoclonal antibodies to type-specific epitopes of the preS2 region (Usuda *et al.*, 1999), using commercial kits (HBV Genotype EIA; Institute of Immunology Co.).

Table 1. The distribution of HBV and HCV serological markers and HBV genotypes among two populations in Cameroon

Population	Bantu ($n=370$)	Pygmies ($n=174$)	*P*
Male/Female*	177/188	87/82	NS†
Age (years); mean ± SD	34·2 ± 14·5	29·9 ± 9·2	<0·05
Anti-HCV	75 (20·3 %)	4 (2·3 %)	<0·0001
HBsAg	33 (8·9 %)	13 (7·5 %)	NS
HBeAg	5 (15·2 %)	0	NS
Anti-HBs	77 (20·8 %)	17 (9·8 %)	0·001
Anti-HBc	322 (87·0 %)	150 (86·2 %)	NS
HBV/A	15 (45·5 %)	5 (38·5 %)	NS
HBV/E	13 (39·4 %)	7 (53·8 %)	NS
HBV/D	5 (15·1 %)	1 (7·7 %)	NS

*Gender and age data were not available for some of the specimens.
†NS, Not significant.

Amplification, quantification of HBV DNA and nucleotide sequencing. DNA was extracted from 27 serum samples, in which HBV/A and HBV/E had been identified by genotyping EIA: 20 (15 from Bantu and five from Pygmies) and seven (only from Pygmies), respectively. Total DNA was extracted from 100 µl serum using a QIAamp DNA mini kit (Qiagen) and suspended in 100 µl storage buffer (supplied by the kit manufacturer). A real-time PCR assay, allowing detection of up to 100 viral DNA copies ml^{-1} (Abe *et al.*, 1999), with slight modifications (Tanaka *et al.*, 2004), was used for HBV DNA screening.

Two overlapping HBV DNA fragments covering the entire genome sequence were amplified using specific primers and PCR conditions that have been described previously (Sugauchi *et al.*, 2001). Amplified HBV DNA fragments were sequenced directly using a Prism Big Dye v3.0 kit (Applied Biosystems) on an ABI 3100 DNA automated sequencer (Applied Biosystems). All sequences were analysed in both the forward and reverse directions. Complete and partial HBV genomes were assembled using GENETYX v11.0 (Software Development). The nucleotide sequence data reported in this paper appear in the GenBank/EMBL/DDBJ nucleotide sequence databases with the accession numbers AB194947–AB194955.

Sequence analysis. Sequences were aligned using the CLUSTAL W software program (Thompson *et al.*, 1997). Phylogenetic trees were constructed using neighbour-joining (NJ) analysis incorporating the six-parameter distance correction method (Gojobori *et al.*, 1982) with bootstrap test confirmation performed on 1000 resamplings using the Online Hepatitis virus database (http://s2as02.genes.nig.ac.jp/). Preliminary trees were constructed for Cameroonian HBV strains obtained in this study and corresponding data of 632 HBV genome sequences available from the GenBank/DDBJ databases (the trees are available from the authors). The final trees presented herein were constructed for Cameroonian strains together with the selected GenBank/DDBJ references including the HBV/A strains of various geographical origins, and representatives of other known human HBV genotypes.

Nucleotide divergence over complete genomes was calculated using the CLUSTAL method implemented in the MEGALIGN software (Clewley & Arnold, 1997).

Detection of recombination. All Cameroonian strains' complete genome sequences were examined for the presence of recombination with other HBV genotypes, as described previously (Robertson *et al.*, 1995). Bootscan analysis implemented in the SimPlot software program (Lole *et al.*, 1999) was performed for each of the strains.

Statistical analysis. All statistical values were calculated using the Mann–Whitney U test, Fisher's exact test and the χ^2 test with Yate's correction, implemented in the STATA v8.0 software program (Stata). Differences were considered significant for P values less than 0·05.

RESULTS

Hepatitis virus serological markers and HBV genotypes in Cameroon

Table 1 summarizes results of the serological screening and HBV genotyping. The overall anti-HCV seroprevalence was very high (14·5 %), and was significantly higher in Bantus (20·3 %) than in Pygmies (2·3 %, $P<0·0001$), demonstrating that transmission networks of the infection are relatively isolated between two populations (blood transfusion and medical procedures probably contributed to transmission in the Bantus but not in the Pygmies; Kowo *et al.*, 1995). Nevertheless, HBsAg prevalence was equally high in both

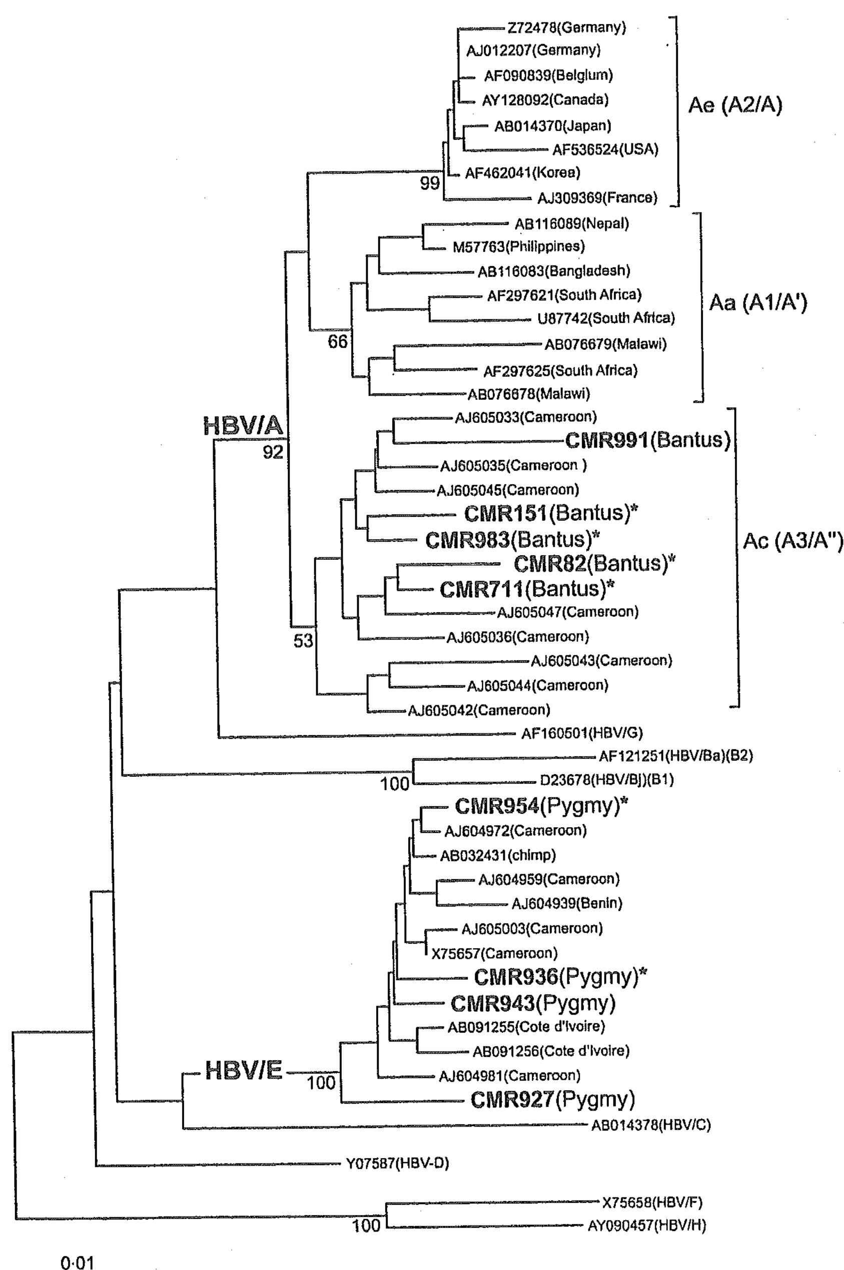


Fig. 1. A phylogenetic NJ tree constructed using the HBV preS2/S nucleotide sequences. Nine strains from Cameroon isolated in this study are indicated in bold. Reference sequences were retrieved from GenBank/EMBL/DDBJ with their accession numbers and origin (in parentheses) indicated. Bootstrap values are indicated in the tree roots. Asterisk (*) marked strains, four HBV/A and two HBV/E, from Cameroon were used for further analyses based on the complete genome sequences.

populations (8·9–7·5 %). HBeAg examined among HBsAg-positive carriers was determined in 10·9 % of cases, all of which were in the Bantus infected with HBV/E (mean age 21·2 years, range 1–30 years). The mean age of HBeAg-negative carriers in the Bantus was 31·6 years, range 17–90 years. All HBsAg-positive carriers among the Pygmies were negative for HBeAg (mean age 29·5 years, range 27–38 years). Thus, the mean age of the HBeAg-negative group was relatively young for HBsAg carriers in both populations in Cameroon, suggesting early HBeAg seroconversion. Anti-HBc seroprevalence was very high in both populations (mean 86·7 %), with no significant difference (86·2 vs 87 %), concordant with a previous report (Ndumbe *et al.*, 1993) and indicating a high incidence of HBV infection in both populations, probably attributable to effective horizontal transmission at a young age, as reported previously in African countries (Kramvis *et al.*, 2005). There was no significant difference in the distribution of the examined viral markers among the Bantu population in different provinces, or among the Pygmies population in the different encampments. A total of 46 serum samples found to be positive for HBsAg were subjected to HBV genotyping using the EIA method. Genotypes A and E identified in 43·5 % of cases were equally predominant in both of the populations, and genotype D was found in a minority (13 %) of cases. No significant difference in distribution of the genotypes was found within the same population in different provinces or between the two populations.

In order to study the molecular genetic characteristics of the prevalent HBV genotypes in Cameroon, 20 HBV/A and seven HBV/E samples, for which sufficient volume was available, were subjected to further investigation. Of the

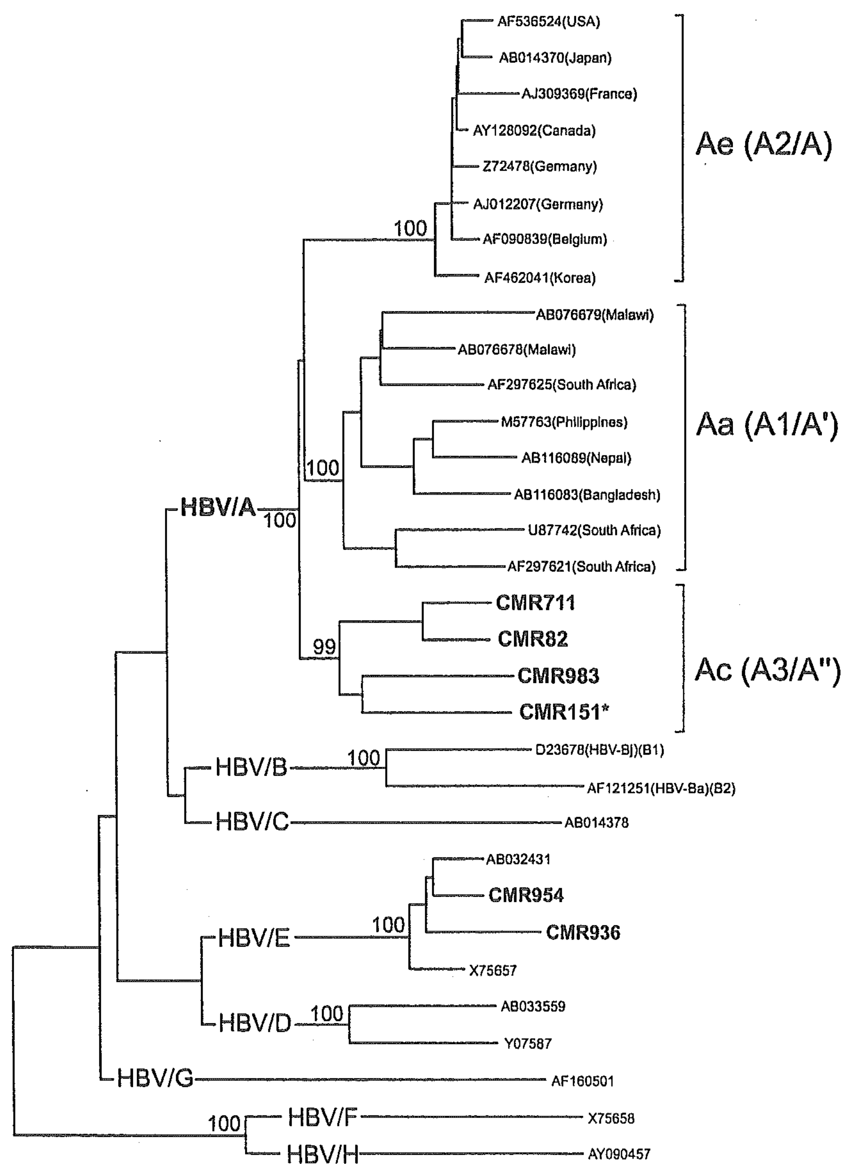
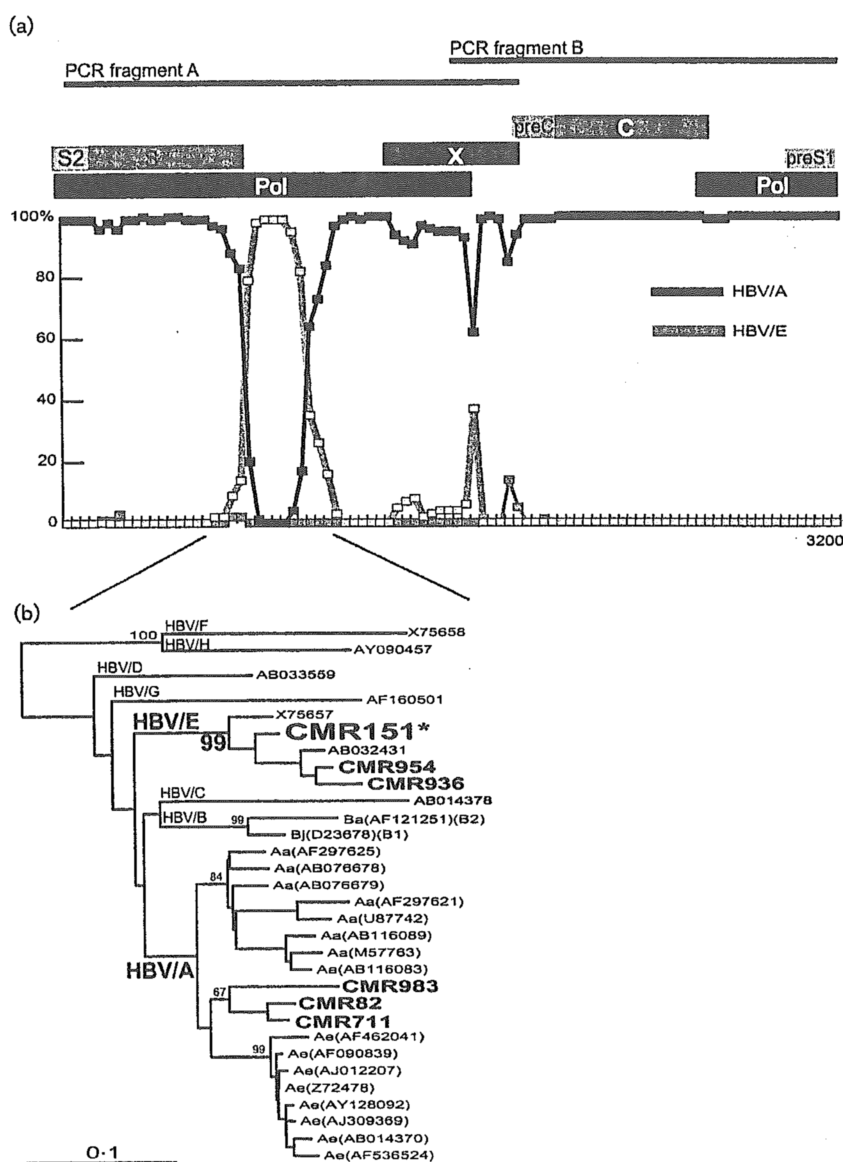


Fig. 2. A phylogenetic NJ tree constructed using the complete HBV genome. Six strains from Cameroon isolated in this study are indicated in bold. Accession numbers are given for reference sequences retrieved from GenBank/EMBL/DDBJ. The origins of the previously published HBV/A strains are indicated in parentheses. Bootstrap values are indicated in the tree roots. The strain from Cameroon with the recombination between HBV/A and HBV/E is marked with an asterisk (*).

samples, only 1/27 was HBeAg-positive (HBV/E by EIA), which was obtained from a 1-year-old child, and the rest (26/27) of the HBsAg-positive carriers had undergone HBeAg seroconversion.

HBV DNA quantification, sequencing, phylogenetic relation and genetic diversity of HBV/A subtypes (subgenotypes)

HBV DNA was detected in only 10/27 serum samples: 5/15 Bantus and 5/12 Pygmies. The highest HBV DNA level ($3{\cdot}4\times10^{10}$ copies ml^{-1}) was detected in the sample obtained from a 1-year-old child. The rest of the nine positive samples were obtained from (mean) 26-year-old carriers (range 21–30 years), with HBV DNA levels ranging from $1{\cdot}1\times10^{3}$ to $7{\cdot}8\times10^{5}$ copies ml^{-1}. HBV DNA-negative carriers were (mean) 30·4 years old, range 17–50 years, showing a general tendency of HBV DNA level to decline with age (not statistically significant, probably due to small numbers). HBV large S coding region sequences were successfully amplified from 9/10 samples. The sequences were subjected to a similarity search throughout GenBank/DDBJ using the BLAST search engine, and the most similar strains were used for phylogenetic analysis together with the reference sequences of all known human HBV genotypes. The phylogenetic relationship of the ~800 nt (positions 31–835) preS2/S sequences of the HBV strains is represented in Fig. 1. Within the HBV/A phylogenetic cluster, the HBV/Aa (A1) and HBV/Ae (A2) strains separated out into two clusters and the five Cameroonian strains sequenced in this study together with other Cameroonian strains retrieved from GenBank/DDBJ clustered separately. The Cameroonian strains retrieved from GenBank/DDBJ were previously designated A″ cluster according to partial (Large S) genome sequence (Mulders *et al.*, 2004). The Cameroonian and HBV/Aa (A1) subclusters, however, did not have significant bootstrap indexes.



The complete genome of six strains (four HBV/A and two HBV/E) were sequenced successfully (marked by asterisks in Fig. 1). The lengths of the complete genomes corresponding to HBV/A and HBV/E were 3221 and 3212 nt, respectively. The phylogenetic analysis of the complete genome sequences (Fig. 2) revealed three distinct bootstrap test supported groups within the HBV/A cluster: HBV/Aa (A1), HBV/Ae (A2), and the third group formed by samples from Cameroon. We denoted the third phylogenetic group as 'HBV/Ac', where 'c' stands for Cameroon and Central Africa. The distinctive grouping of HBV/Ac (A3) strains was also confirmed when preS1/S2, preC/C, and complete Pol genes were analysed phylogenetically. S and X genes were phylogenetically related between the HBV/Aa (A1) and HBV/Ac (A3) groups. Estimated inter-group percentage nucleotide divergence over complete genome sequences consisted of [mean $\pm$SD (range)]: 4·9$\pm$0·4 (4·2–6·1), Aa (A1) versus Ae (A2); 5·1$\pm$0·5 (4·0–6·0), Aa (A1) versus Ac (A3); and 5·2$\pm$0·3 (4·7–5·8), Ae (A2) versus Ac (A3). On the other hand, intra-group percentage nucleotide divergence was similar for HBV/Aa (A1) and HBV/Ac (A3) [mean $\pm$SD (range)]: 3·6$\pm$0·8 (4·0–4·6) and 3·9$\pm$1·1 (1·8–4·8), respectively, and lowest for Ae (A2): 0·9$\pm$0·3 (0·4–1·6) ($P<0·0001$).

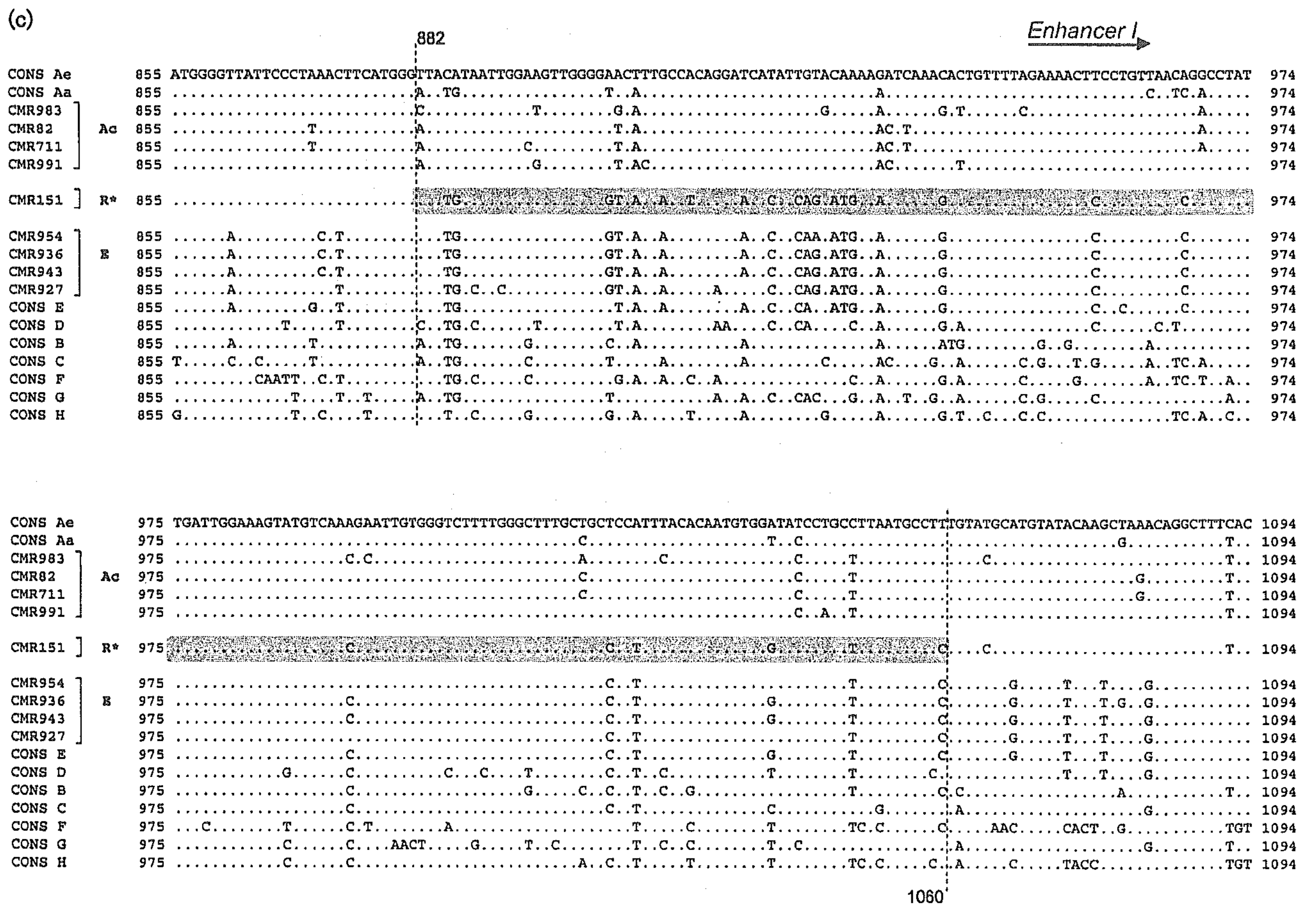


Fig. 3. (a) SimPlot analysis demonstrating the recombination in the non-overlapping part of the polymerase coding region of the CMR151 strain. The strain was subjected to bootscan analysis over the entire genome using the SimPlot program (Lole *et al.*, 1999) with a window size of 300 bp and a step size of 30 bp, under the F84 (ML) model, with bootstrap resampling performed 1000 times. Initially, consensus sequences of each human HBV genotype were used as references; after manual confirmation of the sequence alignment, the final plot was constructed using the consensus of the HBV/A, HBV/E and HBV/D genotypes only. The sequences were obtained from two overlapping PCR fragments, indicated by two lines ('A' and 'B'). HBV genome coding regions are indicated by standard abbreviations (S2, S, Pol, X, preC, C and preS1). (b) The phylogenetic NJ tree constructed using the recombinant segment. Six strains from Cameroon in this study are indicated in bold. Accession numbers are given for reference sequences retrieved from GenBank/EMBL/DDBJ. (c) Alignment of all human HBV genotype genome nucleotide sequences in the region corresponding to the recombination in the Cameroonian strain CMR151 (shaded in grey). Nucleotide positions correspond to the HBV genome reference sequence, GenBank accession no. NC_003977. Dashed lines at 882 and 1060 represent the breakpoints.

Recombination

Evidence of recombination between HBV/A and HBV/E was observed in one of the Cameroonian strains (CMR151, marked by an asterisk in Fig. 2). The result of the bootscan analysis for the complete genome sequence of the strain is presented in Fig. 3(a). The phylogenetic tree constructed using the corresponding sequence segment confirmed the grouping of the CMR151 strain together with the HBV/E strains, with strong bootstrap support (Fig. 3b). The recombinant segment corresponded to a part of the non-overlapping HBV DNA polymerase in the reverse transcriptase (RT) domain and a part of the enhancer I–X promoter. (Fig. 3c). The breakpoints at nucleotide positions 882 and 1060 were estimated by mapping the informative sites and using χ^2 confirmation (Robertson *et al.*, 1995).

Enhancer/promoter elements and amino acid characteristics of the HBV/Ac (A3) strains

A comparison of the nucleotide substitutions within the *cis*-acting elements among the four HBV/Ac (A3) strains and the consensus sequences of the HBV/Aa (A1) and HBV/Ae (A2) subtypes (subgenotypes) as well as the other HBV genotypes (including HBV/Ba, Bj, B2, B1) are summarized in Table 2. Nine specific nucleotide substitutions were found in HBV/Ac (A3) strains: $G^{1173}A$ (enhancer I–X promoter), $C^{1473}G$, $G^{1512}A$ and $C^{1703}T$ (enhancer II–core promoter), $A^{2742}G$ (S1–promoter), $C^{3021}T$, $C^{3042}T$,

Table 2. Subtype (subgenotype) specific sites (bold) within enhancers and promoter regions of HBV/Aa (A1), HBV/Ac (A3) and HBV/Ae (A2)

Nucleotide positions correspond to the HBV genome reference sequence, GenBank accession no. NC_003977. Consensus sequences were composed according to 60 % or higher incidence at the corresponding nucleotide position.

Region	Position (nt)	HBV/Aa (A1)	HBV/Ac (A3)				HBV/Ae (A2)
			CMR711	CMR82	CMR983	CMR151	
Enhancer I–X promoter (950–1350)	963	C	T	T	T	T	T
	1041	T	A	A	A	G	A
	1173	G	–	A	A	A	–
	1320	A	A	A	A	A	G
	1350	T	–	–	–	–	C
Enhancer II–core promoter (1400–1850)	1404	T	–	–	–	–	C
	1464	G	T	T	T	T	T
	1473	C	G	G	–	T	–
	1484	A	–	–	–	–	C
	1511	G	–	–	–	–	A
	1512	T	A	A	–	A	G
	1703	C	T	T	T	A	–
	1727	A	–	–	–	–	G
	1740	T	–	–	–	–	C
	1809–1812	TCAT	-TC-	--C-	----	G--C	G--C
Encapsidation signal (1846–1908)	1888	A	G	G	G	G	G
S1–Promoter (2716–2806)	2720	A	T	T	T	T	G
	2742	A	G	G	G	G	–
	2744	C	A	A	–	–	–
	2777	G	C	T	T	T	T
S2–Promoter (2999–3219)	3013–3014	CA	--	--	--	--	GC
	3021	C	T	T	T	T	–
	3042	C	T	T	T	–	–
	3052	T	–	–	–	–	C
	3057/60	T/C	C/T	C/T	C/T	C/T	C/T
	3069	A	–	–	–	–	C
	3072–3073	TG	A-	C-	--	--	-A
	3076	T	C	C	C	C	–
	3111	T	T	T	T	T	C
	3118	C	–	–	–	–	T
	3121	G	–	–	–	–	A
	3124	G	–	–	–	–	A

$T^{3076}C$ and $C^{3111}T$ (S2-promoter). Interestingly, three of four strains had substitutions in the Kozak sequence (1809–1812) (Ahn *et al.*, 2003; Tanaka *et al.*, 2004), and two had a basal core promoter double mutation (1762/1764).

Although HBV/Ac (A3) amino acid motifs in general were more similar to HBV/Aa (A1) than to HBV/Ae (A2) motifs, HBV/Ac (A3) strains had some specific sites: Thr[84] in preS1, Ala[146] in Pol/terminal protein, Ser[239], Trp[246], Ser[257] in Pol/spacer, Asp[356], Arg[501], Ser[607] in Pol/RT, and Thr[47] in X proteins when compared with consensus sequences composed according to 60 % or higher incidence at the corresponding amino acid position (Table 3). Pre-core/core amino acid patterns had no specific substitutions among HBV/A subtypes (subgenotypes).

Table 3. Subtype (subgenotype) specific sites (bold) in amino acid sequences of HBV/Aa (A1), HBV/Ac (A3) and HBV/Ae (A2)

Consensus sequences were composed according to 60 % or higher incidence at the corresponding amino acid position.

ORF	Position (aa)	HBV/Aa (A1)	HBV/Ac (A3)				HBV/Ae (A2)
			CMR711	CMR82	CMR983	CMR151	
PreS1	54	Q	–	–	–	–	A
	67	F	–	–	–	–	L
	74	V	–	–	–	–	I
	84	I	T	T	T	–	–
	86	A	T	T	T	T	T
	89	P	–	–	–	–	S
	90	A	–	–	–	–	T
	91	V	–	–	–	–	I
PreS2	32	L	V	V	V	V	V
	47	S	–	–	–	–	A
S	209	L	–	–	–	–	V
Pol/terminal protein	17	E	G	G	G	G	G
	33	E	–	–	A	A	A
	74	P	Q	Q	–	–	–
	102	T	N	N	–	–	–
	120	N	–	–	–	–	T
	146	T	A	A	A	A	–
Pol/spacer	236	T	–	–	–	–	S
	239	P	S	S	S	S	–
	246	R	W	W	W	–	–
	257	F	S	S	S	S	–
	269	Y	–	–	–	–	H
	271	A	–	–	–	–	V
	273	S	–	–	–	–	N
	308	S	K	K	–	–	C
	334	Q	–	–	K	K	K
	338	K	E	E	E	E	E
	348	L	–	–	–	–	R
Pol/RT	356	E	D	–	D	D	–
	501	W	R	R	R	R	–
	607	T	S	S	A	S	–
	617	I	L	L	–	–	–
	619	H	–	–	D	D	–
	666	K	R	R	–	–	–
X region	11	S	–	–	–	–	P
	31	A	S	S	S	S	S
	34	L	V	V	–	F	–
	47	S	T	T	–	T	A
	146	S	F	–	–	A	A
	147	S	–	–	–	P	P

DISCUSSION

A previous study carried out in Cameroon among the Bantus and the Pygmies (Kowo *et al.*, 1995) demonstrated a high (18·6 %) overall seroprevalence of HCV, which was significantly higher in Bantus (31·7 %) than in Pygmies (11·1 %). The results of the present study also indicate the very high HCV seroprevalence (14·5 %), and support the difference between the two populations. However, in our study, HCV seroprevalence among the Pygmies was lower (2·3 %), which might be attributed to the younger age of examined subjects compared with the cohort previously studied (Kowo *et al.*, 1995). The difference in HCV seroprevalence between the two populations might be explained by exposure of the Bantus to transmission routes such as medical procedures and blood transfusion, to which the Pygmies are not exposed. However, HBV seroprevalence (HBsAg and anti-HBc) was equally high among the two populations and different regions of the country, which is concordant with previous data (Ndumbe *et al.*, 1993). Further epidemiological investigation is required to evaluate factors contributing to the difference in HBV and HCV transmission in the Pygmies, in contrast with neighbouring Bantus.

The only data available on HBV genotypes in Cameroon demonstrated the predominant prevalence of HBV/A in human immunodeficiency virus-positive cohort (Mulders *et al.*, 2004). The present study revealed that both HBV/A and HBV/E are distributed equally in both native populations in Cameroon. The phylogenetic analysis revealed a close relationship in the large S coding region among the Cameroonian strains sequenced in this study and those from the same country available from previous reports (Mulders *et al.*, 2004; Norder *et al.*, 1992). Based on phylogenetic analysis of the complete genome, including four sequences in this study, the presence of a third phylogenetic cluster was confirmed within HBV/A in this study. The cluster was distinct from known HBV/Aa (A1) and HBV/Ae (A2) subtypes (subgenotypes), and designated HBV/Ac (A3) (where 'c' stands for Cameroon and Central Africa). The inter-subtype (subgenotype) nucleotide divergence over the complete genome sequences falls within the 4–8 % range that justifies the classification of HBV/Ac (A3) into a distinct subtype (subgenotype) according to the recent proposals on HBV nomenclature (Kato *et al.*, 2005; Kramvis *et al.*, 2005). The high intra-subtype (subgenotype) nucleotide divergence of four HBV/Ac (A3) complete genomes suggests a long natural history of this subtype (subgenotype) within the native population of Cameroon, as has been reported for subtype (subgenotype) HBV/Aa (A1) in southern African Blacks (Kimbi *et al.*, 2004). On the other hand, HBV/E strains obtained from the Pygmies did not group together separately from the strains isolated in different geographical regions, even though the Pygmies represent an isolated population in Africa. The presence of low divergent HBV/E genotype among the Pygmies might not support the hypotheses proposed previously that HBV/E has a very short history in humans (Mulders *et al.*, 2004).

The newly described subtype (subgenotype) HBV/Ac (A3) possesses a combination of the sites specific for either HBV/Aa (A1) or HBV/Ae (A2) within the corresponding enhancer/promoter elements and amino acid motifs (Kimbi *et al.*, 2004; Sugauchi *et al.*, 2004; Tanaka *et al.*, 2004). Moreover, the subtype (subgenotype) also has HBV/Ac (A3) unique substitutions. The recombination affecting a short, non-overlapping segment of the polymerase RT domain found in one of the Cameroonian strains is the first event documented to have occurred between HBV/A and HBV/E. The sequencing data generated in the present study could be used to design assays that can discriminate between HBV/Ac (A3) and the other subtypes (subgenotypes) of HBV/A in order to characterize its clinical–virological features. Cohort studies are required to investigate a possible association of HBV/Ac (A3) infection with early HBeAg/anti-HBe seroconversion and low HBV DNA levels in carriers indicated by the tendencies observed on the small number investigated in present study.

At the present time, investigation of HBV molecular heterogeneity, global distribution of HBV genetic forms, including recombination and mutations as well as efficient implications of the data, is one of the major directions in the field of virus research (Kramvis *et al.*, 2005). In this respect, further standardization of the HBV nomenclature and, an efficient and logical classification should be based on a consensus of the accumulated data including recent studies.

In conclusion, the complete genome of the third subtype (subgenotype) of HBV/A, identified in Cameroon, has been analysed and unique nucleotide/amino acid substitutions have been identified within this subtype (subgenotype). The high intra-group divergence suggests that this subtype (subgenotype) represents an indigenous HBV strain with a long natural history. Recombination between this subtype (subgenotype) and genotype E is described.

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REFERENCES

Abe, A., Inoue, K., Tanaka, T., Kato, J., Kajiyama, N., Kawaguchi, R., Tanaka, S., Yoshiba, M. & Kohara, M. (1999). Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. *J Clin Microbiol* **37**, 2899–2903.

Ahn, S. H., Kramvis, A., Kawai, S., Spangenberg, H. C., Li, J., Kimbi, G., Kew, M., Wands, J. & Tong, S. (2003). Sequence variation upstream of precore translation initiation codon reduces hepatitis B virus e antigen production. *Gastroenterology* **125**, 1370–1378.

Arauz-Ruiz, P., Norder, H., Robertson, B. H. & Magnius, L. O. (2002). Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* **83**, 2059–2073.

Bowyer, S. M., van Staden, L., Kew, M. C. & Sim, J. G. (1997). A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. *J Gen Virol* **78**, 1719–1729.

Clewley, J. P. & Arnold, C. (1997). MEGALIGN. The multiple alignment module of LASERGENE. *Methods Mol Biol* **70**, 119–129.

Gojobori, T., Ishii, K. & Nei, M. (1982). Estimation of average number of nucleotide substitutions when the rate of substitution varies with nucleotide. *J Mol Evol* **18**, 414–423.

Kane, M. (1995). Global programme for control of hepatitis B infection. *Vaccine* **13**, S47–S49.

Kato, H., Fujiwara, K., Gish, R. G. & 9 other authors (2005). Classifying genotype F of hepatitis B virus into F1 and F2 subtypes. *World J Gastroenterol* (in press).

Kimbi, G. C., Kramvis, A. & Kew, M. C. (2004). Distinctive sequence characteristics of subgenotype A1 isolates of hepatitis B virus from South Africa. *J Gen Virol* **85**, 1211–1220.

Kowo, M. P., Goubau, P., Ndam, E. C., Njoya, O., Sasaki, S., Seghers, V. & Kesteloot, H. (1995). Prevalence of hepatitis C virus and other blood-borne viruses in Pygmies and neighboring Bantus in southern Cameroon. *Trans R Soc Trop Med Hyg* **89**, 484–486.

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* (in press).

Kramvis, A., Weitzmann, L., Owiredu, W. K. & Kew, M. C. (2002). Analysis of the complete genome of subgroup A′ hepatitis B virus isolates from South Africa. *J Gen Virol* **83**, 835–839.

Kramvis, A., Kew, M. & Francois, G. (2005). Genotypes of hepatitis B virus. *Vaccine* **23**, 2407–2421.

Lole, K. S., Bollinger, R. C., Paranjape, R. S., Gadkari, D., Kulkarni, S. S., Novak, N. G., Ingersoll, R., Sheppard, H. W. & Ray, S. C. (1999). Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. *J Virol* **73**, 152–160.

Miyakawa, Y. & Mizokami, M. (2003). Classifying hepatitis B virus genotypes. *Intervirology* **46**, 329–338.

Mulders, M. N., Venard, V., Njayou, M. & 11 other authors (2004). Low genetic diversity despite hyperendemicity of hepatitis B virus genotype E throughout West Africa. *J Infect Dis* **190**, 400–408.

Ndumbe, P. M., Atchou, G., Biwole, M., Lobe, V. & Ayuk-Takem, J. (1993). Infections among pygmies in the Eastern Province of Cameroon. *Med Microbiol Immunol* **182**, 281–284.

Norder, H., Hammas, B., Lofdahl, S., Courouce, A. M. & Magnius, L. O. (1992). Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. *J Gen Virol* **73**, 1201–1208.

Norder, H., Courouce, A. M. & Magnius, L. O. (1994). Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology* **198**, 489–503.

Okamoto, H., Tsuda, F., Sakugawa, H., Sastrosoewignjo, R. I., Imai, M., Miyakawa, Y. & Mayumi, M. (1988). Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* **69**, 2575–2583.

Robertson, D. L., Hahn, B. H. & Sharp, P. M. (1995). Recombination in AIDS viruses. *J Mol Evol* **40**, 249–259.

Stuyver, L., De Gendt, S., Van Geyt, C., Zoulim, F., Fried, M., Schinazi, R. F. & Rossau, R. (2000). A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* **81**, 67–74.

Sugauchi, F., Mizokami, M., Orito, E. & 7 other authors (2001). A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. *J Gen Virol* **82**, 883–892.

Sugauchi, F., Orito, E., Ichida, T. & 10 other authors (2003). Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. *Gastroenterology* **124**, 925–932.

Sugauchi, F., Kumada, H., Acharya, S. A. & 10 other authors (2004). Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. *J Gen Virol* **85**, 811–820.

Tanaka, Y., Hasegawa, I., Kato, T. & 11 other authors (2004). A case-control study for differences among hepatitis B virus infections of genotypes A (subtypes Aa and Ae) and D. *Hepatology* **40**, 747–755.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Usuda, S., Okamoto, H., Iwanari, H., Baba, K., Tsuda, F., Miyakawa, Y. & Mayumi, M. (1999). Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. *J Virol Methods* **80**, 97–112.

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Variations in the Viral NS5B Region in Japanese Patients with Chronic Hepatitis C Virus Genotype 1b Infection

No Specific Amino Acid Substitution Was Identified as Determinants of Treatment Response to Interferon/Ribavirin Combination Therapy

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Key Words
Hepatitis C virus · Nucleotide · Amino acids, substitutions · Interferon · Ribavirin · Genotype

Abstract
Objective: A recent study suggested that the substitution of amino acid 415 of HCV NS5B from phenylalanine to tyrosine in patients with HCV genotype 1a infection is induced by ribavirin and responsible for resistance to ribavirin therapy. The aim of this study was to evaluate whether specific variations in the HCV NS5B sequence in Japanese patients with HCV genotype 1b (HCV/1b) infection are associated with treatment response or selected by treatment with interferon-α/ribavirin combination therapy. ***Methods:*** Eighteen Japanese patients with HCV/1b infection receiving interferon-α/ribavirin combination therapy for 24 weeks were studied. Five patients treated with interferon-α monotherapy for 24 weeks were also studied as controls. The entire HCV NS5B sequence before and after therapy was determined. ***Results:*** All HCV isolates had tyrosine at position 415 of NS5B before and after therapy. Further analysis showed that no specific amino acid substitutions were identified to associate with clinical response and no specific amino acid substitutions were induced/selected by the clinical treatment. ***Conclusion:*** No specific HCV NS5B nucleotide/amino acid sequence variations, including amino acid 415 of NS5B, were identified as being associated with clinical treatment response or selected by the combination therapy in Japanese patients with HCV/1b infection.

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The sequences reported in this paper have been deposited in the GenBank/DDBJ/EMBL databases (accession numbers AB189078–AB189119).

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Introduction

Hepatitis C virus (HCV) is a single-strand RNA virus of approximately 9,500 nucleotides and is a major etiology of parenteral non-A non-B hepatitis [1, 2]. Natural history studies have shown that a significant proportion of patients with chronic hepatitis C will eventually develop cirrhosis and hepatocellular carcinoma [3]. In Western countries, HCV-related end-stage liver disease is a major indication for liver transplantation [4]. In 1998, the United States Food and Drug Administration approved the interferon-α/ribavirin combination therapy for patients with chronic hepatitis C. A different treatment regimen of interferon-α/ribavirin combination therapy was also approved by the Japanese Health Authority in 2002.

Ribavirin, a synthetic guanosine analog, is an antiviral drug approved for the treatment of respiratory syncytial virus [5] and, in combination with interferon-α, for the treatment of clinically compensated chronic hepatitis C. Ribavirin monotherapy has been shown to reduce serum alanine transaminase (ALT) levels, but no significant reduction in serum HCV RNA levels was observed in most treated patients. Most patients who responded to ribavirin monotherapy relapsed biochemically after cessation of therapy [6]. Subsequently, it was shown that the combination of interferon-α and ribavirin has a much better efficacy than interferon-α monotherapy which was the gold standard of therapy for patients with chronic hepatitis C in the late 1990s [7–9]. However, in patients with HCV genotype-1 infection and high viral load, the response rate to interferon-α/ribavirin combination therapy is still at the 20–30% level, in contrast to a much higher response rate of 80% in patients infected with HCV genotypes 2 and 3 [10].

The non-structural (NS) genomic region of the HCV genome encodes the viral RNA-dependent RNA polymerase, an essential viral replicating enzyme [11]. A recent study suggested that ribavirin treatment in HCV genotype 1a infection might exert selective pressure in favor of a HCV variant with tyrosine (Y) instead of phenylalanine (F) at the 415 position of NS5B. The investigators suggested that this amino acid substitution may lead to viral resistance to ribavirin treatment, as corroborated by their in vitro studies [12].

In Japan, most patients had HCV genotype 1b infection with a high viral titer. The aim of the present study was to evaluate whether variations in the HCV NS5B region play a role in determining the clinical response to interferon-α/ribavirin therapy in Japanese patients with HCV genotype 1b infection.

Patients and Methods

In a clinical study comparing interferon-α/ribavirin combination therapy with interferon-α monotherapy, 70 Japanese patients with HCV genotype 1b infection were recruited in our centers, including 64 patients receiving the combination therapy and 6 patients receiving interferon-α monotherapy. All patients gave written informed consent. All patients were seropositive for anti-HCV and HCV RNA, and genotyping showed HCV genotype 1b infection in all patients. They were all seronegative for HBsAg and anti-HIV, and other causes of liver disease were excluded using standard clinical and laboratory criteria. All patients received treatment in Nagoya City University Hospital and its affiliated hospitals from January 2001 to March 2003. The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki. Treatment regimen consisted of intramuscular injection of interferon-α2b (Schering KK, Osaka, Japan) at a dose of 6 million units (MU) daily for 2 weeks, followed thrice weekly injection for 22 weeks, combined with oral ribavirin 600–800 mg/day (Schering KK) based on body weight for patients with combination therapy. Patients were followed for an additional 24 weeks after cessation of therapy to determine the long-term clinical response. The 24-week treatment protocol was approved by the Japanese Health Authority back in 1999. More recently, the Japanese Health Authority has approved studies based on 48 weeks of treatment.

Clinical treatment response was classified into (1) sustained virologic response (SVR), defined as undetectable serum HCV RNA and normal ALT at week 24 after cessation of the treatment; (2) relapsers (Rel), defined as converting to negative for serum HCV RNA during therapy but relapsed to positive serum HCV RNA within 24 weeks after cessation of treatment, and (3) non-responders (NR), defined as patients who retained serum HCV RNA at the end of treatment. Of the 64 patients who received interferon-α/ribavirin combination therapy, only 45 completed the treatment without a dose reduction/discontinuation, the others had a dose reduction or treatment discontinuation due to side effects. Of the 45 patients who completed their therapy, a SVR was observed in 7 patients (15.6%), 25 patients were Rel (55.6%), and 13 patients were NR (28.9%). There were no significant differences in gender distribution, age, pretreatment ALT level, serum HCV RNA level, blood hemoglobin level, and platelet count between patients in the SVR, Rel and NR groups.

Eighteen patients with different clinical response profiles (SVR 4, Rel 8, NR 6) were randomly selected for this study. Five of the 6 patients who received interferon-α were also included to serve as controls (SVR 1, Rel 1, NR 3). As the Japanese Health Authority did not authorize the ribavirin monotherapy arm in any clinical protocol due to a poor benefit to risk ratio, there was no access to ribavirin monotherapy for Japanese patients as controls. Serum samples before and after therapy were used for this study. The serum samples were collected and serum separated from clots within 3 h and stored at –80°C.

All patients gave written informed consent and this study was approved by the local institutional review board.

Methods

Nucleic acids were extracted using a SepaGean RV-R Nucleic acid extraction kit (Sanko Junyaku Co., Ltd., Tokyo, Japan) in accordance with the manufacturer's protocol. The extracted RNA was reverse-transcribed to cDNA using SuperScript II RNase H^-

Table 1. Background of the patients studied

Patient No.	Clinical treatment response	Gender	HCV genotype	Age years	Pretreatment RNA level KIU/ml	Pretreatment ALT IU/l	Pretreatment Hb mg/dl
M1	SVR	M	1b	43	>850	361	14.0
M2	Rel	M	1b	53	560	54	14.6
M3	NR	M	1b	42	700	212	14.5
M4	NR	F	1b	44	620	209	13.8
M5	NR	M	1b	51	>850	108	13.6
C1	SVR	M	1b	54	830	112	15.1
C2	SVR	F	1b	60	>850	51	14.2
C3	SVR	M	1b	35	>850	134	16.6
C4	SVR	F	1b	54	>850	26	12.6
C5	Rel	M	1b	32	410	51	14.2
C6	Rel	M	1b	67	190	154	13.9
C7	Rel	M	1b	68	460	164	13.0
C8	Rel	M	1b	68	>850	98	12.7
C9	Rel	M	1b	47	560	50	15.6
C10	Rel	F	1b	64	>850	16	13.7
C11	Rel	M	1b	44	>850	56	15.5
C12	Rel	F	1b	70	>850	142	12.2
C13	NR	M	1b	61	>850	61	13.5
C14	NR	M	1b	67	260	139	13.1
C15	NR	M	1b	52	>850	113	11.7
C16	NR	M	1b	70	400	101	16.7
C17	NR	M	1b	29	>850	55	14.1
C18	NR	F	1b	60	>850	90	14.3
Mean ± SD				54 ± 13	697 ± 217	111 ± 77	14.1 ± 1.3

M1–5 = Patients treated with interferon-α monotherapy; C1–18 = patients treated with interferon-α/ribavirin combination therapy; ALT = serum alanine transaminase; Hb = hemoglobin.

Reverse Transcriptase (Invitrogen Corp., Carlsbad, Calif., USA) and random hexamer primer (Takara Shuzo Co. Ltd, Tokyo, Japan) as described previously [13].

The complete HCV NS5B sequence was determined in all samples. Briefly, cDNA was amplified by a long polymerase chain reaction (PCR) with LA Taq (Takara Shuzo Co. Ltd, Tokyo, Japan). The long PCR fragments (around 2.1 kbp) were generated with primers 7355F-NK (CCTGACAGAGTCCACCGTGTCTTCTG CCTT) and 9440R-NK (GTTGGGGAGCAGGTAGATGCCT ACCCCTAC). The first-round PCR product was further amplified using hemi-nested primers including 7378F-NK (AGCTCGC-TACTAAGACCTTTGGCAGCTCCG) and 9440R-NK. The amplicons were then cloned into pCR2.1-TOPO vector (Invitrogen Corp), and nucleotide sequences were determined using Prism Big Dye (Applied Biosystems, Foster City, Calif., USA) with an ABI 3100 DNA automated sequencer.

The sequences generated were used to confirm HCV genotypes and to identify specific nucleotide mutations and amino acid substitutions that may be associated with interferon-α/ribavirin treatment. Additionally, to determine whether ribavirin serves as a RNA mutagen, two models were used to estimate evolutionary distances: Tamura-Nei model for all codon substitutions, and Pamilo-Bianchi-Li model for synonymous and non-synonymous substitutions.

Amino acid sequences of the HCV NS5B region in various HCV genotypes were obtained from GenBank/DDBJ database. They were aligned to show amino acid 415 of the HCV NS5B region.

Statistical Analysis

The χ^2 test, Fisher's exact test and Student's t test were used where appropriate.

Results

Nucleotide Substitutions in the HCV NS5B Region before and after Therapy

The mean pair-wise genetic distances of all codon substitutions among 14 patients (4/18 had a SVR and were negative during follow-up) receiving combination therapy were 0.00668 ± 0.00702, which was not different from

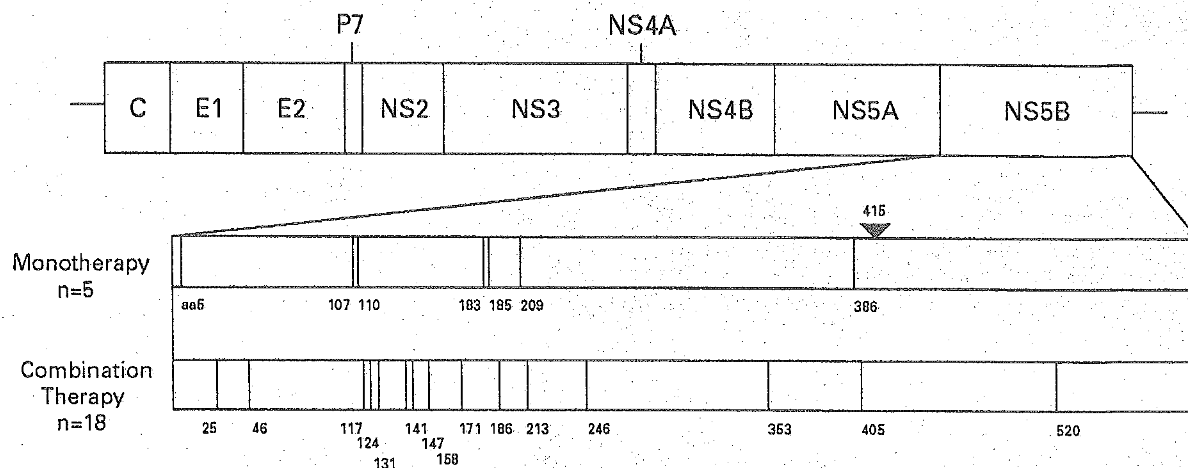

Fig. 1. The site map of amino acid mutations in the NS5B region in patients receiving interferon-α monotherapy versus interferon-α/ribavirin combination therapy. The vertical bars indicate the sites of amino acid substitutions after therapy.

the 4 patients (1/5 had a SVR) receiving interferon-α monotherapy 0.00627 ± 0.00444 (p = NS). The nucleotide substitution rate was also not different between the 2 groups after taking account of the bias towards synonymous substitutions (0.01778 ± 0.0237 vs. 0.01603 ± 0.01208, p = NS).

Amino Acid Substitutions in the NS5B Region

In the 5 patients receiving interferon-α monotherapy (1 was SVR and negative for HCV RNA during follow-up), 7 amino acid substitutions in the NS5B region were detected. In the 18 patients treated with interferon-α/ribavirin combination therapy (4 were SVR and negative for HCV RNA during follow-up), 17 amino acid substitutions were detected (fig. 1; table 2–4). There were no amino acid substitutions specific to the clinical treatment response identified. Note that with reference to the amino acid residue at the NS5B 415th site, all 18 in 23 (since 5/23 were SVRs) patients with HCV genotype 1b infection had Y before and after treatment.

There was also no difference in the number of sites of amino acid substitutions in the HCV NS5B region between 17 in the patients treated with combination therapy and 7 in the patients receiving interferon-α monotherapy (0.2055% (17/591 × 14) vs. 0.2961% (7/591 × 4); the NS5B region consists of 591 amino acids).

Amino Acid Alignment in the NS5B Region of Different HCV Genotypes

A total of 150 complete amino acid sequences derived from the nucleotide sequences of the HCV NS5B region from different HCV genotypes (from genotype 1a to 6) were obtained from the GenBank/DDBJ database. All 17 HCV-1a sequences had F at position 415 of the NS5 region. In contrast, 104/106 HCV genotype 1b sequences had Y at position 415 of NS5B. For other HCV genotypes, only HCV genotype 1c and 1 of 6 genotype 3 sequences had F at position 415 of the NS5B region (fig. 2; table 5).

Discussion

This study showed three important points. First, most HCV genotypes 1b had Y, instead of F in position 415 of the HCV NS5B region. Second, interferon-α/ribavirin treatment did not induce any specific amino acid substitutions in the HCV NS5B region in Japanese patients with HCV genotype 1b infection. Third, interferon-α/ribavirin treatment did not induce more nucleotide mutations or amino acid substitutions compared to interferon-α monotherapy in Japanese patients with HCV genotype 1b infection.

Table 2. Amino acid substitutions in HCV NS5B in the patients studied

NS5B	aa	5th		25th		46th		107th		110th		117th		124th		131th	
		before	after	before	after	before	after	before	after	before	after	before	after	before	after	before	after
M1	SVR	T	ND	A	ND	S	ND	D	ND	N	ND	N	ND	K	ND	E	ND
M2	Rel	T	T	A	A	S	S	D	D	N	N	N	N	K	K	E	E
M3	NR	T	T	A	A	S	S	D	D	N	N	N	N	K	K	E	E
M4	NR	S	T	P	P	G	G	D	N	S	N	N	N	K	K	E	E
M5	NR	T	T	A	A	S	S	D	D	N	N	N	N	K	K	E	E
C1	SVR	T	ND	P	ND	G	ND	D	ND	N	ND	N	ND	K	ND	D	ND
C2	SVR	T	ND	A	ND	S	ND	D	ND	N	ND	N	ND	K	ND	D	ND
C3	SVR	T	ND	A	ND	G	ND	D	ND	S	ND	N	ND	K	ND	E	ND
C4	SVR	T	ND	A	ND	G	ND	D	ND	S	ND	N	ND	K	ND	E	ND
C5	Rel	T	T	A	A	G	G	D	D	N	N	N	N	K	K	D	D
C6	Rel	T	T	P	P	S	S	D	D	S	S	N	N	K	K	E	E
C7	Rel	T	T	A	A	S	S	D	D	N	N	N	N	K	K	E	E
C8	Rel	T	T	P	P	S	S	D	D	N	N	N	N	E	E	E	E
C9	Rel	T	T	P	P	S	S	D	D	N	N	N	N	K	K	E	E
C10	Rel	T	T	A	S	C	S	D	D	N	N	N	N	K	K	E	E
C11	Rel	T	T	A	A	S	S	D	D	N	N	N	N	K	K	E	E
C12	Rel	T	T	A	A	G	G	D	D	S	S	N	N	K	K	E	E
C13	NR	T	T	P	A	S	S	D	D	N	N	D	N	K	E	E	E
C14	NR	T	T	A	P	S	S	D	D	N	N	N	N	E	E	E	E
C15	NR	T	T	A	A	S	S	D	D	N	N	N	N	K	K	V	V
C16	NR	T	T	A	A	S	S	D	D	S	S	N	N	K	K	E	E
C17	NR	T	T	A	A	S	S	D	D	N	N	N	N	K	K	E	E
C18	NR	T	T	A	A	S	S	D	D	N	N	N	N	K	K	D	E

ND = Not detected; M1–5 = patients treated with interferon-α monotherapy; C1–18 = patients treated with interferon-α/ribavirin combination therapy.

Table 3. Amino acid substitutions in HCV NS5B in the patients studied

NS5B	aa	141th		147th		158th		171th		183th		185th		186th		209th	
		before	after	before	after	before	after	before	after	before	after	before	after	before	after	before	after
M1	SVR	K	ND	V	ND	R	ND	E	ND	P	ND	A	ND	V	ND	K	ND
M2	Rel	K	K	V	V	R	R	E	E	P	L	A	A	V	V	K	K
M3	NR	K	K	V	V	R	R	E	E	P	P	A	A	V	V	K	K
M4	NR	K	K	V	V	R	R	E	E	P	P	A	A	V	V	K	K
M5	NR	K	K	V	V	R	R	E	E	P	P	A	V	V	V	R	K
C1	SVR	K	ND	V	ND	R	ND	E	ND	P	ND	A	ND	V	ND	K	ND
C2	SVR	K	ND	V	ND	R	ND	E	ND	P	ND	A	ND	V	ND	K	ND
C3	SVR	K	ND	I	ND	R	ND	E	ND	P	ND	A	ND	V	ND	K	ND
C4	SVR	K	ND	I	ND	R	ND	E	ND	P	ND	A	ND	V	ND	K	ND
C5	Rel	K	V	V	V	R	R	E	E	P	P	A	A	V	V	K	K
C6	Rel	K	K	V	V	R	R	E	E	P	P	A	A	V	V	K	K
C7	Rel	E	K	V	I	R	R	E	E	P	P	A	A	V	V	K	K
C8	Rel	K	K	I	I	R	R	E	E	P	P	A	A	V	V	K	K
C9	Rel	K	K	V	V	R	R	E	E	P	P	A	A	V	V	K	K
C10	Rel	K	K	I	I	R	R	E	E	P	P	A	A	V	V	K	K
C11	Rel	K	K	V	V	R	R	E	E	P	P	A	A	G	G	K	K
C12	Rel	K	K	V	V	R	R	E	E	P	P	A	A	V	V	K	K
C13	NR	K	K	V	V	R	R	E	E	P	P	A	A	V	V	K	K
C14	NR	K	K	V	V	R	R	E	E	P	P	A	A	V	V	K	K
C15	NR	K	K	V	V	R	R	E	E	P	P	A	A	V	V	K	K
C16	NR	K	K	V	V	R	G	E	K	P	P	A	A	V	G	K	K
C17	NR	K	K	V	V	R	R	E	E	P	P	A	A	V	V	K	K
C18	NR	K	K	V	V	R	R	E	E	P	P	A	A	V	V	K	K

ND = Not detected; M1–5 = patients treated with interferon-α monotherapy; C1–18 = patients treated with interferon-α/ribavirin combination therapy.

Table 4. Amino acid substitutions in HCV NS5B in the patients studied

NS5B	aa	213th		246th		353th		386th		405th		415th		520th	
		before	after	before	after	before	after	before	after	before	after	before	after	before	after
M1	SVR	C	ND	A	ND	P	ND	R	ND	V	ND	Y	ND	T	ND
M2	Rel	C	C	A	A	P	P	R	C	V	V	Y	Y	T	T
M3	NR	C	C	A	A	P	P	R	R	V	V	Y	Y	T	T
M4	NR	C	C	A	A	L	L	R	R	V	V	Y	Y	T	T
M5	NR	C	C	A	A	P	P	R	R	V	V	Y	Y	T	T
C1	SVR	C	ND	A	ND	L	ND	R	ND	V	ND	Y	ND	T	ND
C2	SVR	T	ND	A	ND	P	ND	R	ND	V	ND	Y	ND	T	ND
C3	SVR	N	ND	A	ND	P	ND	R	ND	V	ND	Y	ND	T	ND
C4	SVR	C	ND	A	ND	P	ND	R	ND	V	ND	Y	ND	T	ND
C5	Rel	N	N	A	V	P	P	R	R	V	V	Y	Y	T	T
C6	Rel	C	C	A	A	L	L	R	R	V	V	Y	Y	T	I
C7	Rel	N	N	A	A	P	L	R	R	V	V	Y	Y	T	T
C8	Rel	C	C	A	A	L	L	R	R	V	V	Y	Y	T	T
C9	Rel	N	N	A	A	P	P	R	R	V	V	Y	Y	T	T
C10	Rel	N	N	A	A	L	L	R	R	V	V	Y	Y	T	T
C11	Rel	S	S	A	A	P	P	R	R	V	V	Y	Y	T	T
C12	Rel	C	C	A	A	P	P	R	R	V	V	Y	Y	T	T
C13	NR	S	N	A	A	P	P	R	R	V	V	Y	Y	T	T
C14	NR	C	C	A	A	P	P	R	R	V	V	Y	Y	T	T
C15	NR	T	T	A	A	P	P	R	R	V	V	Y	Y	T	T
C16	NR	R	C	A	A	P	P	R	R	I	V	Y	Y	T	T
C17	NR	C	C	A	A	P	P	R	R	V	V	Y	Y	T	T
C18	NR	C	C	A	A	P	P	R	R	V	V	Y	Y	T	T

ND = Not detected; M1–5 = patients treated with interferon-α monotherapy; C1–18 = patients treated with interferon-α/ribavirin combination therapy.

Table 5. Amino acid residues at position 415 of NS5B in various HCV genotypes

HCV genotype	No.	Position 415 of NS5B			
		F	%	Y	%
1a	17	17	100	0	0
1b	106	2	2	104	98
1c	2	2	100	0	0
2a	18	0	0	18	100
2b	3	0	0	3	100
3	6	1	17	5	83
4	1	0	0	1	100
5	2	0	0	2	100
6	5	0	0	5	100

Understanding the molecular mechanism(s) of action of interferon-α/ribavirin therapy and determinants/resistance to therapy may lead to the design of better treatment strategies. The currently proposed mechanisms of action of ribavirin in combination therapy with interferon-α for HCV include: (1) inducing a Th2 to Th1 bias in favor of a host antiviral response; (2) blocking the host enzyme inosine monophosphate dehydrogenase to reduce the availability of the guanosine pool; (3) inhibition of viral RNA-dependent RNA polymerase, and (4) serving as a RNA mutagen to introduce mutations into the HCV genome [14]. Recently, it was observed that a HCV variant with amino acid substitution from F to Y at the NS5B 415th position was consistently detected in patients with HCV genotype 1a infection during ribavirin monotherapy. It was suggested that this amino acid substitution was selected by ribavirin therapy and was responsible for viral resistance during therapy [12].

This study showed that amino acid substitution at the NS5B 415 position is irrelevant in Japanese patients with HCV genotype 1b infection. First, all the Japanese patients with HCV genotype 1b that we studied had Y at the 415 position of the NS5B region. Second, this is confirmed by the HCV gene and deduced amino acid sequences available in the Genbank/DDBJ database. Such a pattern with F at the 415 position of HCV NS5B region was commonly observed in HCV genotypes 1a and 1c, and only occasionally in genotypes 1b and 3. To argue that F at position 415 is an important phenotype as a re-

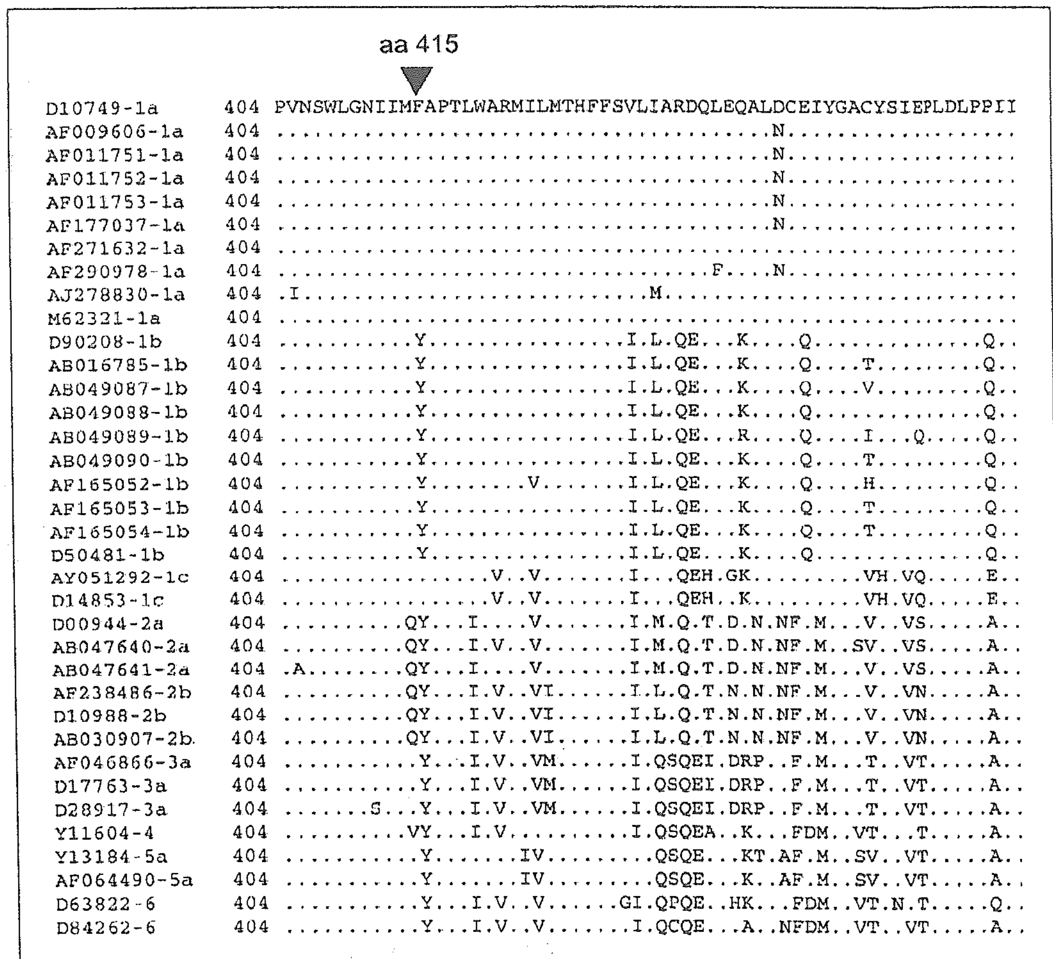

Fig. 2. The alignment of the deduced amino acid sequences of various HCV genotypes in the NS5B region based on 150 sequences available in the GenBank/DDBJ database.

sponse to therapy determinant is difficult since most isolates of HCV genotypes 2 and 3, which are known to respond very well to combination therapy, have Y at position 415 of NS5B. One has to postulate that there are other significant viral or host factors related to HCV genotypes 2 and 3 that make them more susceptible to response to combination therapy to offset the Y factors at position 415 of HCV NS5B. Also, the clinical observation that HCV genotype 1a (with F in position 415) and genotype 1b (with Y in position 415) had a similar clinical treatment response rate to interferon-α/ribavirin therapy also suggests that the amino acid in position 415 is not a key determinant to clinical response to interferon-α/ribavirin combination therapy.

The entire NS5B region was studied before and after therapy and no specific amino acid substitutions were found to be associated with relapse or no response. Therefore, this study ruled out the possibility of a viral amino acid substitution in the NS5B region as a major viral determinant for response to therapy or a viral resistance factor.

Finally, ribavirin has been suggested to be a viral mutagen. In this study, the number of nucleotide mutations and amino acid substitutions in the NS5B region did not increase with interferon-α/ribavirin combination therapy compared with interferon-α monotherapy. Certainly, the best approach is to study ribavirin monotherapy in Japanese patients with HCV genotype 1b infection. However,

the risk to benefit ratio precludes this type of therapy for testing in Japan. Nevertheless, the present study showed that the mutagen effect of ribavirin was not observed in Japanese patients with genotype 1b infection after a 6-month course of interferon-α/ribavirin combination therapy.

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References

1. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M: Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 1989; 244:359–362.
2. Major ME, Feinstone SM: The molecular virology of hepatitis C. Hepatology 1997;25: 1527–1538.
3. Kiyosawa K, Sodeyama T, Tanaka E, Gibo Y, Yoshizawa K, Nakano Y, Furuta S, Akahane Y, Nishioka K, Purcell RH, Alter HJ: Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. Hepatology 1990;12:671–675.
4. National Institutes of Health Consensus Development Conference Statement: Management of hepatitis C: 2002–June 10–12, 2002. Hepatology 2002;36(suppl 1):S3–20.
5. Wyde PR: Respiratory syncytial virus (RSV) disease and prospects for its control. Antiviral Res 1998;39:63–79.
6. Kakumu S, Yoshioka K, Wakita T, Ishikawa T, Takayanagi M, Higashi Y: A pilot study of ribavirin and interferon beta for the treatment of chronic hepatitis C. Gastroenterology 1993; 105:507–512.
7. McHutchison JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, Goodman ZD, Ling MH, Cort S, Albrecht JK: Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. N Engl J Med 1998;339:1485–1492.
8. Brillanti S, Garson J, Foli M, Whitby K, Deaville R, Masci C, Miglioli M, Barbara L: A pilot study of combination therapy with ribavirin plus interferon alfa for interferon alfa-resistant chronic hepatitis C. Gastroenterology 1994;107:812–817.
9. Poynard T, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G, Bain V, Heathcote J, Zeuzem S, Trepo C, Albrecht J: Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). Lancet 1998;352:1426–1432.
10. Davis GL, Esteban-Mur R, Rustgi V, Hoefs J, Gordon SC, Trepo C, Shiffman ML, Zeuzem S, Craxi A, Ling MH, Albrecht J: Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. N Engl J Med 1998;339: 1493–1499.
11. Behrens SE, Tomei L, De Francesco R: Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. EMBO J 1996;15:12–22.
12. Young KC, Lindsay KL, Lee KJ, Liu WC, He JW, Milstein SL, Lai MM: Identification of a ribavirin-resistant NS5B mutation of hepatitis C virus during ribavirin monotherapy. Hepatology 2003;38:869–878.
13. Ohno T, Mizokami M, Wu RR, Saleh MG, Ohba K, Orito E, Mukaide M, Williams R, Lau JY: New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. J Clin Microbiol 1997;35:201–207.
14. Lau JY, Tam RC, Liang TJ, Hong Z: Mechanism of action of ribavirin in the combination treatment of chronic HCV infection. Hepatology 2002;35:1002–1009.

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Two subtypes (subgenotypes) of hepatitis B virus genotype C: A novel subtyping assay based on restriction fragment length polymorphism

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Abstract

Recently hepatitis B virus genotype C (HBV/C) has been classified into geographically typical two subtypes (subgenotypes); HBV/C1 in Southeast Asia (Cs) and HBV/C2 in East Asia (Ce). Our aim is to develop a rapid subtyping assay and to examine the virological features of these two subtypes. Based on 171 HBV/C strains retrieved from the database, 17 single nucleotides polymorphisms (SNPs) were found between two subtypes. Taking advantage of five SNPs in non-overlapping polymerase region, a restriction fragment length polymporphism method with three endonucleases was newly developed for distinguishing between HBV/Cs and HBV/Ce. The method was applied to 49 HBV/C carriers from Japan and Hong Kong. The 24 in Hong Kong were classified into HBV/Cs, and the 25 in Japan were HBV/Ce, confirmed by sequencing. Some specific mutations were detected in the encapsidation signal; precore stop mutation (A1896), accompanied by a C-to-T substitution at nt 1858, was found in HBV/Ce strains, and another precore mutation (A1898), accompanied by a C-to-T mutation at nt 1856, was found in HBV/Cs. Especially, two closely linked mutations (A1896 and A1899) in HBV/Ce could stabilize the epsilon loop structure more efficiently and influece viral replication. Hence, these virological differences between the two subtypes might influence clinical features.
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Keywords: Hepatitis B virus; Single nucleotides polymorphisms; Subgenotypes

1. Introduction

HBV genotypes have a distinct geographical distribution and correlate with severity of liver disease [1,2]. Genotypes B and C are prevalent in Asia, and genotype C causes more serious liver disease than genotype B [3,4]. HBV strains even of the same genotype may differ both virologically and clinically. There are two subtypes (subgenotypes) of genotype B in distinct geographical distributions, designated Ba ("a" standing for Asia) and Bj ("j" for Japan) provisionally [5], and clinical differences between patients infected with HBV/Ba and HBV/Bj are coming to the fore [6,7]. Additionally, there have been some lines of evidence for virological and clinical differences between HBV/Aa in Africa and HBV/Ae in Europe and the US [8,9]. Infection with HBV/Aa is associated with low serum levels of HBV DNA as well as low prevalence of hepatitis B e antigen (HBeAg) in serum, and is implicated in the high incidence of HBV-induced hepatocellular carcinoma (HCC) in Africa [10,11].

Recently, phylogenetic analysis of the pre-S1/pre-S2 genes revealed two major groups within genotype C: one for strains from southeast Asia including Vietnam, Myanmar and Thailand (named HBV/C1) and the other for strains from

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(far) East Asia including Japan, Korea and China (named HBV/C2). This finding was confirmed by phylogenetic analyses based on the complete sequences of 32 HBV/C strains [12], and by a recent independent study in Hong Kong [13]. The latter paper designated the two subtypes (subgenotypes) as HBV/Cs in Southeast Asia and HBV/Ce in the (far) East Asia that have different epidemiological distributions [13]. However, further studies are required to evaluate clinical and virological significance between HBV/C1 (Cs) and HBV/C2 (Ce), and development of a simple and efficient method for classification is essential.

In this study, we investigated single nucleotides polymorphisms (SNPs) between HBV/Cs and HBV/Ce at complete genome levels, and developed a novel polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) method in the non-overlapping polymerase region involving five SNPs to distinguish between HBV/Cs and HBV/Ce precisely.

2. Materials and methods

2.1. Subjects

A total 49 sera containing HBV/C determined by the ELISA on preS2-region products [14,15], with the results confirmed by PCR-RFLP of the S gene [16], were obtained from chronic carriers of HBV who visited Nagoya City University hospital in Japan or Queen Mary Hospital in Hong Kong. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the Ethics Committees of the institutions, and an informed consent was obtained from each HBV carrier. To determine SNPs between HBV/Cs and HBV/Ce, 34 HBV/Cs and 137 HBV/Ce complete sequences were additionally recruited from DDBJ/EMBL/GenBank database.

2.2. PCR-RFLP for distinguishing between subtypes (subgenotypes) Cs and Ce of HBV genotype C

Nucleic acids were extracted from 100 μL of serum using QIAamp DNA Blood Mini Kit (Qiagen Inc., Hilden, Germany). A novel method for specific determination of HBV/C consisted of two PCR cycles with hemi-nested primers followed by RFLP with the restriction site specific for HBV/Cs or Ce. The first-round PCR was performed with a sense primer (HBV964F: 5′-ATT AGA CCT ATT GAT TGG AAA GT-3′ [nt 964-986]) and an antisense primer (HBV1272R: 5′-AGT ATG GAT CGG CAG AGG AG-3′ [nt 1272-1253]) within non-overlapping polymerase region. The second-round PCR was performed with a sense primer (HBV970F2: 5′-CCT ATT GAT TGG AAA GTA TGT CA-3′ [nt 970-992]) and an antisense primer (HBV1272R). To determine HBV/Cs, a portion (5 μl) of the amplification product of 309 base pairs (bp) in size was digested with 5 U of *Ase*I at 37 °C and *Bst*EII at 60 °C for 1 h each. For HBV/Ce digestion, *Nci*I was used at 37 °C for 2 h. Digests with these enzymes were run on electrophoresis in 3.0% (w/v) agarose gel, stained with ethidium bromide and examined for their sizes under the ultraviolet light.

2.3. Amplification and sequencing of the core promoter as well as the precore region plus core gene

To confirm the results by PCR-RFLP, HBV DNA sequences bearing the core promoter and precore/core regions were amplified by PCR with hemi-nested primers by the method described previously [17], with slight modifications. In brief, the first round of PCR was performed with sense primer (HB7F-2: 5′-CAT GGA GAC CAC CGT GAA CGC-3′ [nt 1607–1627]) and antisense primer (HB8R-2: 5′-ATA GGG GCA TTG GTC T-3′ [nt 2314–2299]) for 40 cycles (94 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min [6 min in the last cycle]) in a 96-well cycler (GeneAmp 9700, Perkin-Elmer Cetus, Norwalk, CA). The second round of PCR was performed with sense primer (HB7F-2) and antisense primer (HB7R-2: 5′-CCT GAG TGC TGT ATG GTG AGG-3′ [nt 2072–2052]) for 35 cycles, under the same conditions as in the first-round PCR. The standard precautions for avoiding contamination during PCR were exercised carefully, and a negative control serum was included in each run of tests to ensure the specificity. Thereafter, PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, CA) in the ABI 3100 DNA automated sequencer.

2.4. Molecular evolutionary analyses of HBV

Reference sequences were retrieved from the DDBJ/EMBL/GenBank database and their accession numbers for identification. Nucleotide sequences of HBV were aligned by the program CLUSTAL X, and the genetic distance was estimated with the six-parameter method in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp/). Based on these values, a phylogenetic tree was constructed by the neighbor-joining method with the mid-point rooting option.

3. Results

3.1. SNPs for distinguishing between HBV/Cs and HBV/Ce in complete genomes

When the 171 HBV/C (34 HBV/Cs and 137 HBV/Ce) strains, retrieved from the DDBJ/EMBL/GenBank database were compared over the complete genomes, 17 SNPs were found between two subtypes (subgenotypes) (Table 1). Of them, five SNPs in non-overlapping polymerase region include restricted enzyme sites: *Bst*EII site (nt 1041 of T [T1041] and C1044), *Ase*I site (A1050 and A1053) and *Nci*I site (C1155). Interestingly, the 34 HBV/Cs strains possessed *Bst*EII site (G/GTNACC [nt 1039–1045]) and/or *Ase*I

Table 1
Subtype-specific mutations in the complete genomes of HBV/Cs and HBV/Ce

SNPs no.	Nucleotide position	Cs ($n = 34$)	Unmatched	Amino acids/region	Ce ($n = 137$)	Unmatched	Amino acids/region	Enzymes
1	166	C	0	Thr/S, **His/P**	A	1	Thr/S, **Asn/P**	
2	312	T	2	**Leu/S**, Phe/P	C	1	**Ser/S**, Phe/P	
3	400	C	3	Ile/S, **Leu/P**	A	0	Ile/S, **Ile/P**	
4	**1041**	**T**	**6**	**Gly/P**	**C**	**10**	**Gly/P**	*BstE*II
5	**1044**	**C**	**0**	**Thy/P**	**T**	**1**	**Thy/P**	*BstE*II
6	1047	A	0	Pro/P	T	3	Pro/P	
7	**1050**	**A**	**2**	**Ala/P**	**C**	**20**	**Ala/P**	*Ase*I
8	**1053**	**A**	**1**	**Leu/P**	**A/G**	**1**	**Leu/P**	*Ase*I
9	**1155**	**T**	**0**	**Ala/P**	**C**	**9**	**Ala/P**	*Nci*I
10	1721	A	1	Val/X	G	0	**Leu/X**	
11	2065	A	0	Leu/C	C	7	Leu/C	
12	2158	A	2	Val/C	C	5	Val/C	
13	2559	A	0	Lys/P	C	3	**Gln/P**	
14	2561	A	1	Lys/P	G	5	**Gln/P**	
15	2633	G	0	Leu/P	A	0	Leu/P	
16	2958	T	2	**Phe/P**, Asn/PreS1	C	4	**Leu/P**, Asn/PreS1	
17	3008	C	1	**Ser/P, Ala/PreS1**	A	5	**Arg/P, Asp/PreS1**	

(AT/TAAT [nt 1050–1055]), while the 137 HBV/Ce strains had neither *BstE*II nor *Ase*I sites. On the other hand, 128 of 137 (93%) HBV/C2 strains possessed *Nci*I site (CC/SGG [nt 1154–1158]) and none of the HBV/C1 strains had *Nci*I site due to T1151. Additionally, according to the SNPs, eight amino acids differences were found between two subtypes (subgenotypes) (Table 1).

3.2. PCR-RFLP for distinguishing between HBV/Cs and HBV/Ce

Geographically, typical genetic representatives for HBV/Cs and HBV/Ce (eight strains each) were selected. The partial genome sequence alignment including restriction sites is shown in Fig. 1. HBV/Cs strains were obtained

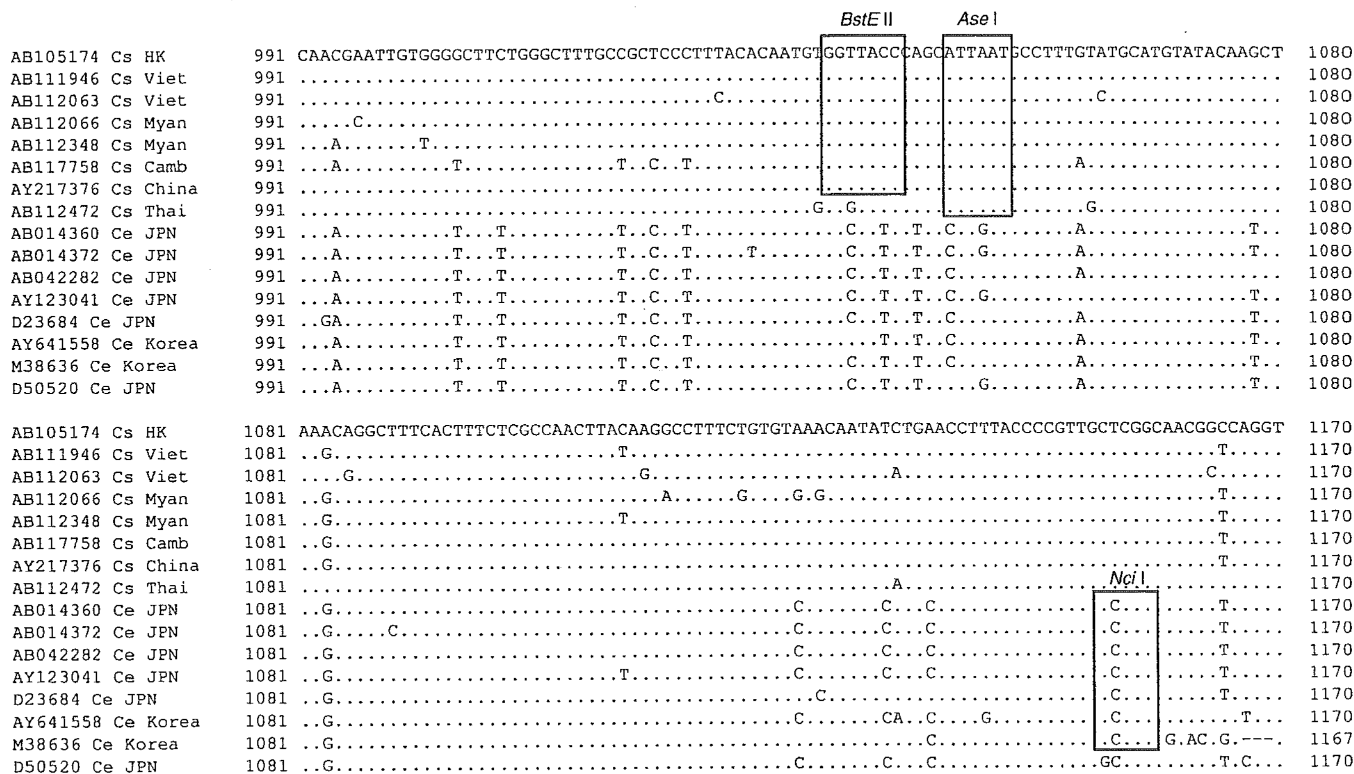

Fig. 1. Alignment of 8 HBV/Cs (C1) and 8 HBV/Ce (C2) sequences in non-overlapping polymerase region. The specific *BstE*II and *Ase*I sites are specific for HBV/Cs strains, while *Nci*I site is found in HBV/Ce strains. All sequences from the database are identified with accession numbers, followed by subtype and the country of origin in abbreviation for Cambodia (Camb), Hong Kong (HK), Japan (JPN), Myanmar (Myan) and Vietnam (Viet).