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Short communication

Novel hepatitis B virus subgenotype A6 in African-Belgian patients

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ABSTRACT

Background: Genome diversity of hepatitis B virus (HBV) is prominent among DNA viruses; which, allowed the virus to be genetically classified into eight genotypes and several subgenotypes.
Objective: To introduce and to characterize a novel subgenotype HBV, classified as A6.
Study design: HBV full-length genomes were isolated and sequenced from three African-Belgian patients chronically infected with the virus. Using phylogenetic reconstruction and genetic distance calculation, the evolutionary relationships of the novel strains were investigated.
Results: Phylogenetic analysis based on complete genome sequences of genotype A strains revealed distinct clusters supported by high bootstrap values. The three African-Belgian strains clustered separately from the other known A subgenotypes (A1–A5) with maximal bootstrap support (100%). The mean inter-subgenotypic nucleotide divergence over the complete genome sequence between the novel A6 strains and A1–A5 was higher than 4%.
Conclusion: Phylogenetic analysis of the complete genome sequences yielded maximal bootstrap value support for nodes that establish the new lineage as a novel subgenotype. In addition, nucleotide divergence more than 4% based on full-length genome of the virus, clearly demonstrated that the three African-Belgian strains belonged to a novel subgenotype of HBV, which was assigned as "A6". Noteworthy, the phylogeny of genotype A demonstrated that the A6 is a basal lineage that diverged earlier from the other African subgenotypes of genotype A.

1. Background

Hepatitis B virus (HBV) exhibits considerable sequence heterogeneity, which is fuelled by the viral polymerase lacking proofreading activity. Phylogenetic analysis has classified HBV into eight "genotypes" defined by an intergroup nucleotide divergence greater than 7.5% in the complete genome sequences.[1–3] "Subgenotypes" are subgroups within the same genotype that need to meet two particular criteria: (i) a nucleotide divergence of more than 4% and less than 7.5% over the full-length genome[4,5] and (ii) a high phylogenetic bootstrap support.[1,2] "Clades" are used for further subdivision within subgenotypes, showing less than 4% nucleotide diversity based on the complete genome sequences.[1,2]

HBV genotypes and subgenotypes are generally characterized by specific geographical distribution patterns.[6] As a consequence, evolutionary analysis of genotypes/subgenotypes may provide important insights into the historical migration or distribution patterns.[2,7,8] This diversification may also be responsible for differences in the clinical outcome, including disease severity as well as response to antiviral therapy. The identification and description of isolates from indigenous populations, particularly in the African continent where hepatitis B infection is hyper-endemic, may help to clarify the HBV origin and may further broaden the knowledge about HBV evolution.[2] Two major HBV genotypes, A and E, are predominant in Africa. To date, five subgenotypes have been reported for HBV genotype A: Aa (A′/A1) prevails in sub-Saharan African and South Asia, Ae (A2) is widely distributed in Europe and USA, Ac (A3) was identified in Cameroon and A4 was introduced from Mali.[9–12] The most recently, identified subgenotype A5 was introduced from Haiti and eastern Africa.[13] Furthermore, several clades and clusters can be distinguished within different subgenotypes of A.[14] Here, we characterized the evolutionary history of three novel HBV strains isolated from African-Belgian hepatitis B infected patients.

Abbreviations: HBV, hepatitis B virus; ORF, open reading frame; anti-HBc, antibody to hepatitis B core antigen; HBeAg, hepatitis B e antigen; anti-HBe, antibody to hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HIV, human immunodeficiency virus; PCR, polymerase chain reaction.

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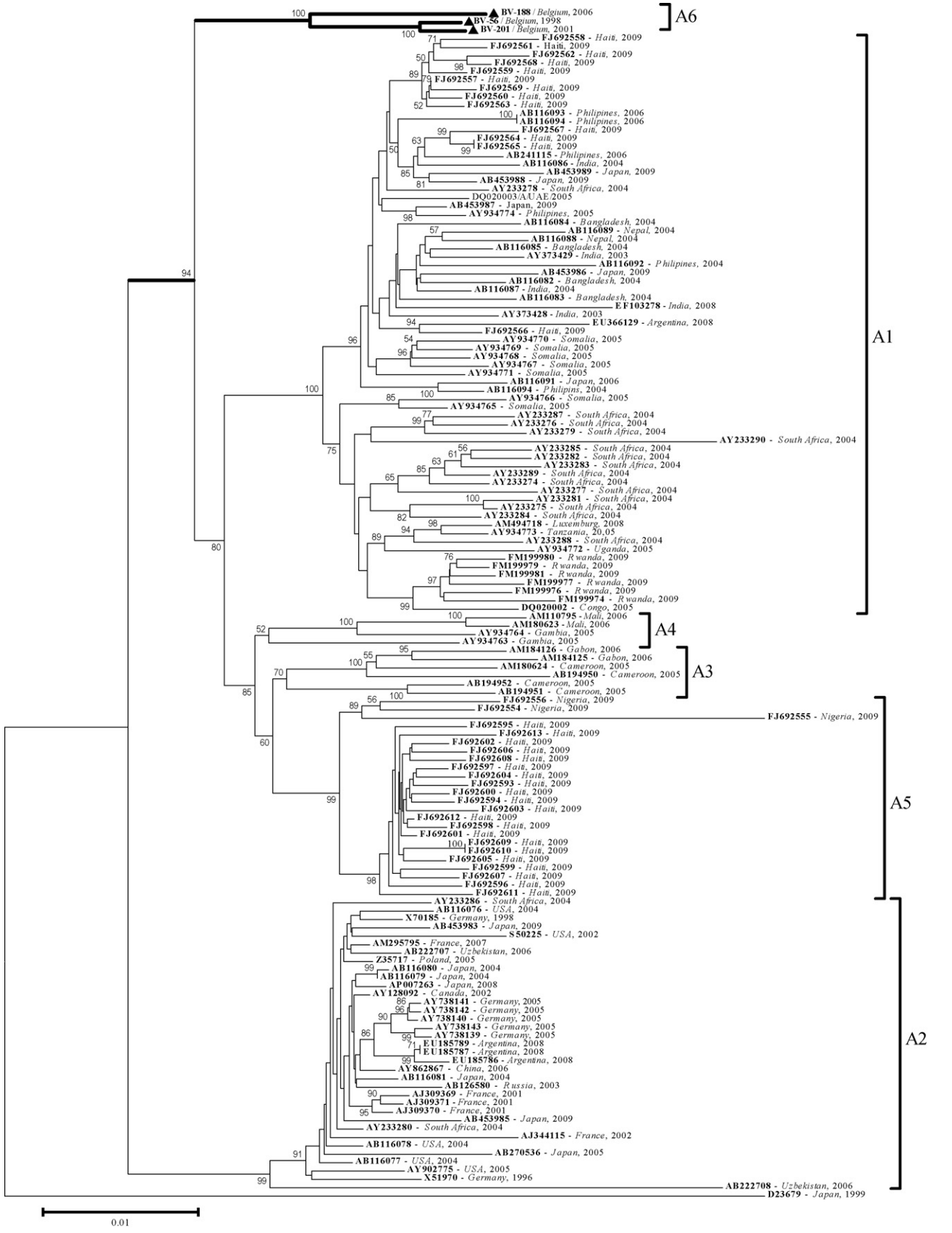

Fig. 1. Neighbour-joining phylogenetic tree constructed based on HBV full-length genomes from three novel subgenotype A6 strains and 137 strains of subgenotypes A1–A5. The novel isolated HBV are marked by ▲ and are introduced as A6 subgenotype. The numbers represent the bootstrap value (1000 replicates) in the main nodes. HBV genotype C was used as an outgroup.

2. Objectives

This study was conducted in order to phylogenetically characterize HBV strains isolated from three African-Belgian patients.

3. Study design

3.1. Amplification and sequencing of the complete HBV genome

Serum samples from three HBV-positive African-Belgian patients (originally from Congo and Rwanda) were collected in 1998, 2001 and 2006, respectively. Viral DNA was extracted from 200 μl of serum using the QIAmp® Viral DNA mini kit (QIAGEN Benelux, Venlo, The Netherlands). Complete HBV genomes were amplified by polymerase chain reaction (PCR) as previously described.[15] To increase the efficiency and fidelity, PCR amplification was done with *pfu* DNA polymerase (Fermentas, St.Leon-Rot, Germany). The PCR products were purified with the QIAquick® PCR purification kit (QIAGEN) and primer-walking was performed to determine the complete genomes.[16] Sequencing reactions were performed using the ABI PRISM Big Dye® Terminator cycle sequencing reaction kit (Applied Biosystems, Foster City, CA, USA) and analysed on an ABI PRISM 3100 automated sequencer. A consensus sequence (whole genome) was generated by assembling different gene segments using the SeqMan II® software (DNAStar Inc., Madison, Wisconsin, USA). Sequences were submitted to the GenBank under accession numbers GQ331046-48.

3.2. Phylogenetic and evolutionary analysis

Multiple alignments were created using the ClustalW software.[17] Possible recombination events were investigated using SimPlot software, version 3.5.1.[18] Phylogenetic trees were constructed using the neighbour-joining method[19] and the reliability of the clusters were assessed by bootstrapping using 1000 replicates.[20] Phylogenetic and molecular evolutionary analyses were conducted using the MEGA software, version 4.0, in which the mean nucleotide divergence (mean ± SD) over the complete genome sequence nucleotide was calculated using the Kimur 2-parameter model.[21]

The analyses were carried out based on HBV full-length genome and we fully adhered to the HBV subgenotyping guidelines which was introduced by Kramvis et al. and Kurbanov et al.[2,11] To obtain the high accurate and conclusive results, three isolated HBV strains were analysed and compared to nearly all reported HBV subgenotypes A worldwide comprising 69 subgenotype A1, 35 subgenotype A2, 6 subgenotype A3, 4 subgenotype A4 and 23 subgenotype A5, which were retrieved from GenBank.

4. Results

During a molecular evolutionary survey in Belgium, HBV full-length genomes from three African-Belgian infected patients were identified as HBV genotype A. Two patients were of Congolese origin (BV-188 and BV-201) and were shown to be dually infected with HIV and HBV, the third patient (BV-56) emigrated from Rwanda. The patients were positive for HBsAg, HBeAg and anti-HBc and negative for anti-HBs. No deletions, insertions and recombinations were identified throughout the complete genome.

Phylogenetic analysis of the complete genome sequences revealed distinct clusters supported by high bootstrap values (Fig. 1). The three African-Belgian strains (marked by ▲) clustered separately from the other known A1–A5 subgenotypes with maximal bootstrap support (100%). The mean inter-subgenotypic nucleotide divergence (mean ± SD) over the complete genome sequence between the novel A6 strains and A1, A2, A3, A4 and A5 was 4.06% ± 0.30, 4.70% ± 0.36, 4.59% ± 0.33, 4.20% ± 0.29 and 4.0% ± 0.33, respectively. The A6 strains presented 1.86 ± 0.21 intra-group nucleotide divergence. Phylogenetic analysis of the complete genome yielding maximal bootstrap value support at the relevant nodes and also nucleotide divergence more than 4% (based on full-length genome) conclusively established that the three African-Belgian strains belong to the novel subgenotype A6 of HBV. Of note, the A6 lineage diverged before the diversification of the other African subgenotype A1, A3, A4 and A5 lineages. This was supported by a high bootstrap value (94%, 1000 replicates) at the main node, where these strains diverged from other A subgenotypes.

HBsAg amino acid analysis identified *ayw1* as the subtype specific motif for the BV-188; whereas, the BV-56 and BV-201 harbored *adw2*. Because of the distinctive clustering of the novel full genomes, we further investigated characteristic amino acid substitutions in different ORFs. In the S ORF, substitutions sQ/A54K, sI/T84L and sA/T90K, and in the POL ORF rtS/T236K, rtS/N273G, rtS/C/K308N and rtH/D619K as subgenotype specific amino acids were identified.

5. Discussion

In order to determine genotype and subgenotype classification of HBV, phylogenetic analysis of full-length genome is considered as the gold standard.[1,22] Such analysis becomes particularly important when subgenotype assignment is the main goal. To maximize the accuracy of the study in our phylogenetic analysis, we considered almost all HBV full-length genomes which have been recently introduced as A3, A4, and A5. Interestingly, the phylogenetic analysis of the three novel strains isolated from African-Belgian infected cases revealed a divergence more than 4% provided with maximal bootstrap support justifying a conclusive assignment as subgenotype A6 of HBV.

The study of Kramvis et al. predicted that by sequencing more HBV isolates, especially from more "remote regions in the world", the number of subgenotypes may expand progressively.[1] Our study underscores such a forecast, since all isolated HBV harboring the novel HBV A6 strains had an African origin (Democratic Republic of Congo and Rwanda), while only subgenotype A1 has been reported in these areas.[23,14] Moreover, considering the African origin of A6 as well as the other African subgenotypes A3, A4, A5 and one of the A1 clusters, it seems very plausible that HBV genotype A originated from Africa.[23] This is also supported by then fact that subgenotype A6 is a basal lineage of African HBV subgenotypes A (see phylogenetic tree).

The existence of a clade distinction between one A6 strain with an *ayw1* subtype and two other A6 strains with the *adw2* subtype highlights the extensive genetic diversity even among A6 subgenotype lineages. Further molecular epidemiologic studies in African HBV carriers may provide more insights into the origin and evolution of HBV.

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Competing interests: None declared.

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