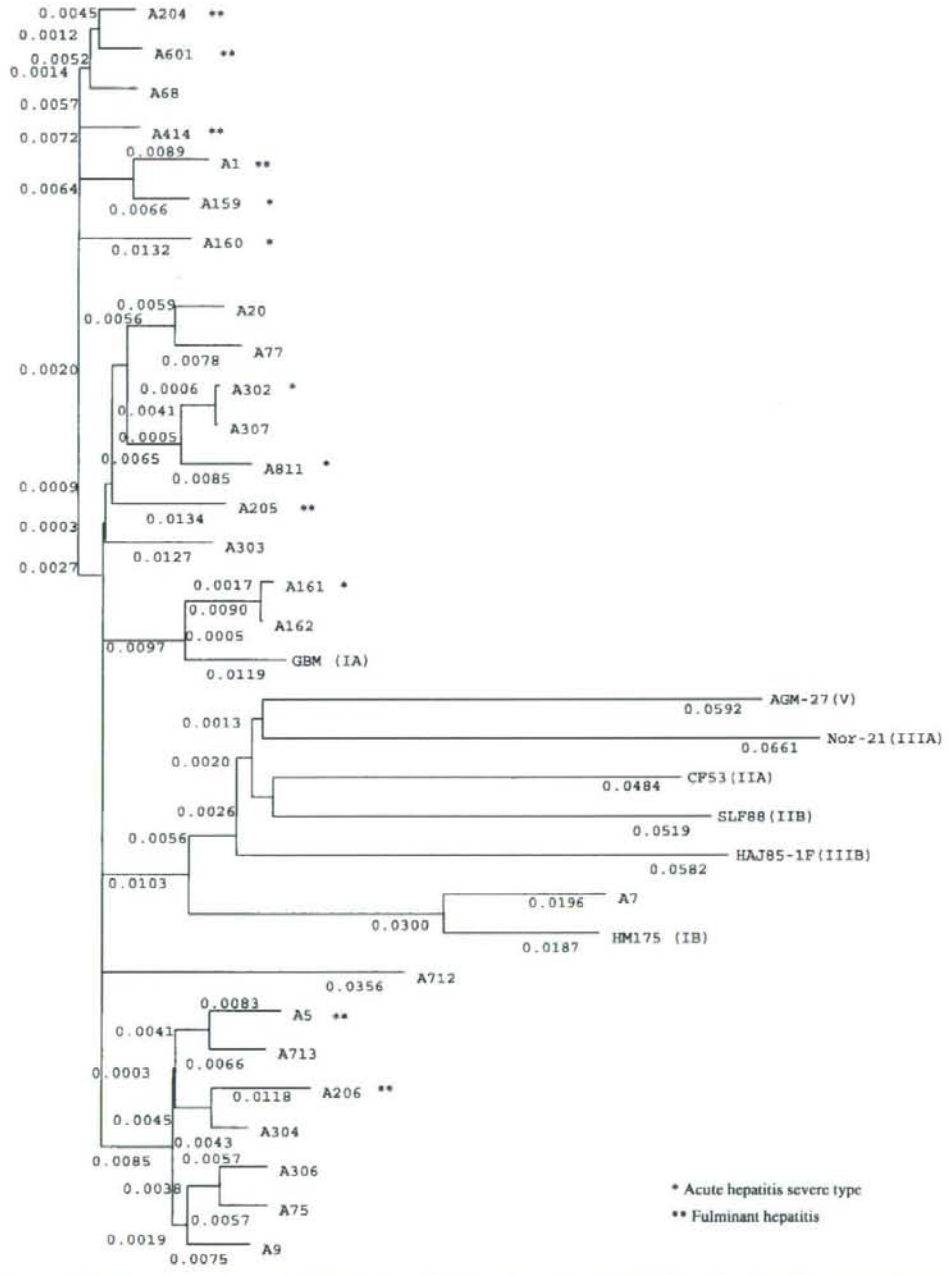

Fig. 2. Genetic relatedness between individual hepatitis A virus (HAV) of entire 2B and 2C recovered from 25 patients and HAV reference strains GBM (subgenotype IA), HM175 (subgenotype IB), CF53 (subgenotype IIA), SLF88 (subgenotype IIB), Nor-21 (subgenotype IIIA), HAJ85-1F (subgenotype IIIB) and AGM27 (genotype V). Numbers beside the phylogenetic roots are the results of bootstrap analyses.

Table 2. Clinical, biochemical and viral characteristics of six patients with fulminant and severe hepatitis located in the near parts of the phylogenetic tree

Patient	Diagnosis	Age/sex	Origin	Onset	Outcome	ALT (IU/L)	T-Bil (mg/dl)	PT (%)	IgM-HA (cut-off index)	Viral load (log copies/ml)	Days from onset
A204	FH	39/F	Tohoku	February 1990	Death	4470	5.3	10	3.8	ND	3
A601	FH	64/F	Shinetsu	January 1997	Death	12 500	7.0	13	2.9	3.7	9
A414	FH	49/M	Shinetsu	January 1989	Recovery	5276	26.3	13	+	5.0	7
A160	AHs	39/M	Kanto	June 1998	Recovery	9164	1.6	38	3.1	5.1	4
A1	FH	29/M	Kanto	March 1992	Recovery	1175	7.3	17	5.1	3.3	6
A159	AHs	50/M	Kanto	May 1998	Recovery	5655	2.5	20	4.6	4.6	5

Patient	Genotype	5′ NTR homology (%)	2B nt homology (%)	2C nt homology (%)
A204	IA	99.0	93.8	90.0
A601	IA	99.3	94.3	89.3
A414	IA	98.7	95.0	88.6
A160	IA	97.7	95.2	88.3
A1	IA	98.7	96.0	88.5
A159	IA	98.7	96.9	88.8

Homology, sequences were compared with wild-type HAV genotype IA strain GBM.

AH, acute hepatitis; AHs, acute hepatitis severe type; ALT, alanine aminotransferase; FH, fulminant hepatitis; 5′NTR, 5′-nontranslated region; ND, not done; nt, nucleotide; Ti-Bil, total bilirubin.

substitutions in 2B, although there were no unique nucleotide or aa substitutions. On the other hand, it was reported that mutations in 5′NTR, 2B and 2C of HAV were associated with cytopathic variants in cultured cells, and virulence in tamarins, as described above (9, 10). These various observations led us to analyse these three regions of HAV in greater numbers of clinical samples (14, 17, 18).

In our analysis of 5′NTR, FH and AHs patients had fewer nucleotide substitutions than AH in the central part of 5′NTR ($P < 0.001$) (14). Several regions of 5′NTR, including the pyrimidine-rich tract and internal ribosomal entry site, have been examined for possible correlations with replication of HAV RNA *in vitro*, and it has been reported that HAV strains adapted to cell culture systems have mutations in 5′NTR and the P2 region (8), and mutations in 5′NTR significantly enhanced growth of the virus in a cell culture system (24). Thus, nucleotide variations in 5′NTR may influence replication of the virus and thereby affect virulence.

In 2B, there seemed to be more mutations in the strains obtained from FH and AHs patients than in those obtained from AH patients in the central part (18). On the basis of cell culture studies, substitutions in the sequence of 2B protein have been suggested to be associated with the replication capability of the virus. One nucleotide substitution at nt 3889 in 2B, which changed Ala to Val in 2B-216, is responsible for differences in the growth rate of the virus along with the nucleotide substitutions in 2C and/or 5′NTR (25, 26). A substitution at the same nt 3889 appeared from the early stage of replication enhancement in cultured cells, and several HAV strains showed a cytopathic effect (8). An Ala-to-Val substitution in 2B-216 was not observed in our study.

In 2C, FH patients had fewer aa substitutions than AH patients ($P < 0.05$) (17). This indicates that viruses with fewer aa substitutions in 2C may be more virulent in comparison with strains with more aa substitutions. 2C is a multifunctional protein and is involved in replication of the viral genome. Analysis of the primary aa sequence of 2C shows homology with a family of proteins that contains a nucleoside triphosphate (NTP)-binding motif. This motif consists of elements 'A' and 'B'. The residues mutated within the conserved A and B sites of the NTP-binding motif are critical in RNA replication and virus proliferation (27). Elements A and B were conserved in all patients except one of FH. 2C is also suggested to be involved in the rearrangement of cellular membranes (28). The simian HAV 2C gene was reported to be required for virulence in tamarins (10). Thus, subtle substitutions in 2C might influence the replication capability of the virus and thereby affect virulence. We could not find specific nucleotide or aa substitutions in any of the regions.

In the present study, patients with FH had fewer nt substitutions in 5′NTR, and had a tendency to have more aa substitutions in 2B, and fewer aa substitutions in 2C, than patients with AH, and four FH and two AHs were located in the near parts of the phylogenetic trees, indicating the association between severity of hepatitis A and genomic variations in 5′NTR, 2B and 2C of HAV. In these patients, HAV load was higher than that of AH patients. Rezende *et al.* (29) reported that HAV-related liver failure is because of an excessive host response associated with a marked reduction in viral load, and there is a discrepancy between their data and ours. But they did not show the time points of serum sampling that represent critical data about viraemia in AH, and so we cannot discuss the discrepancy.

Thus, genetic variations not in one specific region but in 5′NTR, 2B and 2C might cooperatively influence replication of the virus and thereby affect virulence. Our findings are in accordance with the basic reports that the pathogenicity of HAV could be related to cooperative mutations within 5′NTR and P2 in cultured cells and simians, and the clinical finding that there has been only one report about a cluster of fulminant hepatitis A, unlike the many reports of clusters of fulminant hepatitis B.

Our current study suggests that both viral and host factors should be considered and examined when discussing the mechanisms responsible for the severity of hepatitis A. Further, we should examine several portions of the HAV genome including 5′NTR, 2B and 2C rather than focus on one specific region when analysing viral factors. Our study also suggests that

vaccination should be considered all the more if HAV itself is involved in the pathogenicity of hepatitis A, because safe and extremely effective inactivated HAV vaccines are available.

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