Research Paper

Ultra-Deep Sequencing Analysis of the Hepatitis A Virus 5'-Untranslated Region among Cases of the Same Outbreak from a Single Source

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Abstract

Hepatitis A virus (HAV) is a causative agent of acute viral hepatitis for which an effective vaccine has been developed. Here we describe ultra-deep pyrosequences (UDPSs) of HAV 5'-untranslated region (5'UTR) among cases of the same outbreak, which arose from a single source, associated with a revolving sushi bar. We determined the reference sequence from HAV-derived clone from an attendant by the Sanger method. Sixteen UDPSs from this outbreak and one from another sporadic case were compared with this reference. Nucleotide errors yielded a UDPS error rate of < 1%. This study confirmed that nucleotide substitutions of this region are transition mutations in outbreak cases, that insertion was observed only in non-severe cases, and that these nucleotide substitutions were different from those of the sporadic case. Analysis of UDPSs detected low-prevalence HAV variations in 5'UTR, but no specific mutations associated with severity in these outbreak cases. To our surprise, HAV strains in this outbreak conserved HAV IRES sequence even if we performed analysis of UDPSs. UDPS analysis of HAV 5'UTR gave us no association between the disease severity of hepatitis A and HAV 5'UTR substitutions. It might be more interesting to perform ultra-deep sequencing of full length HAV genome in order to reveal possible unknown genomic determinants associated with disease severity. Further studies will be needed.

Key words: Acute liver failure, HAV, IRES, UDPS, 5'UTR.

INTRODUCTION

Hepatitis A virus (HAV) is a leading cause of acute viral hepatitis and occasionally acute liver failure, a life-threatening disease worldwide, and was first identified almost 40 years ago [1]. HAV is still one of the major causative agents of acute hepatitis despite the availability of an effective vaccine [1].

HAV, a member of the *Picornaviridae* family, has a positive-sense single-stranded RNA genome approximately 7.5 kb in length [2,3]. The genome codes a large open reading frame (ORF), which is flanked by 5'-untranslated region (5'UTR) and 3'UTR. The 5'UTR forms the internal ribosomal entry site (IRES), which

mediates cap-independent translation initiation and is important for HAV replication [4-6].

There have been several reports about the association between the severity of hepatitis A and nucleotide variations in 5'UTR of HAV [7-13]. HAV IRES derived from clinical isolates have shown various activities in *in vitro* cell culture [5,12]. Also complicating this issue is the fact that the definition of acute liver failure differs among different countries [11,14]. So, it is unclear whether the HAV genome sequence affects its virulence or not. Here we report on the ultra-deep pyrosequences (UDPSs) of HAV 5'UTR among cases of the same outbreak, which was derived from a single source, a revolving sushi bar.

MATERIALS AND METHODS

Patient Samples

This study was approved by the Ethics Committee, Chiba University, Graduate School of Medicine, Chiba (permission number 1160), and conformed to the Declaration of Helsinki. This HAV outbreak in January/February, 2011, was previously reported in detail [15,16]. Briefly, this outbreak was based in a revolving sushi bar located in a central area of Chiba, Japan. Sixteen patients of this outbreak were included, and one sporadic hepatitis A patient from the same area in June, 2010, was also included in the present study (Table 1). All HAV isolates were classified into HAV IA based on VP1/2A region [15,16]. Fourteen of the 16 outbreak patients were admitted to the National Hospital Organization Chiba Medical Center, Chiba, Japan, and the other patients to Chiba University Hospital, Chiba, Japan. Patient no. 12 was a sushi shop attendant and was expected to be one of the sources of this outbreak. Patients no. 1-16 were outbreak cases. Patient no. 17 was a sporadic case unrelated to this outbreak, he was 59 years old with AST 4313 (IU/L), ALT 5693 (IU/L) and nadir prothrombin level 35 (%), and he was admitted to Chiba University Hospital in June 2010. In this outbreak, no patients with hepatic encephalopathy were observed.

Patients with acute hepatitis A showing a plasma prothrombin level of < 40% without developing hepatic encephalopathy were defined as acute hepatitis 'severe form', and 4 'severe form' patients were included in the present study [14]. Patient no. 1-13 and no. 14-17 were acute hepatitis, non-severe form and acute hepatitis, severe form, respectively.

All patients were positive for immunoglobulin M anti-HAV antibody. Acute viral hepatitis B, C, and E were excluded by serological tests. All were negative for anti-HIV. None of the patients had been taking any medication and none had gone abroad, including to Korea [14] or China [17], within 1 month before disease onset. Sera were obtained at admission and stored at -20°C until analysis.

Table 1. Clinical features of 13 patients with non-severe form and 3 patients with severe form hepatitis A in the outbreak of the present study.

	AH (n = 13)	*P*	AH-S (n = 3)
Mean age (yr)	39.9 ± 14.4	NS#	40.6 ± 11.9
Gender (M/F)	7/6	NS$	2/1
Laboratory data			
ALT level (IU/L)	3392 ± 1886	0.024##	6940 ± 3550
Total bilirubin (mg/dL)	6.3 ± 3.8	NS#	5.7 ± 3.7
Nadir PT (%)	70.5 ± 19.1	0.031##	29.6 ± 8.3

Abbreviations: PT, prothrombin time. Data were expressed as Mean ± SD. #Statistically not significant (NS) by Student's *t* test. $Statistically not significant by Chi-squared test. ##Significant difference between 13 patients with AH (acute hepatitis, non-severe form) and 3 patients with AH-S (acute hepatitis, severe form) in hepatitis A outbreak by Student's *t* test.

UDPSs of HAV 5'UTR

Nucleic acids were extracted from 140 μL of sera using a QIAamp Viral RNA mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions, and subjected to RT-PCR. For the detection of HAV RNA, two sets of amplification primers were made at the position of 5'UTR based on HAV HM175 (M59810) sequences. Complementary DNA was synthesized with primer 1 (5'-AGTACCTCAGAGGCA AACAC-3') for 1 cycle at 55°C for 30 min and at 85°C for 5 min using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Indianapolis, IN, USA), then amplified with primer 1 and primer 2 (5'-TCTTGGAAGTCCATGGTGAG-3') for 35 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec using a FastStart high fidelity PCR system, dNTPack kit (Roche). Then, the first PCR product was further amplified with primers 3 (5'-CCACATAAGGCCC CAAAGAA-3') and 4 (5'- GGGACTTGATACCTCA CCGC-3') for 35 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec. Amplified products were separated by agarose gel electrophoresis and purified using a High pure PCR clean-up micro kit (Roche). Each amplicon was quantified by Nanodrop Lite spectrophotometer (Thermo Scientific, Madison, WI, USA), and all amplicons from a single viral genome were pooled together at equimolar ratios. Each pool was then quantitated, and approximately 500 ng of each was used in a fragmentation reaction mix, using a GS FLX Titanium Rapid Library Preparation Kit (Roche). Final libraries representing each genome were characterized for average size by using an Agilent High Sensitivity DNA kit on Agilent 2100 Bioanalyzer (Agilent Technologies, Loveland, CO, USA). 4×10^7 of molecular DNA libraries were then sub-

jected to emulsion PCR, and enriched DNA beads were loaded onto a picotiter plate and pyrosequenced with a Roche/454 GS Junior sequencer using Titanium chemistry (454 Life Sciences Corp., Branford, CT, USA) [18]. GS Amplicon Variant Analizer Version 2.7 (Roche) was used for read mapping and calculating variant frequencies at each nucleotide position according to the reference sequence.

Reference Plasmid Clone and Sanger Sequencing

We cloned the PCR product from patient no. 12 into the pCR2.1-TOPO vector (Life Technologies, Tokyo, Japan). Sanger sequencing was performed using a BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Life Technologies). Sequences were analyzed using Applied Biosystems 3730xl (Life Technologies).

Statistical Analysis

To obtain the percentage of nucleotide variability in each sample, the total number of nucleotide substitutions was divided by the total number of nucleotides analyzed at each position. Comparison was performed using Fisher's exact test, Chi-squared test, or Student's t-test. All *P-values* were two-tailed, and *P < 0.05* was considered statistically significant.

RESULTS

Calculation of PCR and Roche/454 GS Junior sequencer error rates

In order to ensure that errors introduced by PCR as well as errors inherent to the Roche/454 pyrosequencing technology were below our minimum variant frequency threshold of 1%, we sequenced the PCR products from 10^3 to 10^4 copies of control plasmid and found no mutations, indicating similar error rates lower than 1%. The average read number was 7753.

UDPS of HAV IRES of acute hepatitis, non-severe form derived from this outbreak

A previous study [9] showed one of the different hot-spots between acute hepatitis and fulminant hepatitis was located in HAV 5′UTR, according to analysis of the complete HAV genome. So we performed UDPS in 13 HAV IRES derived from patients with acute hepatitis, non-severe form, who were involved in this outbreak. The sequences were compared with the reference clonal sequence from patient no. 12, the sushi shop attendant. In these patients, 20 nucleotide substitutions at 19 positions and 3 nucleotide insertions at 3 positions were seen (Table 2), while plasmid control possessed no substitutions. In cases of acute hepatitis, non-severe form, 0-5 nucleotide substitutions and 0-1 nucleotide insertions were seen in each

case. In patients no. 5 and no. 13, respectively, 97.8% nucleotide substitution (206C/T) and 18.6% nucleotide substitution (211T/C) were seen, but all other substitutions were lower than 6% at each position.

Table 2. Nucleotide substitutions of HAV IRES from virus with substitutions.

Pa-tient No.	Locations*	Nucleotide Position	Prototype Nucleotide	Nucleotide Substitution
1	Between IIIc' and IIIb'	202	C	T
	IIIa'	220	G	A
	Between IVf and IVi	441	A	G
3	Between IIIa' and IIIe	225	T	C
4	Between IVa' and Va	576	G	A
5	IIIb'	206	C	T
	IIIg	265.5	-	G
8	IIIb'	207	T	C
	IVa	308	A	G
	IVc	335	A	G
	Between IVj' and IVi	482	T	C
	Between IVj' and IVi	484	A	G
10	Between IIIb' and IIIa'	212.5	-	T
	Between IVk and IVk'	466	T	C
	Vb	604	A	G
	Between Vb and Vc	605	T	C
11	IVd	344	T	C
	Vb	597.5	-	A
12	Between IVk and IVk'	471	A	G
	Between IVj' and IVi	484	A	G
	Vc	610	T	C
13	Between IIIb' and IIIa'	211	T	C
	Between IVd and IVe	357	A	G
15	IIIc	157	T	C
	IVf	378	G	A
	IVi	450	G	A
16	IIIf	242	C	T
17	IIIb'	204	A	G
	IIIb'	208	C	T
	Between IIIg and IIIg'	275	A	G
	Between IIIg and IIIg'	276	T	C
	IVf	378	G	A
	Between IVk and IVk'	466	T	C
	Between IVe' and IVd'	527	G	T
	Between IVa' and Va	578	T	C

*Major domains of HAV 5′UTR [19].

UDPS of HAV IRES of acute hepatitis, severe form, derived from this outbreak

We performed UDPS in 3 HAV IRES derived from patients with acute hepatitis, severe form, who were involved in this outbreak. In these patients, 4 nucleotide substitutions at 4 positions and 0 nucleotide insertions were seen (Table 2). In cases of acute hepatitis, severe form, 0-3 nucleotide substitutions were seen in each case. In patient no. 15, 0.49% nucleotide substitution (157C/T), 14.94% nucleotide substitution (378G/A) and 0.37% nucleotide substitution

(450G/A) were observed. In patient no. 16, only 7.94% nucleotide substitution (242C/T) was observed. These results showed no specific mutations in HAV IRES associated with severe form existing in this outbreak.

UDPS of HAV IRES of acute hepatitis, severe form, in sporadic case

We performed UDPS in the one HAV IRES derived from one patient with acute hepatitis, severe form, who was not involved in this outbreak. In patient no. 17, 8 nucleotides at 8 positions were different from the reference sequence (Table 2), and 64.72% (578T/C), 98.14% (208C/T), 99.71% (527G/T), 99.83% (466T/C), 100% (204A/G), 100% (275A/G), 100% (276T/C) and 100% (378G/A) nucleotide substitutions were observed. These results showed that UDPS of the sporadic case was obviously different from those of the outbreak cases.

Comparison of UDPSs among HAV with different severities and HAV from different sources

The substitution rates were analyzed in compar-

ison to the reference sequence [19]. We also counted insertions as substitutions. In HAV IRES (nt. 90-620), total nucleotide substitutions of acute hepatitis, non-severe and severe forms from the outbreak, and sporadic acute hepatitis, severe form, were 0.020, 0.014, and 1.43%, respectively. In HAV IRES domain III (nt. 90-300), nucleotide substitutions of acute hepatitis, non-severe and severe forms from the outbreak, and sporadic acute hepatitis, severe form, were 0.047, 0.013, and 1.88%, respectively. In HAV IRES domain IV (nt. 301-580), nucleotide substitutions of acute hepatitis, non-severe and severe forms from the outbreak, and sporadic acute hepatitis, severe form, were 0.0034, 0.018, and 13.0%, respectively. In HAV IRES domain V (nt. 581-620), nucleotide substitutions of acute hepatitis, non-severe and severe forms from the outbreak, and sporadic acute hepatitis, severe form, were 0.0020, 0, and 0%, respectively. All nucleotide substitutions from acute hepatitis from the outbreak were transition mutations. Three nucleotide insertions were observed in only acute hepatitis, severe form, from the outbreak (Table 3).

Table 3. Nucleotide substitutions of hepatitis A virus (HAV) internal ribosomal entry site (IRES).

Type of Mutations	Insertion	Transition				Transversion	Total Numbers
		A/G	G/A	C/T	T/C	G/T	
Total Numbers	3	10	5	4	12	1	35
Numbers in Outbreak Cases	3	8	4	3	9	0	27
AH of Outbreak Cases	3	8	2	2	8	0	23
AH-S of Outbreak Cases	0	0	2	1	1	0	4
Numbers in Patient no. 17	0	2	1	1	3	1	8

"Numbers" equivalent to "nucleotide substitutions".

DISCUSSION

In the present study, we analyzed the UDPSs of HAV associated with the outbreak and tried to detect specific mutations in HAV 5′UTR associated with hepatitis A, severe form. Our result showed that no specific mutations in HAV 5′UTR associated with severe form existed in this outbreak. UDPS analysis of HAV 5′UTR revealed no association between the disease severity of hepatitis A and HAV 5′UTR substitutions. It might be more interesting to perform ultra-deep sequencing of the full-length HAV genome in order to uncover possible unknown genomic determinants associated with disease severity. Further studies will be needed regarding this point. We also found minor nucleotide sequence variations, which seemed undetectable by the Sanger method. We do not yet know what these variations mean.

The sensitivity of UDPS is higher than that of Sanger sequencing. Next-generation sequencing

technologies are increasingly being used to identify low-abundance minority genetic variants within a heterogeneous pool of amplified DNA molecules, such as those within a virus population, which are especially valuable for the detection of drug resistance mutations [20]. We characterized HAV minority variants in the HAV IRES region.

In general, HAV infection risk is inversely correlated to sanitation and other socio-economic indicators [21]. Although Japan is one of the developed countries in Asia, a universal vaccination program against HAV and HBV has not yet been initiated [17]. Although the present study did not include acute liver failure with hepatic encephalopathy such as fulminant hepatitis A, our study is important because UDPS analysis of these HAV strains reconfirmed that a single source might have caused this outbreak as previously reported [16], suggesting that UDPS analysis might be a new analytical tool for the source of hepatitis A outbreaks.

The effect of mutations in 5′UTR of HAV on the severity of the disease is a long story that has never been clearly proven. Fujiwara et al. reported an association between the severity of hepatitis A and nucleotide substitutions in 5′UTR of HAV RNA [8-10]. However, there have been several contrary observations [7,11-13]. As the definition of acute liver failure is also different among different countries [11,14], it seemed difficult to compare them using different criteria. Then we compared the different HAV IRES sequences derived from a single-source outbreak, based on a single definition of liver failure. In this outbreak, the proportion of acute liver failure was very high (3/29, 10.3%), compared to the previous report [22], although the calculation was performed on the basis of patients admitted into two hospitals [15,16]. The reference clonal sequence from patient 12 had only one different nucleotide from the sequence of the severe hepatitis strain HAV PT (A10), which was reported by Fujiwara et al [10], suggesting that our present study might be conducted among specific HAV strains.

The technical approach used in the present study had the advantage of having great power in detecting mutations present at a very low frequency in the swarm of mutants. However, our results confirm the old paradigm that 5′UTR of picornaviruses and particularly of HAV is highly conserved. This reason might be associated with the function of HAV IRES, preventing the occurrence of variability.

In conclusion, there were no different HAV IRES sequences between severe and non-severe forms in this outbreak. To our surprise, HAV strains in this outbreak conserved HAV IRES sequence even if we performed analysis of UDPSs. Further analysis of HAV UDPSs could give us new information concerning the association between the disease severity of hepatitis A and HAV genome substitutions.

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COMPETING INTERESTS

The authors have declared that no competing interest exists.

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