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Mutation patterns for two flaviviruses: hepatitis C virus and GB virus C/hepatitis G virus

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Abstract We studied the mutation patterns of hepatitis C virus (HCV) and GB virus C/hepatitis G virus (HGV). Although the mutation patterns of the two viruses were similar to each other, they were quite different from that of HIV. In particular, the similarity of the patterns between HCV or HGV and human nuclear pseudogenes was statistically significant whereas there was no similarity between HIV and human nuclear pseudogenes. This finding suggests that the mutation patterns of HCV and HGV are similar to the patterns of spontaneous substitution mutations of human genes, implying that nucleotide analogues which are effective against HCV and HGV may have a side effect on the normal cells of humans.

Key words: Mutation pattern; Relative substitution frequency; Phylogenetic tree; Hepatitis C virus; GB virus C/hepatitis G virus

1. Introduction

Mutation patterns of various human genes, such as functional genes, pseudogenes, viral oncogenes, and major histocompatibility complex genes, have been previously analyzed by Gojobori et al. [1–3]. They showed the mutation patterns as a set of 12 kinds of relative substitution frequencies, which is unaffected by a bias in base composition [1]. As far as mutation patterns of viruses are concerned, Moriyama et al. analyzed the mutation patterns of the human immunodeficiency virus (HIV) genes, using the method of Gojobori et al. [1], and identified a specific mutation pattern of HIV [4]. Based on their results of a high frequency of guanosine (G) to adenosine (A) mutations [4], they inferred that the HIV was likely to misincorporate thymidine (T) into the DNA genome during the reverse transcription process. Their inference is consistent with the high efficacy of 3′-azido-2′,3′-dideoxythymidine (AZT), an analogue of T, for HIV infection [5–7].

Hepatitis C virus (HCV) and GB virus C/hepatitis G virus (HGV) were discovered as the viruses associated with liver diseases [8–10], and they have been vigorously analyzed by many investigators world-wide. A large number of nucleotide sequences related to these viruses has been compiled in the DNA databases. We obtained 27 HCV 1b and 15 HGV nucleotide sequences encompassing the entire coding region. From these nucleotide sequences, we estimated the mutation patterns of HCV and HGV using the method of Gojobori et al. [1], and attempted to infer an efficient therapy for treating the infection by these two viruses using nucleoside analogues, as in the case of HIV. We found that the mutation patterns of HCV and HGV were similar to each other and that these two viruses prefer all four transitional mutations. Therefore, analogues of adenine (A), uracil (U), cytosine (C), and guanine (G) were similarly expected to be efficient in the treatment of the infection by these two viruses. However, we also found that mutation patterns of both HCV and HGV were similar to the patterns of spontaneous substitution mutations of human genes. This finding would suggest that nucleoside analogues being effective against HCV and HGV may have a side effect on normal human cells. Our results may provide pertinent information for the development of a therapy for the infection caused by these viruses.

2. Materials and methods

2.1. HCV and HGV nucleotide sequence data studied

The nucleotide sequence data for 27 HCV 1b strains and an HCV 1a strain (as shown in Fig. 1) and 15 HGV strains (as shown in Fig. 2), encompassing the entire coding region, were collected from the DNA databases (DDBJ/EMBL/GenBank).

2.2. Construction of phylogenetic trees for HCV and HGV strains

The nucleotide sequence alignments were made for the 27 HCV 1b strains with an HCV 1a strain, and for the 15 HGV strains, using the entire coding region. The computer program CLUSTAL W was used to produce sequence alignments [11], and the results were checked by eye. The phylogenetic trees were constructed for both the HCV and HGV strains, by the neighbor-joining method [12], with the evolutionary distances estimated by the 6-parameter method [13]. These analyses were performed on the molecular evolutionary software system, ODEN version 1.1 [14].

As shown on the Web site of the HCV database (http://s2as02.genes.nig.ac.jp), HCV 1b and 1a strains made separate clusters in the phylogenetic tree for HCV strains. Therefore, we regarded an HCV 1a strain, the HCV-1, as an outgroup sequence in the phylogenetic tree for the 27 HCV 1b strains. Concerning HGV strains, African-type strains such as GBV-C reported by Simons et al. has been shown to diverge first from other HGV strains in the phylogenetic analyses for HGV strains [9,15]. Therefore, we regarded the GBV-C as an outgroup sequence in the phylogenetic tree for the remaining 14 HGV strains.

2.3. Analyses of mutation patterns for the 27 HCV 1b and 14 HGV strains

The mutation patterns for the 27 HCV 1b and 14 HGV strains were estimated by the method of Gojobori et al. [1]. In the phylogenetic

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Abbreviations: HCV, hepatitis C virus; HGV, GB virus C/hepatitis G virus; HIV, human immunodeficiency virus; RT, Reverse transcriptase; AZT, 3′-azido-2′,3′-dideoxythymidine

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trees for HCV and HGV strains, the ancestral sequence was inferred at every node by the maximum parsimony method [3]. Then, the numbers for the 12 kinds of mutations, namely, A to U, A to C, A to G, U to A, and so on, were counted for each tree. If the nucleotide at a node had not been determined uniquely, we ignored the mutations that must have occurred on the branches connected with that node. To obtain the relative substitution frequencies among four nucleotides, the numbers of mutations were standardized by the base compositions [1]. The relative substitution frequency indicates the substitution frequency from a particular nucleotide to another in a hypothetical sequence of 100 nucleotides with equal amounts of each nucleotide base (25A, 25U, 25C, 25G). This value is more useful than the raw frequency of nucleotide substitutions, because it is free from the bias in the base compositions in actual sequences [1]. The mutation patterns were estimated at the 1st and 2nd positions, and the 3rd position of the codons, respectively.

2.4. Comparisons among the mutation patterns

By computing the correlation coefficients between the sets of relative substitution frequencies, the mutation patterns for HCV and HGV were compared with one another. In this comparative study, we also included the mutation patterns for the HIV genes, human nuclear pseudogenes, and human functional genes, which were obtained from the literature [1,4].

3. Results

3.1. Mutation patterns of HCV and HGV strains

From the analysis of approximately 250 000 nucleotides of the 27 HCV 1b strains, encompassing the entire coding regions, we detected 8960 mutations at all positions of the codons, out of which 2664 and 6296 were at the 1st and 2nd positions, and at the 3rd position of the codons, respectively. From the analysis of approximately 130 000 nucleotides of the 14 HGV strains, encompassing the entire coding regions, we detected 6653 mutations at all positions of the codons, out of which 1027, and 5626 were at the 1st and 2nd positions, and at the 3rd position of the codons, respectively. For the HCV strains, in 386 mutations of these mutations, which particular nucleotide mutated to another could not be uniquely determined. For the HGV strains, 1043 mutations could not be uniquely determined. We excluded these mutations from our analysis and computed the relative substitution frequencies in the entire coding region of HCV and HGV from the remaining 8574 and 5610 mutations, at the 1st and 2nd positions, and at the 3rd position of the codons, respectively. Table 1 shows the relative substitution frequencies at the 1st and 2nd positions, and at the 3rd position of the codons for the 27 HVC 1b and 14 HGV strains. In HCV 1b strains, the fre-

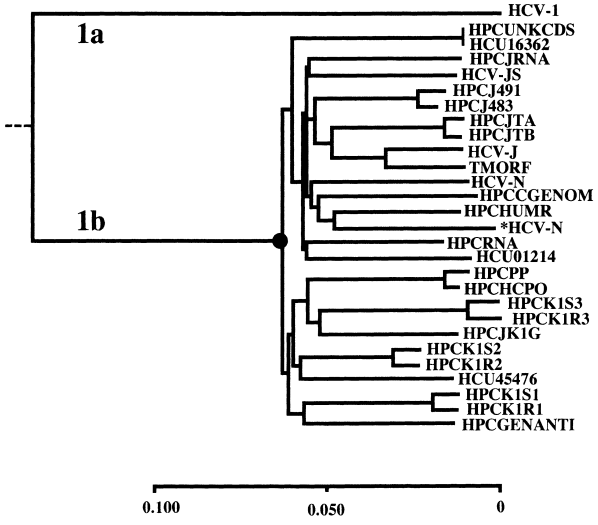

Fig. 1. A phylogenetic tree of the entire coding region of 22 HCV 1b strains with an HCV 1a strain as the outgroup sequence. The sequence data were collected from the DNA databases (DDBJ/EMBL/GenBank). The following are the accession numbers of the HCV strains analyzed: HCV-1: M62321, HCV-JS: D85516, HPCJ491: D10750, HPCJ483: D13558, HPCJTA: D11168, HPCJTB: D11355, HCV-J: D90208, HCV-N: D63857, HPCHUMR: M58335, *HCV-N: S62220, HPCRNA: D10934, HCU01214: U01214, HPCPP: D30613, HPCHCPO: D45172, HPCK1S3: D50484, HPCK1R3: D50482, HPCK1S2: D50485, HPCK1R2: D50481, HPCK1S1: D50483, HPCK1R1: D50480, HCVJK1G: X61596, and HPCGENANTI: M84754, HCU45476: U45476, HPCUNKCDS: M96362, HCU16362: U16362, HPCJRNA: D14484, TMORF: D89872. The tree was constructed by the neighbor-joining method based on the numbers of nucleotide substitutions estimated by the 6-parameter method. The root of the tree was determined at the midpoint of the tree between the 27 HCV 1b strains and an HCV 1a strain. The most recent ancestor for HCV 1b strains is represented by the filled circle. We continued to infer the ancestral nucleotide at every node of the phylogenetic tree in each nucleotide position of the entire coding region of these HCV 1b strains. The numbers of the 12 kinds of mutations in the entire coding region of 27 HCV 1b strains were determined from the tree topology. Subsequently, we computed the relative substitution frequencies from the numbers of the 12 kinds of mutations, corrected by the nucleotide compositions of the studied strains. The scale bar indicates the number of nucleotide substitutions per site.

Table 1
Mutation patterns for HCV 1b and HGV

		1st and 2nd positions		3rd position		All positions	
		HCV 1b	HGV	HCV 1b	HGV	HCV 1b	HGV
A	U	5.0	2.8	1.9	6.7	2.7	4.7
	C	4.9	8.9	2.0	4.4	2.7	4.5
	G	20.2	9.6	24.3	16.2	19.7	11.9
U	A	3.5	3.3	1.6	5.1	2.1	4.5
	C	15.4	23.3	28.8	19.4	22.9	19.1
	G	2.4	2.7	1.8	5.0	1.9	4.3
C	A	4.7	9.6	1.3	3.2	2.5	4.8
	U	16.5	26.0	19.7	18.0	22.6	22.5
	G	5.8	2.3	1.5	3.6	2.9	4.0
G	A	15.3	6.8	14.2	10.6	15.8	11.3
	U	2.0	1.6	1.4	4.5	1.7	4.6
	C	4.4	3.3	1.6	3.5	2.6	3.9
Transition		67.4	65.7	87.0	64.2	81.0	64.8

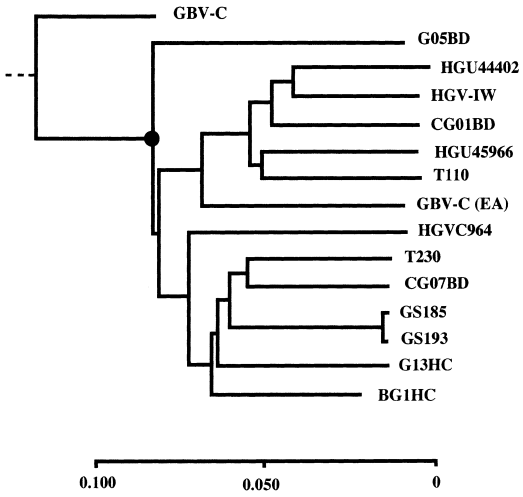

Fig. 2. A phylogenetic tree of the entire coding region of 15 HGV strains. The following are the accession numbers of the HGV strains analyzed: GBV-C: U36380, HGU44402: U44402, HGU45966: U45966, HGV-IW: D87255, G05BD: AB003292, CG01BD: AB003289, CG07BD: AB003290, G13HC: AB003293, BG1HC: AB003288, T110: D90600, T230: D90601, GBV-C(EA): U63715, HGVC964: U75356, GS185: D87262, GS193: D87263. The tree was constructed by the neighbor-joining method based on the numbers of nucleotide substitutions estimated by the 6-parameter method. Tanaka et al. demonstrated that the HGV originated from African-type strains such as GBV-C (accession number: U36380) reported by Simons et al. [9,15]. Therefore, we constructed the phylogenetic tree of 14 HGV strains with GBV-C as the outgroup sequence. The root of the tree was determined at the midpoint of the tree between the 14 HGV strains and GBV-C, and we thought that the node was the ancestral nucleotide sequence of these 14 HGV strains (represented by the filled circle). We continued to infer the ancestral nucleotide at every node of the phylogenetic tree in each nucleotide position of the entire coding region of these HGV strains. The numbers of 12 kinds of mutations in the entire coding region of 14 HGV strains were inferred from the tree topology. Subsequently, we computed the relative substitution frequencies from the numbers of the 12 kinds of mutations, corrected by the nucleotide compositions of the studied strains. The scale bar indicates the number of nucleotide substitutions per site.

quencies of transitions, including A to G, G to A, U to C, and C to U, were high at both the 1st and 2nd positions and at the 3rd position of the codons. The total frequency of the four transitions of HCV at the 3rd position was 87.0%. In HGV strains, the frequencies of transitions, including A to G, G to A, U to C, and C to U, were high at the 3rd position of the codons.

3.2. *Correlation coefficients among mutation patterns of various genes*

To quantify the similarities in mutation patterns between different genes, we calculated the correlation coefficients and P values between the different sets of relative substitution frequencies of the genes (Table 2).

The correlation coefficients between the frequencies at the 1st and 2nd positions and those at the 3rd position of the codons were high in both HCV and HGV strains. The coefficient for HCV was 0.935 ($P < 0.0001$), and that for HGV was 0.833 ($P = 0.0003$). The patterns at the 1st and 2nd positions and at the 3rd position were significantly similar to each other in both HCV and HGV, although the patterns at the 1st and 2nd positions should be skewed by selective forces and the patterns at the 3rd position of the codons should be close to the pattern of spontaneous substitution mutation.

Correlation coefficients between HCV and HGV were very high, at both the 3rd position and all positions. The coefficient at the 3rd position was 0.974 ($P < 0.0001$) and was 0.952 ($P < 0.0001$) at all positions. Since the patterns at the 3rd position of the codons should be close to the pattern of spontaneous substitution mutations [4], the spontaneous substitution patterns of HCV and HGV were similar to each other. The lowest value of the correlation coefficients among the patterns of HCV and HGV was found between the patterns of HCV and HGV at the 1st and 2nd positions. In this case, the correlation coefficient was 0.690 ($P = 0.011$).

The mutation patterns for HIV genomes were analyzed using the phylogenetic tree by Moriyama et al. [4]. We, then, included the mutation pattern for HIV in this comparative analysis. The correlation coefficients between the patterns of HIV genomes were in the range from 0.659 ($P = 0.078$) to 0.909 ($P < 0.0001$), and the patterns between HIV genomes were correlated to one another. Next, we compared the mutation pattern of HCV and HGV with that of HIV. The correlation coefficients in the patterns between the HCV and HIV genomes ranged from 0.471 ($P = 0.1249$) to 0.724 ($P = 0.0059$). The correlation coefficients in the patterns between the HGV and HIV genomes ranged from 0.111 ($P = 0.7390$) to 0.531 ($P = 0.0757$). These results indicate that the patterns of HCV and HGV correlated to each other, more than those of HCV and HIV, or those of HGV and HIV, since the correlation coefficients in the patterns between the HCV and HGV ranged from 0.690 ($P = 0.011$) to 0.976 ($P < 0.0001$).

Moreover, we included the mutation patterns of human nuclear pseudogenes and functional genes reported previously [1], in this study. The mutation pattern of human nuclear pseudogene can be thought of as the pattern of spontaneous substitution mutation of the human gene, because the pseudogenes do not code for any functional proteins and thus can accumulate any kind of mutational change. The correlation coefficient in the patterns between human nuclear pseudogenes and functional genes was 0.496 ($P = 0.1026$), and then, the selective force to functional genes was suggested. We compared the patterns of HCV, HGV, and HIV with those of human genes. The correlation coefficients in the patterns between human nuclear pseudogenes and HCV ranged from 0.562 ($P = 0.0563$) to 0.718 ($P = 0.0067$). The correlation coefficients in the pattern between human nuclear pseudogene and HGV ranged from 0.626 ($P = 0.0276$) to 0.755 ($P = 0.0032$). The correlation coefficients in the pattern between human nuclear pseudogenes and HIV ranged from 0.453 ($P = 0.1430$) to 0.660 ($P = 0.0174$). These observations indicate that the correlations between the patterns of HCV of HGV and human nuclear pseudogenes were stronger than the correlation between those of HIV and human nuclear pseudogenes. Therefore, the mutation patterns of HCV and HGV were similar to the pattern of spontaneous substitution mutation of the human genome.

4. Discussion

In functional genes, the mutation patterns at the 3rd position of the codons can reflect, to some extent, the patterns of spontaneous substitution mutations, since the mutations at the 3rd position among such genes are mostly free from func-

Table 2
Correlation coefficient of mutation patterns for various genes

	HCV 1st and 2nd[a]	HCV 3rd[b]	HCV all[c]	HGV 1st and 2nd[a]	HGV 3rd[b]	HGV all[c]	HIV env 3rd[b]	HIV env all[c]	HIV gag 3rd[b]	HIV gag all[c]	Human nuclear pseudogene
HCV 3rd[b]	0.935 (<0.0001)										
HCV all[c]	0.957 (<0.0001)	0.976 (<0.0001)									
HGV 1st and 2nd[a]	0.690 (0.011)	0.783 (0.0016)	0.828 (0.0004)								
HGV 3rd[b]	0.907 (<0.0001)	0.974 (<0.0001)	0.976 (<0.0001)	0.833 (0.0003)							
HGV all[c]	0.839 (0.0003)	0.896 (<0.0001)	0.952 (<0.0001)	0.927 (<0.0001)	0.948 (<0.0001)						
HIV env 3rd[b]	0.615 (0.314)	0.471 (0.1249)	0.505 (0.0956)	0.111 (0.7390)	0.355 (0.2658)	0.308 (0.3401)					
HIV env all[c]	0.724 (0.0059)	0.534 (0.0738)	0.550 (0.0634)	0.174 (0.5985)	0.411 (0.1895)	0.320 (0.3201)	0.909 (<0.0001)				
HIV gag 3rd[b]	0.680 (0.0129)	0.574 (0.0500)	0.642 (0.0225)	0.285 (0.3790)	0.531 (0.0757)	0.520 (0.0837)	0.887 (<0.0001)	0.756 (0.0031)			
HIV gag all[c]	0.747 (0.0037)	0.803 (0.0009)	0.788 (0.0014)	0.503 (0.0967)	0.734 (0.0049)	0.674 (0.0142)	0.764 (0.0025)	0.659 (0.0178)	0.856 (0.0001)		
Human nuclear pseudogene	0.709 (0.0079)	0.562 (0.0563)	0.718 (0.0067)	0.628 (0.0267)	0.626 (0.0276)	0.755 (0.0032)	0.462 (0.1337)	0.456 (0.1395)	0.660 (0.0174)	0.453 (0.1430)	
Human functional genes	0.462 (0.1333)	0.199 (0.5411)	0.298 (0.3573)	−0.028 (0.9340)	0.103 (0.7560)	0.144 (0.6643)	0.812 (0.0007)	0.803 (0.0009)	0.636 (0.0243)	0.373 (0.2394)	0.496 (0.1026)

The values whose P values are less than 0.001 are underlined.
[a]1st and 2nd: 1st and 2nd positions.
[b]3rd: 3rd position.
[c]all: all positions

tional constraints [4]. We have shown that the mutation patterns of HCV and HGV were similar to each other, especially, at the 3rd codon position. Therefore, the patterns of spontaneous substitution mutations for HCV and HGV were suggested to be similar. In the case of viruses, it is believed that the incidence of mutations is mainly related to the fidelity of replicase [16–18]; thus, the characteristics of the virus replicase may reflect the patterns of spontaneous substitution mutation [4]. As far as HCV and HGV were concerned, they are replicated by their own RNA-dependent RNA polymerases [19,20]. Therefore, the similarity of the mutation patterns between HCV and HGV at the 3rd codon position, characterized by a high frequency of transitions, may imply that the natures of RNA-dependent RNA polymerase of the two viruses are similar to each other. HCV and HGV were reported to be evolutionarily related to each other and to belong to the Flaviviridae family [9,10]. Both are positive-strand RNA viruses with a similar genomic organization [20]. This may be the reason why HCV and HGV have common mutation patterns that reflect the characteristics of each RNA-dependent RNA polymerase.

Nucleoside analogues are known to inhibit replicase of genomes as substrate competitors. Replication or mutation rate of HCV [21,22], HGV [23], and HIV genomes were reported to be higher than that of the human genome [24,25]. Therefore, nucleoside analogues are supposed to inhibit the replicase of these viruses more efficiently than DNA polymerase of the host's cells. In the therapy for HIV infection, AZT is more effective than other nucleoside analogues, and is the most appropriate agent among nucleoside analogues for combination therapy against HIV infection [5,6,26]. Moriyama et al. estimated the mutation patterns of HIV and suggested that a high incidence of G to A mutation is one of the reasons for the greater efficacy of AZT, when compared with other nucleoside analogues. During HIV replication, normally, G in HIV RNA is replicated to complementary C in DNA by reverse transcriptase (RT), and the C is replicated to complementary G in a new virus particle by DNA-dependent DNA polymerase of the host. In the G to A mutation, G is supposed to be replicated to incorrect T in DNA by RT, since the high incidence of mutations of HIV is due mainly to the low fidelity of RT. Therefore, a high incidence of G to A mutations implies that incorrect T is more easily misincorporated into DNA by RT. Thus, Moriyama et al. explained why AZT, an analogue of T, is more effective than other analogues.

Using nucleoside analogues from our observations, we attempted to infer an efficient therapy for HCV and HGV infection. In the analysis of the mutation patterns of HCV and HGV, we found that these two viruses prefer to mutate by all four transitional patterns (Table 1). Therefore, analogues of A, U, C, and G were equally incorporated into their next generation during the replication process, and were expected to be efficient in the inhibition of the replication of these two viruses. However, the mutation patterns of HCV and HGV were similar to the patterns of spontaneous substitution mutations of human genes. This finding suggests that nucleoside analogues efficient for inhibiting the replication of HCV or HGV could also inhibit the replication of human genes, and that severe side effects may result as a consequence.

Although HCV and HGV had a similar mutation pattern, as described above, humans infected by these viruses show different clinical manifestations. HCV has been a causative agent of chronic liver disease [27] and is associated etiologically with hepatocellular carcinoma [28]. In regard to the therapy for chronic hepatitis C, the current standard therapy using interferon is rather unsatisfactory [29–31]. Therefore, the search for additional therapies needs to be pursued. On the other hand, the association of HGV infection with liver

disease has recently become doubtful [32,33], although HGV has been identified as a new virus that causes hepatitis [9,10]. HGV may be an innocent by-stander virus and a therapy for HGV infection may not be indicated at all. The benefits of a therapy for HCV or HGV infection, using nucleoside analogues, must be weighed carefully, considering the side effects of nucleoside analogues and the pathogenesis of these two viruses. Therefore, it is imperative that further studies in this area be conducted.

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