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SHORT COMMUNICATION

Analysis of synonymous codon usage in enterovirus 71

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Abstract Enterovirus 71 (EV71) is the major cause of hand-foot-and-mouth disease in children. In our study, using the complete genome sequences of 42 EV71 representing all three genotypes, we analyzed synonymous codon usage and the relative dinucleotide abundance in EV71 genome. The general correlation between base composition and codon usage bias suggests that mutational pressure rather than natural selection is the main factor that determines the codon usage bias in EV71 genome. Furthermore, we observed that the relative abundance of dinucleotides in EV71 is independent of the overall base composition but is still the result of differential mutational pressure, which also shapes codon usage. In addition, other factors, such as hydrophobicity and aromaticity, also influence the codon usage variation among the genomes of EV71. This study represents the most comprehensive analysis of EV71 codon usage patterns and provides a basic understanding of the mechanisms for codon usage bias.

Keywords EV71 · Synonymous codon usage · Mutational bias · Dinucleotide bias · Subgenotype

Introduction

Enterovirus 71 (EV71) is a member of the *Enterovirus* genus of the *Picornaviridae* family, which is the major cause of hand-foot-and-mouth disease (HFMD) in children. EV71 is a small, non-enveloped virus with a positive-stranded RNA genome size of about 7.4 kb. The virus has a single-stranded positive-sense RNA containing a single open reading frame (ORF) encoding a polyprotein that, following viral protease mediated co- and post-translational processing, gives rise to 4 capsid proteins (VP1, VP2, VP3, and VP4) and 7 nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D) [26]. Studies on the phylogenetic relationship of EV71 have divided the viruses into genotypes A, B and C [2], which has been further divided into subtypes, designated A, B1-5, and C1-4 based on sequencing the region encoding the VP1 major capsid protein [16, 20]. Synonymous codons are not used randomly [14, 15]. Mutational pressure and translational selection were thought to be the main factors that account for codon usage variation among genes in some human RNA virus [12, 13, 29]. Studies the extent and causes of biases in codon usage is essential to the understanding of viral evolution, particularly the interplay between viruses and the immune response [4, 17]. Recently, recombination was found to play a more important role than positive selection in the formation of genetic diversity in EV71 virus. Positive selection was only detected at site 145 of VP1, but most amino acid sites of nonstructural proteins were under negative selection [5]. Previous studies of EV71 have mainly been limited to phylogenic analysis, and few synonymous codon usage analyses have been applied. In order to better understand the characteristics of the EV71 genome and to reveal more information about the viral genome, we have analyzed the codon usage and dinucleotide composition. In this report, we sought to address the issues concerning codon usage in EV71 virus.

A total of 42 publicly available complete human EV71 RNAs isolated from China, Taiwan, Malaysia, Singapore, Australia, Japan, South Korea, Viet Nam, America and Norway were download from GeneBank and sequence

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Table 1 List of EV71 strains used for analysis of synonymous codon usage in this study

Genebank accession	Strain	Country	Date	Genotype	GC3s	ENC	Mononucleotide frequencies (%)				Reference
							C	T	A	G	
AB204852	BrCr-Tr	Japan	2008	A	0.473	56.09	0.2172	0.2476	0.2795	0.2385	[1]
AB204853	BrCr-Ts	Japan	2008	A	0.473	56.19	0.2174	0.2475	0.2795	0.2385	[1]
AM396587	UH1/PM/1997	Malaysia	1997	B	0.480	55.97	0.2201	0.2481	0.2744	0.2399	[27]
U22522	MS/7423/87	America	2001	B	0.478	55.94	0.2198	0.2488	0.2742	0.2394	[3]
AM396586	EV71/SAR/SHA66	Malaysia	2006	B	0.473	56.82	0.2212	0.2482	0.2754	0.2373	[27]
DQ341362	SB12736-SAR-03	Malaysia	2007	B	0.479	56.20	0.2218	0.2476	0.2739	0.2396	Unpublished
DQ341365	PP37-MAL-01	Malaysia	2007	B	0.484	56.55	0.2221	0.2465	0.2736	0.2409	Unpublished
DQ341366	SB2864-SAR-00	Malaysia	2007	B	0.477	56.19	0.2212	0.2470	0.2750	0.2397	Unpublished
DQ341354	3799-SIN-98	Singapore	2007	B	0.470	56.63	0.2216	0.2478	0.2769	0.2356	Unpublished
DQ341364	5511-SIN-00	Singapore	2007	B	0.479	55.59	0.2234	0.2449	0.2757	0.2388	Unpublished
EU364841	26M/AUS/4/99	Australia	2008	B	0.479	56.80	0.2206	0.2485	0.2756	0.2374	[6]
AB469183	EV71-GFP	Malaysia	2008	B	0.520	55.82	0.2319	0.2380	0.2715	0.2413	Unpublished
EF373575	E2002042-TW-CDC	Taiwan	2008	B	0.473	56.19	0.2213	0.2468	0.2765	0.2380	Unpublished
AF176044	1245a/98/tw	Taiwan	1999	C	0.493	56.43	0.2207	0.2427	0.2762	0.2440	Unpublished
AF119796	TW/2086/98	Taiwan	2000	C	0.494	56.40	0.2209	0.2418	0.2748	0.2453	[19]
AF304458	Tainan/6092/98	Taiwan	2001	C	0.493	56.42	0.2210	0.2424	0.2763	0.2438	[26]
AY465356	SHZH03	China	2003	C	0.467	56.97	0.2210	0.2499	0.2751	0.2364	Unpublished
DQ452074	804/NO/03	Norway	2003	C	0.479	57.07	0.2187	0.2455	0.2780	0.2414	[22]
DQ133458	984	Taiwan	2004	C	0.478	56.96	0.2221	0.2482	0.2737	0.2377	Unpublished
DQ133459	1235	Taiwan	2004	C	0.472	56.72	0.2206	0.2494	0.2756	0.2371	Unpublished
DQ060149	pinf7-54A	Taiwan	2005	C	0.492	56.83	0.2199	0.2430	0.2763	0.2446	Unpublished
JQ965759	540V/VNM/05	Viet Nam	2005	C	0.473	57.05	0.2222	0.2484	0.2756	0.2365	[28]
AM396584	ENT/PM/SHA52	Malaysia	2006	C	0.486	56.56	0.2204	0.2430	0.2785	0.2421	[27]
DQ341357	7F-AUS-6-99	Australia	2007	C	0.497	57.22	0.2216	0.2421	0.2759	0.2447	Unpublished
DQ341361	1M-AUS-12-00	Australia	2007	C	0.480	56.90	0.2192	0.2447	0.2783	0.2415	Unpublished
DQ341358	S40221-SAR-00	Malaysia	2007	C	0.482	56.76	0.2187	0.2449	0.2779	0.2426	Unpublished
DQ341359	S10862-SAR-98	Malaysia	2007	C	0.486	56.89	0.2209	0.2432	0.2783	0.2415	Unpublished
DQ341360	J115-MAL-01	Malaysia	2007	C	0.487	57.12	0.2198	0.2438	0.2777	0.2426	Unpublished
DQ341355	06-KOR-00	South Korea	2007	C	0.484	56.41	0.2181	0.2465	0.2754	0.2434	Unpublished
EF063152	E2005125-TW	Taiwan	2007	C	0.477	57.41	0.2158	0.2479	0.2762	0.2437	unpublished
FJ194964	EV71/GDFS/3/2008	China	2008	C	0.479	57.11	0.2207	0.2499	0.2733	0.2386	Unpublished
FJ194965	EV71/DGSC117/2008	China	2008	C	0.478	56.99	0.2236	0.2462	0.2756	0.2373	Unpublished
FJ360544	GZ08-01	China	2008	C	0.475	56.82	0.2224	0.2485	0.2739	0.2377	Unpublished
FJ360545	GZ08-02	China	2008	C	0.482	56.54	0.2239	0.2470	0.2745	0.2374	Unpublished
FJ439769	Fuyang-0805	China	2008	C	0.481	56.65	0.2260	0.2452	0.2751	0.2368	[24]
EF373576	E2004104-TW-CDC	Taiwan	2008	C	0.478	56.96	0.2230	0.2478	0.2751	0.2368	Unpublished
EU131776	N3340-TW-02	Taiwan	2008	C	0.478	56.55	0.2236	0.2465	0.2771	0.2354	[11]
FJ606447	BJ08-Z004-3	China	2009	C	0.466	56.63	0.2199	0.2509	0.2757	0.2362	[8]
FJ606449	BJ08-Z020-1	China	2009	C	0.477	56.76	0.2250	0.2461	0.2759	0.2362	[8]
FJ606450	BJ08-Z025-5	China	2009	C	0.477	56.34	0.2244	0.2461	0.2756	0.2373	[8]
FJ713137	Shanghai036-2009	China	2009	C	0.477	56.91	0.2230	0.2478	0.2734	0.2385	[25]
JQ806378	35/Jingdezhen/China/HFMD_Severe/2011	China	2011	C	0.479	56.79	0.2244	0.2473	0.2739	0.2373	Unpublished

Table 2 Synonymous codon usage in EV71 viruses

AA	Codon	*N*	RSCU	AA	Codon	*N*	RSCU
Phe	UUU	1,918	0.99	Ser	UCU	1,168	1.01
	UUC	**1,938**	**1.01**		**UCC**	**1,294**	**1.12**
Leu	UUA	1,004	0.77		UCA	1,502	1.30
	UUG	**1,572**	**1.21**		UCG	436	0.38
Tyr	UAU	1,654	0.91	Cys	UGU	868	0.99
	UAC	**1,970**	**1.09**		**UGC**	**880**	**1.01**
ter	UAA	20	0.00	ter	UGA	1	0.00
ter	UAG	21	0.00	Trp	UGG	1,302	1.00
Leu	**CUU**	**1,489**	**1.15**	Pro	CCU	1,409	1.08
	CUC	1,435	1.10		CCC	1,189	0.91
	CUA	1,054	0.81		**CCA**	**2,084**	**1.59**
	CUG	1,242	0.96		CCG	547	0.42
His	CAU	771	0.73	Arg	CGU	311	0.45
	CAC	**1,335**	**1.27**		**CGC**	**716**	**1.05**
Gln	**CAA**	**1,938**	**1.09**		CGA	368	0.54
	CAG	1,608	0.91		CGG	276	0.40
Ile	**AUU**	**2,220**	**1.25**	Thr	ACU	1,874	1.18
	AUC	1,887	1.06		**ACC**	**1,953**	**1.23**
	AUA	1,233	0.69		ACA	1,894	1.20
Met	AUG	2,083	1.00		ACG	611	0.39
Asn	AAU	1,924	0.92	Ser	**AGU**	**1,373**	**1.19**
	AAC	**2,277**	**1.08**		AGC	1,136	0.99
Lys	AAA	2,145	0.91	Arg	**AGA**	**1,261**	**1.84**
	AAG	**2,576**	**1.09**		AGG	1,170	1.71
Val	GUU	1,574	0.97	Ala	**GCU**	**2,121**	**1.21**
	GUC	1,423	0.88		GCC	1,803	1.03
	GUA	795	0.49		GCA	2,194	1.25
	GUG	**2,676**	**1.65**		GCG	908	0.52
Asp	**GAU**	**2,666**	**1.11**	Gly	**GGU**	**1,799**	**1.10**
	GAC	2,151	0.89		GGC	1,614	0.99
Glu	GAA	2,135	0.93		GGA	1,474	0.90
	GAG	**2,479**	**1.07**		GGG	1,637	1.00

The preferentially used codons for each amino acid are displayed in bold

AA Amino acids, *N* number of codons, *RSCU* cumulative relative synonymous codon usage

with >99 % sequence identities were excluded. The EV71 genome represented 3 genotypes (A, B and C). The serial number (SN), mononucleotide composition of each genome, GenBank accession numbers, genotype, and other detail information are listed in Table 1. Relative synonymous codon usage (RSCU) values are largely independent of amino acid composition and are particularly useful in comparing codon usage between genes, or sets of genes that differ in their size and amino acid composition. To examine synonymous codon usage without the confounding influence of amino acid composition of different EV71 virus, RSCU values of each

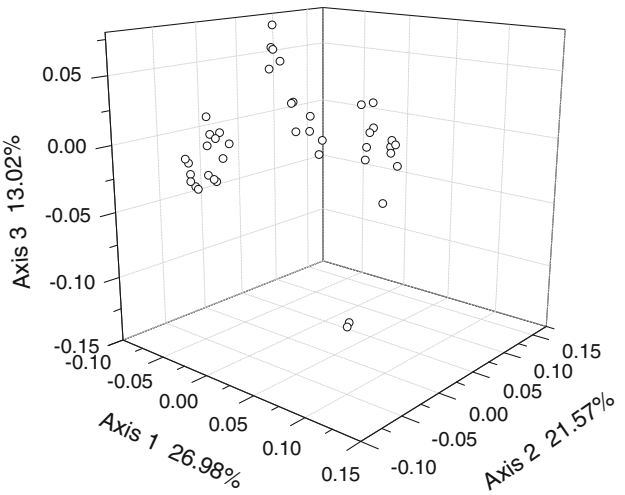

Fig. 1 A plot of value of the first, second and third axis of each ORF in COA. The first axis accounts for 26.98 % of all variation among ORFs, the second axis accounts for 21.57 % and third axis accounts for 13.02 % of total vibrations

codon in each ORF were used to measure the synonymous codon usage [18]. RSCU values of different codon in each ORF were calculated to investigate the extent of codon bias in EV71. The details of each ORF and the overall RSCU values of 59 codons in 42 EV71 genomes are, respectively, represented in Tables 1 and 2. The preferentially used codons were A-ended (3 ones), U-ended (6 ones) codons, C-ended (8 ones), codons G-ended (4 ones) codons. The average GC content of all EV71 viruses was 46.09 % (From 45.57 to 47.32 %, with a S.D. of 0.32 %), and the average GC3s content in codons was 48.05 % (From 46.62 to 51.98 %, with a S.D. of 0.94 %). This is consistent with our previous observations that EV71 viruses are GC-moderate genomes, and so it is expected that the third-ended codons are not preferentially used. In order to investigate whether these 42 coding sequences of EV71 display similar compositional features, ENC values were calculated (Table 1). The effective number of codons of a gene (ENC) is generally used to quantify the codon usage bias of a gene, which is essentially independent of gene length. The ENC values range from 20 to 61. The larger the extent of codon preference in a gene, the smaller the ENC value is. In an extremely biased gene where only one codon is used for each amino acid, this value would be 20; in an unbiased gene, it would be 61 [7]. The ENC values of different EV71 genes vary from 55.59 to 57.41, with a mean of 56.62 and S.D. of 0.4039. We found that all the ENC values for EV71 ORFs are much higher (ENC > 55). Based on this finding, together with published data on codon usage bias among some RNA viruses [10, 21, 29], we conclude that the codon usage bias in EV71 genome is slight.

Spearman's rank correlation analysis and multiple regression analysis were performed to determine the role of different factors in shaping the codon usage biases in the various EV71 viruses. All statistical analyses, as well as cluster analysis, were carried out using the statistical analysis software SPSS Version 15.0. To investigate synonymous codon usage variation among EV71 viruses, COA was implemented for all 42 EV71 ORFs selected for this study. Figure 1 depicts the position of each ORF on the plane defined by the first, second and third principal axes generated by COA on RSCU values of ORFs. The first principal axis accounts for 26.98 % of the total variation. The next three axes account for 21.57, 13.02, and 8.05 % of the variation, respectively. This observation indicates that although the first major axis explains a substantial amount of variation in trends in codon usage, the second major axis also has an appreciable impact on total variation in synonymous codon usage. If not specifically mentioned, the values of the first two axes of this COA were used for correlation and regression analysis hereafter.

Mutational pressure and translational selection were thought to be the main factors that account for codon usage variation among genes in some human RNA virus [12, 13, 29]. Therefore, we compared the G+C content at the first and second codon positions (GC12s) with that at the synonymous third position (GC3s) to investigate which factor in EV71 can explain their codon usage. It was found that GC12s and GC3s are significantly correlated ($r = 0.393$, $P < 0.01$). This suggests that they are most likely the result of mutational pressure, as natural selection would be expected to act differently on different codon positions. The plot of ENC and GC3S is another effective way to explore codon usage variation among genes [23]. In order to further find whether codon usage variation among EV71 virus is determined by mutational bias, ENC values of each virus gene were plotted against its corresponding GC3s. Genes, whose codon choice is constrained only by a G+C mutational bias, will lie on or just below the curve of the predicted values. All of the spots lie below the expected curve as shown in Fig. 2. In addition, a significantly positive correlation between GC3s and ENC ($r = -0.048$, $P < 0.001$) values was observed. The results indicated that the codon usage bias in these 42 EV71 genomes is greatly influenced by the G+C mutation bias.

Multivariate statistical analysis can be used to explore the relationships between variables and samples. The major trend in codon usage variation among ORFs can be investigated using correspondence analysis (COA). In order to minimize the effects of amino acid composition on codon usage, each ORF is represented as a 59-dimensional vector; each dimension corresponds to the RSCU value of one sense codon (excluding AUG, UGG, and stop codons). Major trends within this dataset can be determined using

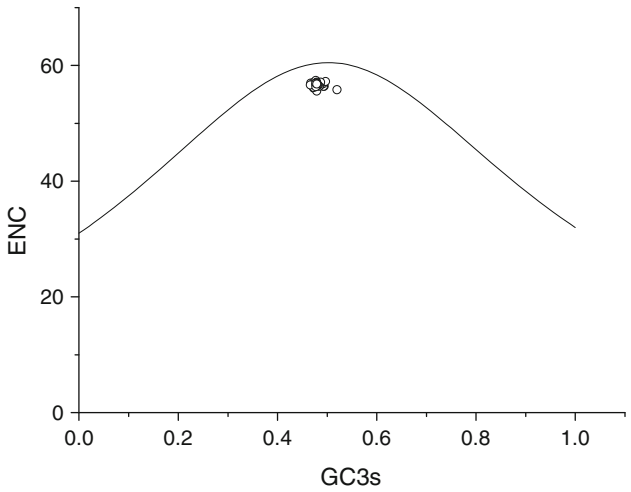

Fig. 2 Effective number of codons used in each ORF plotted against the GC3s. The continuous curve plots the relationship between GC3s and NEC in the absence of selection. All of spots lie below the expected curve

Table 3 Summary of correlation analysis between the first two axes in COA and GC12s, GC3s, GRAVY, or aromaticity in the selected 42 EV71 ORFs

	GRAVY	Aromaticity	GC3s	GC12s
Axis 1				
r	0.450**	−0.104	0.342*	0.628**
P	0.003	0.513	0.027	<0.001
Axis 2				
r	0.446**	−0.653**	0.208	−0.373*
P	0.003	<0.001	0.186	0.018
ENC				
r	−0.073	0.500**	−0.048**	
P	0.644	<0.001	<0.001	

* *P* value ≤0.05; ** *P* value ≤0.01

measures of relative inertia and genes ordered according to their positions along the axis of major inertia. We analyzed the correlation between the first or second axis values in COA and GC12s or GC3s values of each strain. The first axis value in COA of each selected genome, which contains most of the variation in synonymous codon usage bias between these genomes, is closely correlated with the GC composition at the first, second, and third codon position (Table 3). The second axis in the COA of each gene is also closely correlated with the GC12s. This analysis indicated that most of the codon usage bias among different ORFs is directly related to the nucleotide composition. Therefore, the compositional constraint is the main determinant of the variation in synonymous codon usage among different EV71 ORFs. These results were similar with previously

study described by Liu et al. that the interaction between mutation pressure from virus and natural selection from host exists in the processes of evolution of EV71 [13]. However, other factors, such as hydrophobicity and aromaticity, whether influence the codon usage variation among the genomes of EV71 need to be elucidated.

The GC index was used to calculate the overall GC content in the ORF, while the index GC3s was used to calculate the fraction of GC nucleotides at the synonymous third codon position (excluding Met, Trp, and the termination codons). At the amino acid level, the general average hydrophobicity score (GRAVY) and the frequency of aromatic amino acids (Aromaticity) in the putative gene product were also analyzed. All the indices mentioned were calculated using the analysis program CodonW, version 1.4 [9]. As showed in Fig. 2, the majority of the actual ENC values are slightly lower than the expected ones, which indicated that other factors may also influence the codon usage in EV71 viruses. To test whether selection pressure contributes to the codon usage variation among EV71 viruses, we performed a correlation analysis to evaluate whether GRAVY and Aromaticity values were related to first two axes of COA and ENC values (Table 3). Our results showed that GRAVY was correlated with both axis 1 and axis 2, while Aromaticity was correlated with ENC and axis 2, indicating that the degree of hydrophobicity and the frequency of aromatic amino acids (Phe, Tyr, Trp) were also associated with the codon usage variation.

In our study, the synonymous codon usage biases in 42 EV71 genomes were analyzed. We found that both EV71 genes had low codon usage bias, and mutational pressure rather selection pressure is the main factor determining the codon usage biases. Moreover, aromaticity and hydrophobicity could be partially accounting for the codon usage variation.

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