

Differences in the Hemagglutinin Amino Acids of Japanese and US Influenza A/H3N2 Viral Isolates and their Relationship with Vaccine Strain Differences

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Received date: January 06, 2018; Accepted date: January 10, 2018; Published date: January 17, 2018

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Abstract

The effects of vaccination on the dynamics of influenza virus remain largely unknown. Different A/H3N2 vaccine strains were used in Japan and the United States (US) in the 2014-2015 season. To examine how different vaccine strains affect the selection of surviving variants, we compared hemagglutinin (HA) sequences in Japan with those in the US. A total of 85 A/H3N2 samples from 38 vaccinated and 47 unvaccinated Japanese patients (33 from the 2013-2014 and 52 from the 2014-2015 Japanese seasons) were isolated and genetically analyzed using a next-generation sequencer for comparison with 113 US isolates (30 from the 2013-2014 season and 83 from the 2014-2015 season) referenced from the GenBank database. HA1 amino acid (AA) differences between Japan and the US for the 2014-2015 vaccine strains were found at sites 128 (epitope B), 142 (A), 145 (A) and 198 (B). 145S and 198S in Japanese isolates, which matched with those of the 2014-2015 vaccine strain, significantly decreased between the two seasons, contrary to the full maintenance of US isolates (57.6% vs. 5.8% for 145S, $P < 0.0001$; 100.0% vs. 75.0% for 198S, $P = 0.0012$). The predominance of 128A and 142G was also lost in Japanese isolates, as observed while matching these AAs with those of the 2014-2015 vaccine strain (72.7% vs. 19.2% for 128A, $P < 0.0001$; 75.8% vs. 15.4% for 142G, $P < 0.0001$). Our data suggest that vaccine selection might be associated with the emergence of influenza variants genetically distant from vaccine strains.

Keywords: Influenza; Vaccination; Vaccine selection; Mutation; Hemagglutinin

Introduction

Annual outbreaks of influenza viruses result in a high morbidity and mortality in humans. A/H3N2 viruses are the most common and virulent of the influenza subtypes [1,2]. Antigenic drift, changes in antigenicity through the accumulation of mutations in the hemagglutinin (HA) gene, which encodes a major surface protein, is chiefly responsible for the continuing circulation of these viruses [1,2]. As a result, influenza vaccines must be frequently updated based on new variant analyses. In recent seasons, however, the protection by influenza vaccines has been suboptimal [3-5].

It is possible that the immune pressure elicited by vaccination (vaccine pressure) has an effect on the selection of influenza variants that become epidemic in the following season; thus, understanding the mechanisms would contribute to more accurate HA prediction, which would be helpful for counteracting future epidemic viruses. Based on this hypothesis, we have been investigating the influence of vaccine pressure on the epidemic dynamics of influenza viruses.

In Japan, recent influenza vaccine strains have been selected according to the World Health Organization (WHO) recommendations; however, in the 2014-2015 Japanese season, an A/H3N2 vaccine strain different from that recommended by the WHO was chosen in order to more efficiently avoid a decrease in antigenicity due to "egg-adaptation", as explained by the National Institute of

Infectious Diseases [6]. This circumstance presented us an opportunity to analyze how different vaccine strains affect the selection of surviving variants in different regions, based on the assumption that vaccine pressure has a certain effect on influenza epidemics.

The necessary data was gathered through our network of physicians throughout Japan who routinely collect influenza virus samples, along with patient information that includes vaccination history [7-10]. In this study, we determined the full-length sequence of HA genes of influenza A/H3N2 viruses isolated from both vaccinated and unvaccinated patients in the 2013-2014 and 2014-2015 seasons, and compared amino acid (AA) mutations at different HA1 AA sites between Japanese and the United States (US) vaccine strains, using these sequences for the Japanese isolates and the reference sequences for the US isolates.

Materials and Methods

Sample collection

Nasopharyngeal swabs for influenza virus isolation were collected and vaccination history was determined from patients who had a positive result on a rapid influenza antigen test, given at one of the member clinics of our nation-wide study network of general practitioners [7-10]. Informed consent was obtained from all patients. All patients were outpatients, and this study did not include any patients with severe chronic respiratory diseases, renal diseases, liver diseases, or heart failure. All viral samples were collected before the initiation of neuraminidase inhibitors (NAIs). Background

information on these patients, including their vaccination history, was also collected. In this study, the isolates of 85 samples (33 from the 2013-14 season and 52 from the 2014-15 season) were genetically analyzed. In total, 38 isolates were obtained from vaccinated patients and 47 from unvaccinated patients. The seasonal influenza vaccine is available to all residents of Japan. All viral samples from vaccinated patients were collected more than four weeks after vaccination. A/Texas/50/2012 (X-223) and A/NewYork/39/2012 (X-233A) were used in the Japanese influenza A/H3N2 vaccines for the 2013-14 and 2014-15 seasons, respectively. GenBank accession numbers of A/Texas/50/2012 and A/NewYork/39/2012 are KC892248 and KF790277, respectively. For the US data, 113 US isolates (30 from the 2013-2014 season and 83 from the 2014-2015 season) were referenced from the GenBank database. For the 2014-2015 season, all states of the US were searched for isolates and one to six were retrieved from all but thirteen states.

Viral RNA extraction and RT-PCR

Nasopharyngeal swabs from patients were soaked in virus transport medium and 75 µL of the medium was cultured using Madin-Darby canine kidney (MDCK) cells. Viral RNA was extracted from infected MDCK cell culture supernatants using the Maxwell 16 LEV simply RNA Cells Kit (Promega, Madison, WI). The A/H3N2 subtype was determined by PCR [11]. RT-PCR was performed using the H3N2 RNA samples. PCR primers, synthesized based on the 3' and 5' terminal nucleotides that are common to all human influenza A virus segments [12] were as follows: forward primer (Uni-12), 5'-ACGCGTGATCAGCAAAAAGCAGG-3' and reverse primer (Uni-13), 5'-ACGCGTGATCAGTAGAAACAAGG-3'. The PCR consisted of 31 cycles of a denaturing step at 94°C for 30 sec, an annealing step at 57°C for 30 sec, and an extension step at 72°C for 2 min.

Next generation sequencing

A DNA library for Illumina sequencing was prepared using the Nextera XT DNA Sample Prep kit (Illumina, San Diego, CA).

Sequencing was conducted *via* a paired-end, 2 × 250 bp cycle run, using the Illumina MiSeq sequencing system and MiSeq Reagent Kit version 2 (300 Cycle) (Illumina) [13].

Bioinformatic analysis

Data processing was performed using the pipeline prepared by Amelieff Co [13]. The reference sequence was A/New York/396/2005 (H3N2) (GenBank accession numbers for the eight gene segments: CY002079, CY121123, CY121122, CY121117, CY121120, CY121119, CY121118, and CY121121). The HA amino acid (AA) sequence was deduced from the obtained nucleotide sequence.

Nucleotide sequence accession number

The sequence data from this study were deposited into the DDBJ/EMBL/GenBank nucleotide sequence databases under the following accession numbers: LC111574-LC111606, and LC155845-LC155896.

Statistical analysis

Categorical variables between groups were tested using the Fisher's exact test. $P < 0.05$ was considered to be statistically significant. All statistical analyses were performed using the JMP Pro software, version 11 (SAS Institute, Inc., Cary, NC, USA).

Results

Table 1 shows the influenza A/H3N2 vaccine strains selected in the US and Japan from the 2011-2012 to the 2015-2016 season. Different vaccine strains were used only in the 2014-2015 season during this five year period. In the US, A/Texas/50/2012 was chosen for the 2014-2015 season as well as in the previous season (2013-2014). In parallel, A/New York/39/2012 was selected for the 2014-2015 season in Japan (Table 1).

AA (epitope)		AA128 (epitope B)			AA142 (epitope A)		AA145 (epitope A)		AA198 (epitope B)		
Season (country)	Vaccine strain	N	A	T	R	G	N	S	P	S	A
2011-2012 (US and JPN)	A/Victoria/210/2009			T	R		N				A
2012-2013 (US and JPN)	A/Victoria/361/2011			T	R		N			S	
2013-2014 (US and JPN)	A/Texas/50/2012	N			R		N		P		
2014-2015 (US)	A/Texas/50/2012	N			R		N		P		
2014-2015 (JPN)	A/NewYork/39/2012		A			G		S		S	
2015-2016 (US and JPN)	A/Switzerland/9715293/2013		A			G		S		S	

AA: Amino Acid; US: United States; JPN: Japan

Table1: Influenza A/H3N2 vaccine selection in Japan and the United States.

Of the AA sites known to be susceptible to mutation induced by egg-adaptation (sites 156, 186, and 219) [14], sites 186 and 219 have the possibility of different AAs between the Japanese and US vaccine strains of the 2014-2015 season, but these AA differences were not

definite based on the result of reference sequencing. Identical AAs (156H, 186G, and 219S) were detected in all of the isolates from Japan and the US in both the 2013-2014 and 2014-2015 seasons. Based on these results, these three AA sites were excluded from our analysis.

AA145				AA198				
		2013-2014 season (145N in US and JPN ^a)	2014-2015 season (145N in US and 145S in JPN)			2013-2014 season (198P in US and JPN)	2014-2015 season (198P in US and 198S in JPN)	
Country	AA	% (No. of isolates/ total isolates)	% (No. of isolates/ total isolates)	P value ^b	AA	% (No. of isolates/ total isolates)	% (No. of isolates/ total isolates)	P value
US	S	100 (30/30)	98.8 (82/83)	1	S	100 (30/30)	96.4 (80/83)	0.564
	N	0 (0/30)	1.2 (1/83)	1	A	0 (0/30)	0 (0/83)	1
					Other	0 (0/30)	3.6 (3/83) ^c	0.564
JPN	S	57.6 (19/33)	5.8 (3/52)	<0.0001	S	100 (33/33)	75 (39/52)	0.0012
	N	42.4 (14/33)	94.2 (49/52)	<0.0001	A	0 (0/33)	25 (13/52)	0.0012
Vaccinated	S	43.8 (7/16)	4.5 (1 / 22)	0.0054	S	100 (16/16)	63.6 (14/22)	0.0119
	N	56.2 (9/16)	95.5 (21/22)	0.0054	A	0 (0/16)	36.4 (8/22)	0.0119
Unvaccinated	S	70.6 (12/17)	6.7 (2/30)	<0.0001	S	100 (17/17)	83.3 (25/30)	0.1435
	N	29.4 (5/17)	93.3 (28/30)	<0.0001	A	0 (0/17)	16.7 (5/30)	0.1435

^aThis indicates AAs of vaccine strains in each season.
^bThis indicates a comparison of the frequency of isolates displaying the indicated AA between the 2013-2014 and 2014-2015 seasons.
^cThis indicated 198P in two isolates and 198L in one isolate.
AA: Amino Acid; US: United States; JPN: Japan

Table 2: Amino acid mutation at Hemagglutinin 1 (HA1) sites 145 and 198 of influenza A/H3N2 viruses isolated in Japan and the United States during the 2013-2014 and 2014-2015 seasons.

We examined definitely different HA1 AA sites between the two vaccine strains selected in the 2014-2015 season and found four AA sites (sites 128,142,145 and 198) (Table 1). The frequencies of AA mutations at sites 145 and 198 were compared for the isolates from Japan and the US (Table 2). In the vaccine strains of the 2013-2014 season, 145N was present in both countries in the 2013-2014 season. In the 2014-2015 season, AA145 changed from N to S in the Japanese vaccine strain. 145S was detected in all but one of the US isolates during the two seasons. In contrast, the predominance changed from 145S to 145N in Japan during this season (57.6% for 145S in the 2013-2014 vs. 94.2% for 145N in the 2014-2015, P<0.0001). In addition, the rate of 145S in the 2014-2015 season was lower in the isolates from vaccinated than from unvaccinated patients (4.5% vs. 6.7%), although this difference was not statistically significant. AA198 changed from P to S in the Japanese vaccine strain for the 2014-2015 season, while the US vaccine strain remained unchanged (198P). 198S was found in all of the US and Japanese isolates in the 2013-2014 season. In the 2014-2015 season, 198A emerged only in Japan (0.0%, 0/33 isolates in the 2013-2014 vs. 25.0%, 13/52 isolates in the 2014-2015, P=0.0012). The rate of 198S in the 2014-2015 season was lower in the isolates from vaccinated than from unvaccinated patients (63.6% vs. 83.3%).

The analysis of sites 128 and 142 is shown in Table 3. In the 2013-2014 season, 128N was present in the vaccine strains of both countries. In the 2014-2015 season, AA128 changed from N to A only in the Japanese vaccine strain. In the 2013-2014 season, the dominant AAs at site 128 were 128T (63.3%) in the US and 128A (72.7%) in Japan. In the 2014-2015 season, the 128A found in the isolates from Japan decreased in frequency from 72.7% (24/33 isolates) to 19.2% (10/52 isolates) (P<0.0001). In addition, 128A dominance was lost in both the isolates from vaccinated and unvaccinated patients (from 93.8% to 22.7% in the vaccinated; from 52.9% to 16.7% in the unvaccinated); however, the rate of 128A in the 2014-2015 season was higher in the isolates from vaccinated than from unvaccinated patients (22.7% vs. 16.7%). In the 2014-2015 season, AA142 changed from R to G in the Japanese vaccine strain. AA combination at sites 128 and 142, 128A-142G, was found, as reported in our previous study [13]. Therefore, the predominance of 142G, similar to that of 128A, was lost in the Japanese 2014-2015 season. In addition, the rate of 142G in the 2014-2015 season was lower in the isolates from vaccinated than from unvaccinated patients (13.6% vs. 16.7%), although this difference was not statistically significant.

AA128			AA142				
		2013-2014 season	2014-2015 season (128N in US and 128A in JPN)			2013-2014 season (142R in US and JPN)	2014-2015 season (142R in US and 142G in JPN)

		(128N in US and JPNa)						
Country	AA	% (No. of isolates/total isolates)	% (No. of isolates/total isolates)	P value ^b	AA	% (No. of isolates/total isolates)	% (No. of isolates/total isolates)	P value
US	A	36.7 (11/30)	4.8 (4/83)	0.0001	G	36.7 (11/30)	4.8 (4/83)	0.0001
	T	63.3 (19/30)	95.2 (79/83)	0.0001	R	63.3 (19/30)	86.8 (72/83)	0.0132
					Other	0.0 (0/30)	8.4 (7/83) ^c	0.1866
JPN	A	72.7 (24/33)	19.2 (10/52)	<0.0001	G	75.8 (25/33)	15.4 (8/52)	<0.0001
	T	24.2 (8/33)	80.8 (42/52)	<0.0001	R	24.2 (8/33)	84.6 (44/52)	<0.0001
Vaccinated	A	93.8 (15/16)	22.7 (15/ 22)	<0.0001	G	93.8 (15/16)	13.6 (3/22)	<0.0001
	T	6.2 (1/16)	77.3 (17/22)	<0.0001	R	6.2 (1/16)	86.4 (19/22)	<0.0001
Unvaccinated	A	52.9 (9/17)	16.7 (5/30)	0.0182	G	58.8 (10/17)	16.7 (5/30)	0.0077
	T	41.2 (7/17)	83.3 (25/30)	0.0182	R	41.2 (7/17)	83.3 (25/30)	0.0077
aThis indicates AAs of vaccine strains in each season.								
bThis indicates a comparison of the frequency of isolates displaying the indicated AA between the 2013-2014 and 2014-2015 seasons.								
cThis indicated 142K in seven isolates.								
AA: Amino Acid; US: United States; JPN: Japan								

Table 3: Amino acid mutation at Hemagglutinin 1 (HA1) sites 128 and 142 of influenza A/H3N2 viruses isolated in Japan and the United States during the 2013-2014 and 2014-2015 seasons.

As shown in Table 2, 198A was detected in 13 of 52 isolates (25.0%) in the Japanese 2014-2015 season. Sites 198 and 128 were both within antigenic epitope B. 128A remained detectable in 10 of 52 isolates (19.2%), as observed when including the isolates with 198A (Table 4).

On the other hand, 128A was detected in only one isolate (2.6%, P=0.021), when excluding the isolates with 198A (Table 4). In addition, none of 128A was detected in the vaccinated patients under the condition of 198A exclusion.

AA128					
		2013-2014 season (128N in US and JPNa)		2013-2014 season (128A in JPN)	
				Including 198A	Excluding 198A
Country	AA	% (No. of isolates/total isolates)	% (No. of isolates/total isolates)	% (No. of isolates/total isolates)	P value ^b
JPN	A	72.7 (24/33)	19.2 (10/52)	2.6 (1/39)	0.021
	T	24.2 (8/33)	80.8 (42/52)	97.4 (38/39)	0.021
Vaccinated	A	93.8 (15/16)	22.7 (15/ 22)	0.0 (0/14)	0.1336
	T	6.2 (1/16)	77.3 (17/22)	100.0 (14/14)	0.1336
Unvaccinated	A	52.9 (9/17)	16.7 (5/30)	4.0 (1/25)	0.2045
	T	41.2 (7/17)	83.3 (25/30)	96.0 (24/25)	0.0077
aThis indicates AAs of vaccine strains in each season.					
bThis indicates the comparison between "including 198A" and "excluding 198A".					

AA: Amino Acid; JPN: Japan

Table 4: Amino acid mutation at Hemagglutinin 1 (HA1) site 128 of influenza A/H3N2 isolates with or without 198A in the Japanese 2014-2015 season.

Discussion

Vaccine mismatch was of concern because of the antigenic change of vaccine strains due to egg-adaptation; however, the indicated adaptation sites, particularly sites 156,186 and 219, exhibited identical AAs in all of the Japanese and US isolates. Thus, these egg-adaptation sites, included within known antigenic epitopes, did not seem to function as the main antigenic sites recognized by the host during the 2013-2014 and 2014-2015 seasons.

In the field of avian influenza, antigenic drift-related HA mutation due to escape from vaccine-induced immunity has been reported to cause vaccine failure [15,16]; however, the presence of vaccine-induced antigenic drift has not been demonstrated in humans. We recently reported evidence showing that vaccine pressure works to select influenza variants genetically distant from vaccine strains and affects the dynamics of the epidemic variants for humans [13]. At the beginning of the previous study, the phylogenetic method did not segregate viruses isolated from vaccinated and unvaccinated patients, as indicated in the report [17]. One possible explanation for the vaccine pressure not being reflected in the phylogenetic tree is that viruses that are able to infect vaccinated persons will become epidemic, irrespective of vaccination status. Viruses containing mutations that evade host immunity will be isolated from both the vaccinated and unvaccinated patients, resulting in no segregation in the phylogenetic tree of the viruses isolated from the vaccinated patients. We were first able to suggest the possible effect of vaccine pressure on HA mutation and its relation to the emergence of epidemic variants by directly comparing AA differences from the corresponding vaccine strains between isolates from vaccinated and unvaccinated patients. As shown in the phylogenetic tree in the previous study [13], the epidemic viruses in the Japanese 2013-2014 and 2014-2015 seasons formed clades, which were segregated by AA mutations other than the four AA sites that were examined in this study. Thus, if we had based this study only on the results of phylogenetic tree analysis, we would have missed HA mutations possibly associated with the emergence of local variants.

Our present study allowed the examination of the influence of different vaccine strains on the HA mutations of influenza epidemic viruses, because different vaccine strains were selected between Japan and the US in the 2014-2015 season, in contrast to the 2013-2014 season. In the four different AA sites between Japanese and the US vaccine strains, the isolates from Japan showed more predominant AA mutations that were genetically distant from the corresponding vaccine strain, as compared to those from the US (Tables 2 and 3). Particularly, 145N, which was not detected in the US, was extremely predominant in Japan. 198A was observed in the isolates from Japan but was not detected in those from the US. HA AA site 145 is within antigenic epitope A and 198 within epitope B. Thus, genetic variation was generated in important antigenic sites, such as epitopes A and B. This variation may be attributed to AA differences in the vaccine strains used in the two countries. As a result, these findings suggest the influence of vaccination on drift mutation.

AA sites 128 and 198 are both within antigenic epitope B [18]. In our analysis (Table 4), 128A was detected in 10 (19.2%) of 52 Japanese

isolates, when including the isolates with 198A. On the other hand, 128A was detected in only one isolate (2.6%, 1/39 isolates), when excluding the isolates with 198A. Thus, 128A was detected in 9 (69.2%) of 13 Japanese isolates with 198A. In contrast, almost all isolates without 198A exhibited 128T (97.4%, 38/39 isolates). The acquisition of 198A or 128T appears to be alternative. These mutations within epitope B may have a similar effect as the structural change for antigenic drift.

The mechanisms responsible for HA mutation in influenza viruses seem to be very complicated, with many potentially contributing factors. Therefore, it was anticipated that not all HA mutations would be explained only by the immune pressure elicited by vaccination. The global epidemiological situation of H3N2 might also be associated with HA mutations, as indicated by global surveillance [19]. Thus, in the analysis of HA mutation in each region, the presence of local variants itself is not included as a prerequisite. In this study, we have obtained findings suggesting that there are local variants within epidemic H3N2 viruses in Japan. The emergence of 145N and 198A is especially intriguing (Table 2). The AA patterns of sites 128 and 142 appear to be similar in both the Japan and US samples, irrespective of AA differences at these sites between the samples of the two countries in the 2014-2015 seasonal vaccine (Table 3). Indeed, this result might be associated with the global spread of seasonal epidemic viruses. On the other hand, in Japan the emergence of 198A appears to be related to the mutation of site 128 (Table 4). Japan-specific selection of a vaccine strain in the 2014-2015 season might have accelerated the emergence of 198A and also affected the mutation pattern of site 128. Thus, our approach to the analysis of HA mutation revealed the presence of local variants that were not reflected by a phylogenetic analysis, and the emergence of these variants may be attributed, at least in part, to vaccine pressure.

The vaccine strain (A/Switzerland/9715293/2013) was chosen for the 2015-2016 season in both Japan and the US (Table 1). Interestingly, the A/NewYork/39/2012 strain used in the 2014-2015 season in Japan matched the four AA sites (128A, 142G, 145S, and 198S) with those of the 2015-2016 vaccine strain. As mentioned above, these four sites appeared to exhibit AA mutations that were genetically distant from the vaccine strain in the Japanese 2014-2015 season. These mutations, particularly 145N and 198A, would be anticipated to emerge along with the match of the vaccine strain. Although our results are not conclusive, we are looking forward to investigating the HA AA sequences of the Japanese and US isolates in the 2015-2016 season, for confirmation.

A limitation of this study is that viral isolation could cause AA mutations during cultivation (MDCK passage). Direct sequencing from clinical samples would resolve this issue, although the direct use of respiratory samples involves the risk of detecting defective viruses with no growth activity potential.

It is unlikely that epidemic viruses in the 2014-2015 season were formed solely by AA mutations at the four sites that were different in the Japanese and US vaccine strains. It is unknown to what extent AA mutations at the four sites would be associated with the selection of

epidemic variants in the two countries. Further study of the antigenic changes caused by AA mutations at these four sites will be necessary to determine the precise mechanisms of this challenging issue. However, the difference in the composition of epidemic viruses between Japan and the US is apparent. It would be natural to attribute this variation to AA differences in the vaccine strains, which might have induced different vaccine pressure.

Acknowledgements

We thank Kenjiro Shirane, Shinya Matsumoto, and Nozomi Noda for their technical support. We also thank Hiroyuki Sasaki and Donchon Kang for their support on this study.

We thank the following doctors for participating in this study: Shunsuke Akimitsu, Masaaki Chinen, Ken-ichi Doniwa, Keisuke Egashira, Yasuhiko Hirata, Miki Hirata, Nobuo Hirotsu, Tsuneo Inoue, Norio Iwaki, Kyosuke Kaji, Seizaburo Kashiwagi, Naoki Kawai, Takashi Kawashima, Kunio Kondo, Hiroko Kondo, Haruo Kuroki, Woon Joo Lee, Tetsunari Maeda, Koji Maehara, Tsuyoshi Makino, Shinro Matsuura, Akiko Miyata, Masashi Miyazaki, Kouichi Mochizuki, Kazuo Mori, Atsuko Nabeshima, Shin Nagao, Masatoshi Nakao, Kiyoshi Nishikawa, Yoshinori Nishimoto, Tadahiko Ogasawara, Jun Ogawa, Kaoru Oguchi, Yasuo Ontachi, Ietaka Sato, Yasuo Sato, Keigo Shibao, Kunihisa Shimomura, Shizuo Shindo, Kotoko Sumimoto, Yoshio Takasaki, Takeshi Tana, Osame Tanaka, Yuriko Tarukawa, Noriko Tateno, Keita Tatsushima, Satuki Tomita, Hiroaki Tomori, Hiroshi Ukai, Yutaka Wakasa, Masahiro Yaekashiwa, Norio Yamaguchi, Hareaki Yamamoto, Yasuhito Yamanishi, Yuji Yamashita, Satoshi Yamauchi, Takashi Yokoyama, Takato Yokoyama, Gen Yoshida, Hiroyuki Yoshimine, and Midori Yoshimura.

Funding

This work was supported by a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (26461505 to Y.C.)

Authors' Contribution

Yong Chong: Designed the study, carried out data analysis and drafted the manuscript.

Hideyuki Ikematsu: Designed the study, carried out data analysis and drafted the manuscript.

Conflicts of Interest

The authors indicate no potential conflicts of interest.

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