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Molecular Analysis of Genome of the Pandemic Influenza A(H1N1) 2009 Virus Associated with Fatal Infections in Gunma, Tochigi, Yamagata, and Yamaguchi Prefectures in Japan during the First Pandemic Wave

Masatsugu Obuchi[1]*, Shoichi Toda[2], Hiroyuki Tsukagoshi[3], Teruko Oogane[4], Chieko Abiko[5], Keiji Funatogawa[4], Katsumi Mizuta[5], Komei Shirabe[2], Kunihisa Kozawa[3], Masahiro Noda[6], Hirokazu Kimura[6], and Masato Tashiro[1]

[1]*Influenza Virus Research Center and*
[6]*Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Tokyo 208-0011;*
[2]*Yamaguchi Prefectural Institute of Public Health and Environment, Yamaguchi 753-0821;*
[3]*Gunma Prefectural Institute of Public Health and Environmental Science, Gunma 371-0052;*
[4]*Tochigi Prefectural Institute of Public Health and Environmental Science, Tochigi 329-1196; and*
[5]*Yamagata Prefectural Institute of Public Health, Yamagata 990-0031, Japan*

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In May 2009, a novel swine-origin pandemic influenza A(H1N1) 2009 virus [A(H1N1)pdm09] was first identified in Osaka and Hyogo prefectures of Japan, which then spread to other prefectures in the next several weeks (1). During the summer, pandemic influenza activity remained low; however, it subsequently increased and reached a peak in November 2009 (2). In most cases, infection by A(H1N1)pdm09 caused mild disease, though there have been sporadic cases with severe or fatal outcomes (2). One hundred and ninety-eight fatal cases were reported during the first pandemic wave in Japan (2). Several countries report that an amino acid substitution of aspartic acid by glycine at position 222 (D222G) in hemagglutinin (HA) is associated with disease severity; however, many A(H1N1)pdm09 isolates without this mutation have been identified in severe and fatal cases (3–5). To further explore the molecular determinants of A(H1N1)pdm09 that are associated with severity, we performed whole genome analysis on virus isolates obtained from the fatal cases identified in Gunma, Tochigi, Yamagata, and Yamaguchi prefectures between May 2009 and March 2010.

Nasal swabs were collected from hospitalized influenza patients and sent to the district's prefectural public health institute for diagnosis and viral strain surveillance. Clinical specimens were first inoculated onto Madin-Darby canine kidney (MDCK) cells for virus isolation. Viral RNA was then extracted from the virus culture supernatant using QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, Calif., USA) according to the manufacturer's instructions. Reverse transcription-polymerase chain reaction was carried out using the One Step RNA PCR Kit (AMV) (TaKaRa Bio Inc., Otsu, Japan) and virus-specific primers established by the National Institute of Technology and Evaluation and the National Institute of Infectious Diseases, Japan (6). Amplicons were purified using ExoSAP-IT (USB Corp., Cleveland, Ohio, USA) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif., USA). The sequence data were assembled and analyzed using the Sequencher for Macintosh V4.9 software (Hitachi Software Engineering Co., Tokyo, Japan) and GENETYX-Mac Ver. 13.0.17 (Genetyx Corp., Tokyo, Japan).

In Gunma, Tochigi, Yamagata, and Yamaguchi prefectures, a total of 12 fatal cases were reported to the Ministry of Health, Labour and Welfare of Japan during the first pandemic wave. A(H1N1)pdm09 isolates were successfully obtained from 9 fatal cases; the patients were aged between 4 and 85 years (Table 1). Of these patients, 2 developed pneumonia (Patients 6 and 9) and 1 patient developed multiple organ failure (Patient 3). Patient 8 suffered a comorbidity, namely a subarachnoid hemorrhage. Moreover, 5 patients had underlying diseases: 3 had chronic obstructive pulmonary disease, 1 had multiple myeloma and diabetes mellitus, and 1 had lung cancer. Of the 7 patients taking medication, 4 were taking oseltamivir and 3 zanamivir. In this study, no pandemic influenza-related deaths were reported in pregnant women. For comparison, we obtained 6 isolates from influenza patients presented with mild conditions (Patients 10–15).

Nucleotide sequencing of entire viral genome segments (i.e., polymerase basic 2 [PB2], polymerase basic 1 [PB1], polymerase acidic [PA], HA, nucleoprotein [NP], neuraminidase [NA], matrix [M] protein, and nonstructural [NS] protein genes) was conducted for all 15 viral isolates. The amino acid sequences of viral proteins were deduced from the nucleotide sequences. Table 2 shows the amino acid differences between the virus isolates analyzed in this study and A(H1N1)pdm09 isolates (as consensus sequences) collected worldwide between April 2009 and March 2010. The consensus

*Corresponding author: Present address: Department of Virology, Toyama Institute of Health, 17-1 Nakataikoyama, Imizu-shi, Toyama 939-0363, Japan. Tel: +81-766-56-8143, Fax: +81-766-56-7326, E-mail: masatsugu.obuchi@pref.toyama.lg.jp

Table 1. Fatal and mild cases of A(H1N1)pdm09 infection and virus isolates in this study

Patient	Sex/age (yr)	Clinical feature	Severity	Medication	Underlying disease	Sampling date	Virus isolate	GenBank accession no.
1	M/85	Influenza	Fatal	Oseltamivir	None	Nov. 25, 2009	A/Gunma/287/2009	AB704443-AB704450
2	M/60	Influenza	Fatal	Zanamivir	Multiple myeloma, diabetes mellitus	Dec. 15, 2009	A/Gunma/293/2009	AB704451-AB704458
3	M/83	Multiple organ failure	Fatal	Oseltamivir	COPD	Oct. 29, 2009	A/Tochigi/350/2009	AB704459-AB704466
4	F/8	Influenza	Fatal	Zanamivir	None	Nov. 24, 2009	A/Tochigi/445/2009	AB704467-AB704474
5	M/56	Influenza	Fatal	None	None	Jan. 13, 2010	A/Tochigi/2/2010	AB704475-AB704482
6	F/62	Pneumonia	Fatal	Oseltamivir	COPD	Nov. 9, 2009	A/Yamagata/473/2009	AB704491-AB704498
7	F/13	Influenza	Fatal	Zanamivir	COPD	Nov. 21, 2009	A/Yamaguchi/217/2009	AB704507-AB704514
8	M/4	Subarachnoid hemorrhage	Fatal	None	None	Dec. 2, 2009	A/Yamaguchi/247/2009	AB704515-AB704522
9	M/60	Pneumonia	Fatal	Oseltamivir	Lung cancer	Dec. 5, 2009	A/Yamaguchi/248/2009	AB704523-AB704530
10	M/10	Influenza	Mild	None	None	Nov. 19, 2009	A/Gunma/262/2009	AB704419-AB704426
11	M/9	Influenza	Mild	Zanamivir	None	Nov. 16, 2009	A/Gunma/263/2009	AB704427-AB704434
12	M/4	Influenza	Mild	Oseltamivir	None	Nov. 25, 2009	A/Gunma/267/2009	AB704435-AB704442
13	M/56	Influenza	Mild	Unknown	None	Jan. 12, 2010	A/Tochigi/10/2010	AB704483-AB704490
14	F/50	Influenza	Mild	None	None	Nov. 27, 2009	A/Yamagata/674/2009	AB704499-AB704506
15	M/7	Influenza	Mild	Oseltamivir	None	Dec. 18, 2009	A/Yamaguchi/273/2009	AB704531-AB704538

COPD, chronic obstructive pulmonary disease.

sequences of each segment consist of 2728 (PB2), 2312 (PB1), 2366 (PA), 4733 (HA), 2440 (NP), 4401 (NA), 3449 (MP), and 2376 (NS) nucleotide sequences, which were downloaded from the Global Initiative on Sharing All Influenza Data EpiFlu database (http://platform.gisaid.org/epi3/frontend#34c9cf). Amino acid substitutions, namely S203T in HA, V100I in NP, V106I and N248D in NA, and I123V in NS1, which are known as specific markers for cluster 2, were present in all the analyzed sequences, indicating that these virus isolates belonged to the cluster containing a large majority of circulating A(H1N1)pdm09 strains in Japan, during the peak phase of the pandemic (7). There was no reassortment with other seasonal (either H1N1 or H3N2), swine, or avian influenza A viruses. A total of 39 unique amino acid differences were found in 9 isolates obtained from fatal cases: 5 in PB2, 4 in PB1, 9 in PA, 6 in HA, 2 in NP, 6 in NA, 3 in M2, 2 in NS1, and 2 in nuclear export protein (NEP). Of these differences, only V19I in HA was common to 2 of the isolates (i.e., A/Yamaguchi/217/2009 and A/Yamaguchi/248/2009). A marker for oseltamivir resistance, H275Y in NA, was identified in A/Yamaguchi/248/2009 that was derived from an oseltamivir-treated patient. Frequently observed changes in the fatal cases, defined as more than 3 out of 9 isolates, were commonly observed in mild cases (i.e., T257A, I435V, and N537S in PB1; S69L, D274N, and E374K in HA; and G41E in NA), suggesting that these amino acid substitutions are unlikely to be associated with the level of severity.

The amino acid location at position 222 in the receptor binding site of HA predicts that alterations to this position would influence the binding specificity of viruses. Previous studies have reported that a D222G substitution confers enhanced binding to α2,3-linked (avian-like) rather than α2,6-linked (human-like) sialic acids, suggesting an augmented ability to bind to lung cells in the lower respiratory tract in humans and cause an exacerbation of the disease (3). No D222G substitution in the HA was observed in any of the isolates analyzed in this study. Alternatively, a D222E substitution was found in 1 of the isolates, A/Yamaguchi/247/2009, which was derived from a fatal infection. This substitution, however, seems to be unrelated to the disease severity of the A(H1N1)pdm09 as previously reported (4,8). Another V132E amino acid mutation in the receptor binding site was found in 2 isolates, a fatal case-strain (i.e., A/Tochigi/2/2010) and a mild case-strain (i.e., A/Yamaguchi/273/2009); however, the precise impact of this mutation is unclear. A mixed population of viruses possessing 163K/E in an antigenic site was found in the isolate A/Yamaguchi/217/2009, which was also derived from a fatal infection. However, the antigenicity of this isolate was similar to that of the vaccine strain, A/California/07/2009 (data not shown).

Virus isolates derived from patients with fatal infection manifested sporadic amino acid changes in the PB2 and PA proteins more frequently than those derived from patients with mild infections (Table 2). Notably, 6 of the 9 isolates from fatal cases had 1 or 2 amino acid substitutions in the PA, e.g., E2K, A70V, P325L, V387I, S405A, V432F, L589I, S594G, and A598P. The RNA-dependent RNA polymerase of influenza viruses is a complex of 3 viral proteins, PB1, PB2, and PA, and

Table 2. Amino acid differences in the viral proteins of A(H1N1)pdm09 isolates obtained from fatal and mild cases

Virus isolate[1)]	Viral protein[2)]																																		
	PB2									PB1										PA															
	121	251	368	495	588	616	660	673	682	36	76	94	257	383	393	435	537	609	652	2	70	224	325	387	405	432	578	589	594	598					
Consensus	K	R	R	V	T	I	K	G	G	T	D	F	T	E	R	I	N	V	A	E	A	S	P	V	S	V	G	L	S	A					
A/Gunma/287/2009					**I**				**R**			**L**													**A**			**I**							
A/Gunma/293/2009							**R**	**R**	**R**				**A**																**P**						
A/Tochigi/350/2009																**V**	**S**																		
A/Tochigi/445/2009																**V**	**S**																		
A/Tochigi/2/2010							**R**						**A**							**K**						**F**									
A/Yamagata/473/2009			**K**													**V**	**S**																		
A/Yamaguchi/217/2009				**I**																			**L**												
A/Yamaguchi/247/2009	**R**													**D**					**V**					**I**											
A/Yamaguchi/248/2009		**K**											**A**			**V**		**A**			**V**								**G**						
A/Gunma/262/2009							R																												
A/Gunma/263/2009																V	S																		
A/Gunma/267/2009								R	R		N				T	V	S																		
A/Tochigi/10/2010							R			M																									
A/Yamagata/674/2009							R																												
A/Yamaguchi/273/2009						V	R						A														S								
A/California/07/2009																						*P*													

Virus isolate	Viral protein																									
	HA																	NP								
	19	69	83	132	163	171	197	203	222	258	274	297	304	321	339	374	393	31	100	119	297	363				
Consensus	V	S	S	V	K	K	A	T	D	E	D	P	P	V	G	E	V	R	I	V	Y	V				
A/Gunma/287/2009													**S**		**R**						**H**	**I**				
A/Gunma/293/2009																**K**										
A/Tochigi/350/2009		**L**									**N**					**K**										
A/Tochigi/445/2009		**L**									**N**					**K**										
A/Tochigi/2/2010				**E**												**K**										
A/Yamagata/473/2009		**L**									**N**					**K**										
A/Yamaguchi/217/2009	**I**				**K/E**																					
A/Yamaguchi/247/2009									**E**			**S**					**I**									
A/Yamaguchi/248/2009	**I**																									
A/Gunma/262/2009						R	T													I						
A/Gunma/263/2009		L									N					K										
A/Gunma/267/2009		L								V	N					K										
A/Tochigi/10/2010						R	T								R											
A/Yamagata/674/2009						R	T											K								
A/Yamaguchi/273/2009				E												K										
A/California/07/2009			*P*					*S*						*I*					*V*							

Table 2. Continued

Virus isolate	Viral protein																														
	NA															M1	M2				NS1							NEP			
	41	62	76	82	106	110	140	221	248	275	351	381	382	416	465	64	39	50	51	63	2	64	93	100	112	122	123	2	89	105	
Consensus	G	V	A	S	I	S	L	N	D	H	F	T	G	D	F	F	I	C	I	P	D	I	M	D	I	A	V	D	A	L	
A/Gunma/287/2009						**C**											**V**	**Y**		**A**		**T**									
A/Gunma/293/2009																							**I**								
A/Tochigi/350/2009	**E**																														
A/Tochigi/445/2009	**E**							**S**																							
A/Tochigi/2/2010																							**I**								
A/Yamagata/473/2009	**E**																													**I**	
A/Yamaguchi/217/2009																													**V**		
A/Yamaguchi/247/2009												**N**			**L**											**T**					
A/Yamaguchi/248/2009				**P**						**Y**																					
A/Gunma/262/2009		I					I						E	N		Y															
A/Gunma/263/2009	E																														
A/Gunma/267/2009	E																														
A/Tochigi/10/2010													E	N		Y					G			N				G			
A/Yamagata/674/2009													E	N		Y			T						M						
A/Yamaguchi/273/2009			T																				I								
A/California/07/2009					*V*				*N*		*Y*																*I*				

[1)]: Virus isolates obtained from fatal cases and their amino acid substitutions are indicated with a bold font. The vaccine strain for the 2009–2010 influenza season and its amino acid substitutions are indicated in an italicized font.

[2)]: PB2, polymerase basic protein 2; PB1, polymerase basic protein 1; PA, polymerase acidic protein; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; M1, matrix protein 1; M2, matrix protein 2; NS1, nonstructural protein 1; NEP, nuclear export protein. Fatal case-specific substitutions are indicated with opened boxes. Positions of antigenic and receptor binding sites in the HA are indicated with gray and dotted backgrounds, respectively.

plays a key role in viral growth within the mammalian host cells. Recent studies demonstrated that mutations within PB2 or PA lead to increased pathogenicity of the A(H1N1)pdm09 in mice (9–11). A limitation of this study is the relatively small size of the analyzed data set. In addition, the pathogenetic role of the observed viral mutations remains to be elucidated.

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Conflict of interest None to declare.

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