Intrahost emergent dynamics of oseltamivir-resistant virus of pandemic influenza A (H1N1) 2009 in a fatally immunocompromised patient

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Abstract The oseltamivir-resistant pandemic influenza virus A (2009 H1N1) with H275Y mutation in neuraminidase (NA) has been sporadically reported, and its wide spread remains a potential threat. Here we detected the uneven distribution of H275Y mutant virus in a patient who received a 21-day long-term administration of oseltamivir. Intrahost variation of the virus showed that the H275Y mutant virus was the predominant population in both nasopharynx and right lung, whereas the oseltamivir-sensitive virus comprised half the population in the left lung. By constructing minimum spanning trees, it is proposed that the H275Y mutant might be generated primarily in the nasopharynx, then spread to the right and left lungs.

Keywords Pandemic influenza A virus (H1N1) 2009 · Oseltamivir resistance · Quasi-species · Minimum spanning tree

Introduction

In March 2009, a new swine-origin influenza A (H1N1) virus (S-OIV) emerged first in Mexico and spread next to

the United States [1]. Within a half-year, S-OIV expanded worldwide. Fortunately, most S-OIV was susceptible to oseltamivir, which is an effective anti-influenza drug, although most seasonal influenza A (H1N1) viruses had already developed resistance to this drug [2]. However, an oseltamivir-resistant pandemic influenza virus has rarely appeared in patients [3], even those who were under intensive care. To determine the appropriate antiviral treatment and eventually control the outbreak, it is important to elucidate the mechanism by which the oseltamivir-resistant influenza virus appears, as well as to monitor the antiviral susceptibility profile of that influenza virus.

Although molecular investigation of S-OIV was extensively performed worldwide, the evolutionary dynamics of this virus in a single patient, especially in connection with oseltamivir resistance, were not yet elucidated [4]. In this report, we investigated that the dynamics of these influenza viruses in a single patient, and we discuss the mechanisms by which mutant clones appeared.

Materials and methods

The data were analyzed anonymously, and all clinical investigations have been conducted according to the principles expressed in the Declaration of Helsinki. Furthermore, this study (Number 11162) was approved by The Ethical Committee of Kurume University.

Patients and specimen collection

Case 1

On December 23, 2009 (day 1; Table 1), a 56-year-old man with multiple myeloma (IgA kappa) complained of fever

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Table 1 Clinical examination and treatment of patients infected with 2009 H1N1 influenza virus

Case	Age, sex	Underlying disease	Days (h) after the onset	Specimen taken	Influenza diagnosis		Treatment	Outcome
					Rapid test	RT-PCR (virus population)		
1	56, male	Multiple myeloma	2	Nasopharyngeal	+	nt	Day 2–22: oseltamivir (75 mg twice daily)	Died on day 27
			8	Nasopharyngeal	+	nt		
			13	Nasopharyngeal	+	+ (100 % H275Y)		
			20	Nasopharyngeal	–	nt		
			22	Nasopharyngeal	+	nt		
			27	Nasopharyngeal	nt	–	Day 23: zanamivir (10 mg twice daily)	
			Right lung	nt	+ (100 % H275Y)			
	Left lung	nt	+ (25 % H275Y)					
2	66, female	Asthma COPD	4	Nasopharyngeal	+	+ (100 % wild type)	Day 1–7: oseltamivir	Died on day 9
3	20, female	None	1 (12 h)	Nasopharyngeal	+	+ (100 % wild type)	Day 1–5: oseltamivir	Recovered
4	9, female	None	1 (1 h, 24 h)	Nasopharyngeal	–, +	+ (100 % wild type)	Day 1–5: zanamivir	Recovered

(37.8 °C) and cough. A nasopharyngeal swab specimen was positive for influenza virus type A by the rapid diagnosis kit (Esplein Influenza A&B–N; Fujirebio, Tokyo, Japan) on day 2. Oseltamivir was administered on day 2. Administration was then maintained for 21 days, until January 13, 2009, because of prolonged disease. Methylprednisolone pulse therapy was initiated on day 2 for the treatment of acute respiratory distress syndrome (ARDS). On day 6, a chest radiograph showed an interstitial pattern in both lungs. The patient was intermittently placed on noninvasive positive pressure ventilation (NIPPV). On day 20 the patient improved, and a rapid test for influenza virus A showed negative. On day 22, the patient exhibited a positive cytomegalovirus (CMV)-antigenemia (more than 53 cells) as well as being positive for influenza A rapid test. Furthermore, it was reported that the specimen collected on day 13 showed oseltamivir-resistant influenza A infection. Therefore, zanamivir instead of oseltamivir as well as ganciclovir were administered on day 23. The patient died of respiratory failure on day 27.

Case 2

A woman aged 66 years with asthma and chronic obstructive pulmonary disease (COPD) complained of fever (37.5 °C) and difficulty in breathing on September 12, 2009. A nasopharyngeal swab specimen was positive for influenza virus type A by the rapid diagnosis kit. The nasopharyngeal specimen was collected on September 15. The patient died of myocarditis on September 20, 2009.

Case 3

A woman aged 20 years complained of fever (38.2 °C) on November 26, 2009. A nasopharyngeal swab specimen collected on November 27 was positive for influenza virus type A by the rapid diagnosis kit.

Case 4

A girl aged 9 years complained of fever (37.0 °C) on December 9, 2009. Nasopharyngeal swab specimen [designated as Naso (pre) in Fig. 4] was negative for influenza virus type A by the rapid diagnosis kit. On the next day, a nasopharyngeal swab specimen [designated as Naso (after) in Fig. 4] was positive for influenza virus type A by the rapid diagnosis kit.

RNA extraction, RT-PCR, cloning, and sequencing

An autopsy of the patient of case 1 was immediately performed. About 1 g of tissue from the lower part of the lung was collected. Total RNA of the lung tissue was extracted using TRIAZOL by the manufacturer's instructions. Nasopharyngeal swab specimens from patients were subjected to RNA extraction with a QIAamp viral RNA kit (Qiagen, Hilden, Germany). Influenza virus genomic RNA was reverse transcribed using RT primer mixture (RT1: AGCAAAAGCAGGT; RT2: AGCGAAAGCAGGT; RT3: AGCAAAAGCAGGC; RT4: AGCGAAAGCAGGC; RT5: AGCAAAAGCAGGG; RT6: AGCGAAAGCAGGG), followed by the first polymerase chain reaction (PCR) using

NA/F primer ATGAATHCAAHCADADDAT and the RT primer mixture. A second PCR was performed using NA/F primer and NA/R primer HHACTTGTCAATDGTDAATG. The amplicon fragment (1,411 bps) was extracted using GeneClean II (MP-Biomedicals, San Francisco, CA, USA). Sequence determination was outsourced to FASMAC, Kanagawa, Japan. The extracted amplicon fragment was ligated to pT7Blue-T vector (Novagen, Germany), and the recombinant plasmid was transformed into *Escherichia coli* (DH5 α). The colonies were picked up and each NA sequence in the plasmid was determined as already described.

Sequence analysis

Parsimony-informative sites, which are parsimony informative in alignment of nucleic acid sequence if it contains at least two types of nucleic acids, and at least two of them occur with a minimum frequency of two, were extracted by the MEGA4 program [5] using all sequences determined by this study (Table 2). The proportion of nonsynonymous substitution (dN) and synonymous substitution (dS), and the ratio of dN to dS, were calculated using the SNAP program on the website (<http://www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html>). A minimum spanning tree was constructed using the mst program of APE package in R software (R Development Core Team). In this program, each clone not only was mutually connected but also was plotted according to the parameters determined by additional nonsymmetrical correspondence analysis.

All sequences obtained in the present study have been submitted to GenBank under accession numbers HQ286390 to HQ286476.

Results

Intrahost variation of influenza virus under long-term oseltamivir treatment

The patient of case 1 was infected with the 2009 H1N1 virus and received oseltamivir (75 mg twice daily) for 21 days and zanamivir for another day (Table 1). He died of interstitial pneumonia on day 27 of his illness. We detected the H275Y mutation by direct sequencing from the nasopharyngeal samples on day 13 (Table 1) and confirmed it was resistant to oseltamivir [6]. The H275Y mutation was also detected in autopsy tissues from the right lung. In addition, we found a small but apparent signal of A (estimated 25 %) in the left lung (Fig. 1), indicating the presence of H257Y mutant population. To investigate the intrahost variation of influenza virus, RT-PCR products were cloned into a TA cloning vector and

the NA gene from independent colonies was sequenced. The H275Y mutation was detected in all clones tested in 12 clones from the right lung and 11 clones from the nasopharynx (Table 2). However, the H275Y mutation was detected in 13 of 22 clones from the left lung. These results indicated that the H275Y mutant was the predominant population in both nasopharynx and right lung, whereas the H275Y mutant virus in the left lung was distributed with an equivalent population of oseltamivir-sensitive clones.

Intrahost evolution of H275Y mutant

To analyze the intrahost evolution of H275Y oseltamivir-resistant virus in case 1, we constructed a minimum spanning tree that provides a better understanding of sequence relationships for microevolution than standard phylogenetic tree representation [7]. Figure 2 shows the minimum spanning tree of all 45 clones from right lung, left lung, and nasopharynx. All H275Y mutants, except for the L7 clone, had additional common mutations at V80M and S82P, which were parsimony-informative sites (Table 2), suggesting that V80M and S82P might be important for the microevolution of oseltamivir-resistant virus. From the tree, the H275Y mutant was predicted to be derived from an oseltamivir-sensitive L2 clone, because the L2 clone was most closely related to the H275Y mutant and had common mutations at V80M and S82P. The L1 clone, in addition, was located to the center of all oseltamivir-sensitive clones. The L2 clone was supposed to be derived from L1, which is most similar to the putative origin of the Eurasian avian-like swine strain of pandemic influenza A (H1N1) 2009. The oseltamivir-resistant clone L7 was located in the same clade including oseltamivir-sensitive clones L6, L7, L8, L9, and L10 (Fig. 2), indicating that the L7 clone was originally derived from this oseltamivir-sensitive clade. The parsimony-informative amino acid of this L7 clone was the same as those of L1, except H275Y.

Intrahost variation of oseltamivir-sensitive virus in other cases

We further extended this approach to three other patients infected with the 2009 H1N1 influenza virus. Samples were taken from nasopharynx during acute phase before (cases 3 and 4) or after (case 2) oseltamivir administration. No H275Y mutant was detected in all 42 clones tested (Table 2). The clones were segregated into three major branches from the clone 33, which was most closely related to the putative origin of 2009 H1N1, European avian-like swine A/swine/Belgium/WVL1/1979 (Fig. 3). Clone 33 was also located between the L1 and L2 clones of case 1.

Table 2 Parsimony-informative sites of NA extracted by alignment of nucleotide sequences of all influenza virus clones determined by this study

Case	Region ^a and/or clone number	Position number of parsimony-informative site and assigned amino acid of clone 33												
		29 I	53 V	80 ^a V	82 ^a S	94 V	142 D	212 I	218 S	221 N	225 R	237 S	275 ^a H	307 N
1	R1–R2			M	P								Y	
	R3			M	P	A							Y	
	R4–R5			M	P								Y	
	R6			M	P								Y	D
	R7			M	P					D			Y	
	R8		A	M	P								Y	
	R9–R11			M	P								Y	
	R12	R		M	P								Y	
	L1													
	L2			M	P									
	L3			M	P									
	L4		A											S
	L5										G			S
	L6													
	L7												Y	
	L8–L10													
	L11–L20			M	P								Y	
	L21			M	P							F	Y	
	L22			M	P								Y	
	N1–N8			M	P								Y	
	N9			M	P				G				Y	
	N10–N11			M	P								Y	
2	1–3								G					
	4											F		
	5–6													
	7						G							
	8–9													
	10						G	M						
3	11													
	12					A								
	13–14													
	15										K			
4	16–21													
	22	R						M						
	23		I											
	24							M		D				
	25–26							M						
	27			A				M						
	28							M						
	29							M		D				
	30–31							M						
	32–37													
	38										K			
	39–42													

^a Key mutations are designated with bold font

Discussion

Oseltamivir is a first-line treatment of patients infected with pandemic 2009 H1N1 virus, and the majority of 2009 H1N1 viruses remain susceptible to oseltamivir, although oseltamivir-resistant strains have been sporadically reported [3]. In this study, we first detected the H275Y mutation in the right lung but not in the left lung. There are few reports of the uneven distribution of oseltamivir-sensitive and oseltamivir-resistant viruses in a single patient [8]. Further analysis of intrahost variation of the virus in the left lung demonstrated that a part of the population was H275Y mutant and was predicted to be derived from oseltamivir-sensitive clone L1 (Fig. 2), which might be a descendant of

[illegible]

Fig. 3 Minimum spanning tree of intrahost variants of influenza virus in cases 2–4. Eleven independent clones from case 2 (1–11), 10 clones from case 3 (12–21), and 21 clones from case 4 (22–31, 32–42) were analyzed as in Fig. 1. All specimens were taken from the nasopharynx. Oseltamivir-sensitive clones are shown in blue, and the

tree was overlapped with that of case 1 (faint color). A train of parsimony-informative amino acids extracted using all sequences from cases 1 to 4 is indicated beside the circles, and nonsynonymous mutations are shown in **bold type**. The train of clone 33 was set as the starting point for the assessment for mutation (color figure online)

Fig. 4 Proportions of nonsynonymous substitution (dN) and synonymous substitutions (dS) of intrahost variants of influenza virus in cases 1 to 4. Data are shown as average $\pm$ SD. The dN/dS ratio is also shown. Asterisk indicates statistically significant by Student's *t* test. RL right lung, LL left lung, Naso nasopharynx

the putative oseltamivir-sensitive clone in the nasopharynx. Considering the route of virus spread from upper respiratory tract to lower respiratory tract, it is likely that the H275Y mutant is generated primarily in the nasopharynx, then spreads to the right and left lungs. In a study of other patients (cases 2–4), all intrahost species were oseltamivir sensitive, possibly because oseltamivir was administered briefly (case 2) or not administered at all (cases 3 and 4). Minimum spanning trees of all clones in this study determined clone 33, from the case 4 clones, as the core of oseltamivir-sensitive clones, indicating an origin of all intrahost species of oseltamivir-sensitive clones (Fig. 3). Interestingly, the oseltamivir-sensitive L1 clone of case 1 was also closely located to clone 33 and had common parsimony-informative amino acids (Table 2), which would predict a high degree of relatedness. In addition, clone 33 was most closely related to the putative origin of 2009 H1N1, European avian-like swineA/swine/Belgium/WVL1/1979. The dN/dS ratio of left lung clones from case 1 was smallest (Fig. 4). We also found a further decrease of the ratio of the oseltamivir-sensitive clone (dN/dS = 0.27), which was half of the population in the left lung, indicating

comparatively strong purifying selection occurred in the left lung [9]. It might be speculated that the oseltamivir-sensitive clone of the left lung is in a stable phase of evolution [9]. It is likely that case 1 might have been infected primarily with the oseltamivir-sensitive virus and that long-term administration of oseltamivir seems to have induced the generation of the H275Y mutant. Unfortunately, specimens were not available at the early phase of oseltamivir therapy, and we could not determine when the H275Y mutant was generated during therapy. Taken together, we supposed that the patient was infected with the L1 lineage first, which then spread to right and left lungs; it mutated independently in the left lung to L4–L10 strains but died out in the right lung (Fig. 2). In the nasopharynx, major clones having 80M, 82P, and 275Y appeared at about day 13 and spread to the right and left lungs at a later stage (Table 2). Thereafter, a small number of sensitive strains persisted stubbornly in the left lung although oseltamivir had been administered. It is still unclear, however, why the oseltamivir-sensitive clone still constituted half the total clones in the left lung despite the long-term administration of oseltamivir (21 days).

In summary, our study highlights the generation and intrahost variation of oseltamivir-resistant 2009 H1N1 virus under long-term oseltamivir therapy. This information allows us to predict the process of microevolution of an influenza virus within a host.

References

1. Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, et al. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature (Lond)*. 2009;459:1122–5.
2. Baranovich T, Saito R, Suzuki Y, Zaraket H, Dapat C, Caperig-Dapat I, et al. Emergence of H274Y oseltamivir-resistant A (H1N1) influenza viruses in Japan during the 2008–2009 season. *J Clin Virol*. 2010;47:23–8.
3. Campanini G, Piralla A, Rovida F, Puzelli S, Facchini M, Locatelli F, et al. First case in Italy of acquired resistance to oseltamivir in an immunocompromised patient with influenza A/H1N1v infection. *J Clin Virol*. 2010;48:220–2.
4. Hoelzer K, Murcia PR, Baillie GJ, Wood JL, Metzger SM, Osterrieder N, et al. Intrahost evolutionary dynamics of canine influenza virus in naive and partially immune dogs. *J Virol*. 2010;84:5329–35.
5. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol*. 2007;24:1596–9.
6. Ministry of Health: LaWoJ. Oseltamivir-resistant pandemic influenza virus in Kurume City. Press Release 26 February 2010.
7. Spada E, Sagliocca L, Sourdis J, Garbuglia AR, Poggi V, De Fusco C, et al. Use of the minimum spanning tree model for molecular epidemiological investigation of a nosocomial outbreak of hepatitis C virus infection. *J Clin Microbiol*. 2004;42:4230–6.
8. Furuse Y, Suzuki A, Kishi M, Nukiwa N, Shimizu M, Sawayama R, et al. Occurrence of mixed populations of influenza A viruses that can be maintained through transmission in a single host and potential for reassortment. *J Clin Microbiol*. 2010;48:369–74.
9. Kryazhimskiy S, Plotkin JB. The population genetics of dN/dS. *PLoS Genet*. 2008;4:e1000304.