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Emergence of multidrug resistant influenza A(H1N1)pdm09 virus variants in an immunocompromised child treated with oseltamivir and zanamivir

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Abstract

Prolonged treatment of an immunocompromised child with oseltamivir and zanamivir for A(H1N1)pdm09 virus infection led to the emergence of viruses carrying H275Y and/or E119G in the neuraminidase. When phenotypically evaluated by neuraminidase inhibition, the dual H275Y-E119G substitution caused highly reduced inhibition by four neuraminidase inhibitors including oseltamivir, zanamivir, peramivir and laninamivir.

INTRODUCTION

Neuraminidase (NA) inhibitors (NAIs) are vital for treating influenza infections among immunocompromised patients at high risk for influenza-associated complications and mortality. Because currently circulating influenza viruses are resistant to M2 blockers, NAIs are the only effective FDA-approved drugs for treating influenza. The NAIs, orally administered oseltamivir and inhaled zanamivir, are licensed in most countries, while intravenous peramivir (1) is approved in the USA, Japan, South Korea, China, and inhaled laninamivir (2) in Japan. Intravenous (IV) zanamivir is undergoing evaluation for treatment of hospitalized patients with severe influenza (3). Use of NAIs in immunocompromised patients who often have sustained viral replication is associated with emergence of drug resistance resulting from subtype-specific neuraminidase (NA) mutations (4).

The H275Y substitution in the N1 NA is the most commonly observed change associated with resistance to oseltamivir and peramivir, and can emerge and persist following oseltamivir treatment in immunocompromised patients infected with influenza A(H1N1)pdm09 (5). Use of zanamivir in patients infected with H275Y viruses is associated with reduced viral shedding and provides an alternative treatment (6). Resistance to zanamivir has rarely been detected in clinical settings, and was first reported in an influenza B virus with R152K substitution, isolated from an immunocompromised patient (7). The emergence of I223R has been reported in A(H1N1)pdm09 viruses infecting immunocompromised patients, often in combination with or after the H275Y substitution has been detected (8) and results in reduced susceptibility to zanamivir. The variant has emerged after sequential oseltamivir and zanamivir treatment. By itself, I223R causes minimal effects on zanamivir susceptibility, but synergizes with H275Y to produce a greater effect. An I223R-H275Y double mutant exhibited ~20-fold and ~10-fold increases in zanamivir IC_{50},compared to the wild type virus and the H275Y mutant, respectively (8).

We report the emergence of an influenza A(H1N1)pdm09 virus carrying a newly described substitution in the NA from an immunocompromised infant following prolonged zanamivir treatment.

METHODS

Patient Information

The patient was an 8-month-old infant with familial Hemophagocytic Lymphohistiocytosis (HLH). The patient had initially developed upper respiratory symptoms and tested positive for influenza A by PCR (xTAG^{TM} RVP, Luminex Molecular Diagnostics, Inc., Ontario, Canada) at 4 months of age, after being hospitalized for two months to receive immunosuppressive treatment for HLH. The patient received 5 days of oral oseltamivir and symptoms resolved. Two months later (64 days prior to cord blood transfusion [day -64]), the patient was re-admitted with diarrhea and mild respiratory symptoms and restarted on oseltamivir after testing positive for influenza A (Table 1). The patient's respiratory status worsened and treatment with IV zanamivir was initiated under an emergency investigational new drug request (day –57). Transplantation was delayed due to renal and electrolyte issues. While on zanamivir, the patient's respiratory status improved and the patient eventually underwent cord blood transplantation for severe HLH that was not responsive to chemotherapy after receiving zanamivir for 57 days. On day 9 following transplantation, he was diagnosed with pulmonary aspergillosis based on bronchoalveolar lavage, and received antifungal therapy. Zanamivir was discontinued after 73 days of treatment (17 days following transplantation), when results of the NI assay performed on his clinical isolate revealed a multi-drug resistant profile. He received intravenous immunoglobulin therapy throughout the treatment course. He had lymphocyte engraftment on day 27 following transplantation, but eventually expired on day 69 due to progressive respiratory failure and pulmonary hemorrhage. No autopsy was performed.

A nasal wash specimen collected from the infant was submitted to the Centers for Disease Control and Prevention (CDC) for antiviral susceptibility testing 10 days prior to cord blood

transplantation (day -10). Additional clinical specimens were submitted to CDC for evaluation after this specimen was fully characterized.

Virus Characterization

The CLIA-certified CDC pyrosequencing assay was used on the day -10 specimen to detect amino acid substitutions at the NA residues H275, I223, N295 and D199, and was performed on the PyroMark PSQ96™ MA platform (Qiagen, Valencia, CA). A pyrosequencing assay targeting residue E119 was specifically designed for this study (Supplemental Table S1).

Virus propagation was performed in Madin-Darby canine kidney (MDCK) cells (ATCC, Manassas, VA). Plaque purification of viruses was performed in accordance with standard procedures.

The fluorescent NI assay was used to determine susceptibilities of virus isolates to oseltamivir carboxylate (Hoffmann-La Roche, Basel, Switzerland), zanamivir (GlaxoSmithKline, Uxbridge, UK), peramivir (BioCryst Pharmaceuticals, Birmingham, AL) and laninamivir (Biota, Melbourne, Australia) using the NA-Fluor™ Influenza Neuraminidase Assay Kit (Applied Biosystems, Foster City, CA). Drug concentrations required to inhibit NA activity by 50% (IC_{50}) were determined using JASPR v1.2 curve-fitting software (CDC, Atlanta, GA). Fold changes in IC_{50} were determined by comparing IC_{50}s of test viruses with a wildtype reference virus, A/California/12/2012 (H1N1)pdm09, and interpreted according to classification criteria of the World Health Organization Influenza Antiviral Working Group (WHO-AVWG).

Sanger sequencing of NA genes was performed using standard procedures, and NA sequence data was deposited to the Global Initiative on Sharing All Influenza Data (GISAID), under the accession number EPI531845.

Quantitative PCR analysis of respiratory specimens was performed as previously described (9).

RESULTS

The respiratory specimen submitted to CDC was obtained 10 days pre-transplantation (-10), after 47 days of zanamivir treatment and was tested with pyrosequencing for markers of NAI resistance (Table 1). The proportion of H275Y in the virus quasi-species was 23%, which was ~2-fold above the limit of detection in the pyrosequencing assay (cutoff 10%). The H275Y substitution is a marker of resistance to oseltamivir and peramivir. No amino acid changes were detected at residue I223, N295, or D199.

The virus isolate from this specimen (passage C1) was tested by NI assay (Table 2) and showed slightly reduced inhibition by oseltamivir (7-fold increase in IC_{50}) and highly reduced inhibition by zanamivir (~1300-fold), peramivir (~170-fold) and laninamivir (~330-fold), compared to the wildtype reference virus, A/California/12/2012 (H1N1)pdm09. Notably, pyrosequencing of this isolate (C1) revealed only one NA virus variant, H275Y (5%) and no variants with the other 3 markers. To identify molecular changes responsible for the unusual NI assay profile, virus isolate (C1) was subjected to Sanger sequencing and the E119G substitution was detected.

New primers were designed to extend the pyrosequencing assay to residue E119 (Supplemental Table S1). Using this assay, the E119G was detected in both the clinical specimen and virus isolate (passage C1). The clinical specimen contained both H275Y (23%) and E119G. Plaque purification of the original clinical sample in MDCK cells identified a H275Y-E119G double NA variant. When tested by NI assay (Table 2), the virus carrying H275Y-E119G exhibited highly reduced inhibition by oseltamivir (~2500-fold), zanamivir (~1500-fold), peramivir (>10,000-fold) and laninamivir (~600-fold), compared to the wildtype reference virus, A/California/12/2012 (H1N1)pdm09. Peculiarly, plaque-purification of the virus isolate (passage C1) yielded viruses containing only E119G (in 39 (46%) of the 84 clones that were picked), and none with H275Y, suggesting that the virus carrying E119G had

growth advantage over the one with the dual substitution when propagated in MDCK cells in the absence of NAIs. The phenotypic effect of the H275Y-E119D double variant (day -32 sample) on drug susceptibility was not determined due to unsuccessful attempts to recover the virus in cell culture.

Additional clinical specimens (n=19) collected from the patient from day -64 to day +45, relative to transplantation, were tested by pyrosequencing at residues E119 and H275 (Table 1). The H275Y substitution alone, in proportions of 95-100%, was detected in the first four samples collected between day -64 and day -46; zanamivir treatment commenced on day -57. Five samples collected between day -39 and day -10 (the initial sample), had changes at residue E119, in addition to H275Y. Of note, the samples collected on day -25 and day -10 contained only 16% and 23% H275Y, respectively, while those collected on day -39, -32, and -18 had 100% H275Y. A mix of E119G and E119D variants (E119G/D) in addition to H275Y (100%) was detected in the sample from day -9, while that collected on day -3 had only E119G/D, and those collected on day +3 and day +17, respectively, had E119G/D with 34-37% H275Y. Two samples collected day +24 and day +34, respectively had E119G alone. The viral RNA titers varied and did not directly reflect the detection of E119D/G (Table 1).

DISCUSSION

We report the emergence of an influenza A(H1N1)pdm09 virus infecting an immunocompromised infant after oseltamivir and zanamivir treatment, that carried the E119G substitution in the NA. This change confers highly reduced inhibition to zanamivir, peramivir and laninamivir. Moreover, a dual variant containing H275Y-E119G was detected and was highly resistant to these three NAIs, in addition to oseltamivir. The presence of E119G potentiated the effects of H275Y substitution, increasing IC_{50} values against oseltamivir by 2.5-fold, compared to the H275Y reference virus A/Texas/32/2012 (H1N1)pdm09.To our knowledge, neither E119G nor the above combination of substitutions, have previously been reported in A(H1N1)pdm09 viruses.

No established laboratory test criteria exist for defining clinically relevant resistance to NAIs, but highly reduced inhibition in the neuraminidase inhibition (NI) assay likely correlates with failure to control virus replication. Four NA substitutions, H275Y, I223R, D199N, and N295S, are recommended by the WHO-AVWG for monitoring influenza antiviral drug susceptibility in viruses carrying the N1 NA; H275Y and I223R have been described most frequently. The pyrosequencing assay targeting residue E119 will increase the scope of detecting neuraminidase (NA) markers of drug resistance in A(H1N1)pdm09 viruses. There are no previous reports regarding the emergence of E119G or E119D in influenza A(H1N1)pdm09 viruses in patients following zanamivir treatment. The E119G mutation has been reported in N2 (e.g. A(H3N2)) (10), and A(H5N1) viruses (11) following serial passages in the presence of zanamivir and is associated with zanamivir-resistance (10,11). Recombinant A(H1N1)pdm09 viruses with E119G mutation exhibited highly reduced inhibition by zanamivir and peramivir, but were normally inhibited by oseltamivir (12), while those with E119D had zanamivir and peramivir profiles similar to the E119G variant, but also exhibited reduced inhibition by oseltamivir. Finally, these substitutions have been reported infrequently from viruses infecting patients. The substitutions E119K and E119D were identified by next-generation sequencing (NGS) in A(H1N1)pdm09 viruses collected from patients hospitalized with influenza and treated with IV zanamivir (13); only E119D was present in proportions detectable by Sanger sequencing. The E119G mutation was detected during virological surveillance in 2011-12, in an influenza A(H1N1)pdm09 virus isolate from Chile, A/Iquique/44427/2012 that exhibited highly reduced inhibition by zanamivir (743-fold) and laninamivir (113-fold), reduced inhibition by peramivir (85-fold), but normal inhibition by oseltamivir (3-fold); treatment history was not available (L. Gubareva, personal communication).

The mechanisms by which mutations at residue 119 of A(H1N1)pdm09 viruses affect NAI susceptibility remain unclear. The molecular structure of zanamivir, peramivir and laninamivir includes a guanidine group (14), which interacts with the conserved E119 residue without undergoing a side chain conformational change. Mutations at residue E119, such as E119D (12) that involves replacement

of glutamic acid with aspartic acid, with a shorter side chain, may cause an unfavorable interaction with NAIs and their natural substrate sialic acid. This may confer reduced sensitivity to the drugs. Furthermore, viruses carrying substitutions at residue 119 may have poor replication kinetics (15), suggesting low potential for transmission. Studies demonstrate that E119G is associated with a significant reduction of total NA activity (15), which alters NA enzymatic properties to levels that would significantly compromise viral viability and transmissibility in ferrets. A recombinant A(H1N1)pdm09 virus with E119D showed impaired viral replicative fitness, but retained its growth properties and pathogenicity in mice (12). Such findings may explain the low frequency of mutations at residue E119 of the NA, especially in the N1 subtype.

Although uncommon, substitutions at residue E119 (e.g. E119G and E119D), can confer a multidrug resistance phenotype in influenza A(H1N1)pdm09 viruses and may represent a problem for immunocompromised patients on prolonged zanamivir treatment; further investigations of virus virulence and transmission are warranted. In this infant, additional experimental therapies, such as convalescent plasma, DAS181, or T705, were not tried due to signs of engraftment, a stable respiratory status, and lack of product safety and efficacy data for infants. This report highlights the importance of continuous surveillance and characterization of potential NAI multidrug-resistant influenza variants as well as the continued development of alternative therapeutic options for influenza.

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Conflict of Interest: We declare that we have no potential conflicts of interest. R.L.D. is site PI for the multicenter GSK zanamivir study. The subject was not in this GSK study, but received zanamivir under an emergency investigational new drug (EIND) request.

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Table 1. Characterization of A(H1N1)pdm09 clinical specimens collected from an immunocompromised patient treated with oseltamivir and zanamivir

Day Relative to CBT[b]	Clinical Influenza Detection Type (Subtype)	NAI Treatment	Specimen Source	Quantitative PCR (copies/ml)	Changes at NA Residue	
					275 [a]	119
-121	A[c](H1N1)pdm09	Oseltamivir x 5 days	NP			
-72	A[c](H1N1)pdm09	Oseltamivir x 5 days	NP			
-64	A	Oseltamivir restarted	NW	5.23 x10^6	H275Y (100)	E119
-60	A	Oseltamivir	NW	2.24 x10^6	H275Y (95)	E119
-57		Zanamivir IV started				
-53	Negative	Zanamivir IV	NP			
-51		Zanamivir IV	NP	0.21 x10^6	H275Y (100)	E119
-49	A	Zanamivir IV	NP	0.31 x10^6		
-46		Zanamivir IV	Swab		H275Y (100)	E119
-39	A	Zanamivir IV	NP		H275Y (ND)	E119G (100)
-32	Negative	Zanamivir IV	NP			
-32	A	Zanamivir IV	NP	0.28 x10^6	H275Y (100)	E119D (100)
-25		Zanamivir IV	Swab		H275Y(16)	E119G (100)
-21	A	Zanamivir IV	BAL			
-18	A	Zanamivir IV	NP	66.2 x10^6	H275Y (100)	E119G (100)
-10[d, e]	A	Zanamivir IV	NP [d, e]	0.022 x10^6	H275Y (23) [c]	E119G (100) [c]
-9		Zanamivir IV	NP		H275Y (100)	E119G (40)/D (60)
-3 [e]		Zanamivir IV	NP [e]	0.015 x10^6	--	E119G (71)/D (29)
0, CBT						
+3	A	Zanamivir IV	NP	0.96 x10^6	H275Y (34)	E119G (39)/D (61)
+12	A	Zanamivir IV	NP			
+17 [e]	A	Zanamivir stopped	NW, NP [e]	43.4 x10^6	H275Y (37)	E119G (74)/D (26)
+24	A		NP	27.0 x10^6	H275Y (7)	E119G (100)
+27, Engraftment						
+28			CSF	0		
+31	A		BAL			
+32			BAL	0		
+34			BAL		--	E119G (100)
+39			ETT		--	--
+39			NP	0	--	--
+45			ETT		--	--
+59	Negative		ETT			
+66	Negative		ETT			

Abbreviations: CBT: cord blood transplantation; NW: nasal wash; NP: nasopharyngeal swab; CSF: celebrospinal fluid; ETT: endotracheal tube aspirate;

"--" Pyrosequencing assay failed at respective residue.

[a] Proportion of NA variants versus wildtype (in parenthesis) determined using single nucleotide polymorphism (SNP) pyrosequencing analysis.

[b] Only dates with data or a significant event are included in the table; other days were left off the Table.

[c] The Respiratory Virus Panel assay: xTAG™ (Luminex Diagnostics) detected Type A, non H3N2 and non-seasonal H1N1. It was assumed to be A(H1N1)pdm09.

[d] Initial sample received at CDC.

[e] Two separate specimens were tested for virus RNA load and NA substitutions.

Table 2. NAI susceptibility of influenza A(H1N1)pdm09 virus isolates assessed in the fluorescent NI assay

Virus	Passage	NA changes (Frequency of variants, %)[a]		Mean IC_{50} ± SD[b], nM (Fold change)[c]			
		275	119	Zanamivir	Oseltamivir	Peramivir	Laninamivir
A/District of Columbia/02/2014	C1[d]	H275Y (5)	E119G (100)	235.05±27.17 (1306)	1.19±0.05 (7)	30.33±0.39 (167)	49.01±5.89 (327)
A/District of Columbia/02/2014, Clone 1	C2[e]	H275Y (100)	E119G (100)	278.34±69.91 (1546)	422.10±93.93 (2483)	4671.66±191.02 (93433)	90.78±6.91 (605)
Reference viruses							
A/California/12/2012, oseltamivir-susceptible		H275	-	0.18±0.02 (1)	0.17±0.01 (1)	0.05±00 (1)	0.15±0.02 (1)
A/Texas/32/2012, oseltamivir-resistant		H275Y	-	0.23±0.03 (1)	176.26±22.4 (1036)	15.66±2.05 (313)	0.35±0.03 (2)

[a] Proportion of NA variants versus wildtype, determined using SNP pyrosequencing analysis.
[b] Based on at least three independent experiments.
[c] Fold increase (in bold) based on comparison to the respective IC_{50} values of the reference virus, A/California/12/2012.
[d] C1 - Original clinical specimen passaged once in Madin Darby Canine Kidney (MDCK) cells at the CDC laboratory.
[e] C2 - Original clinical specimen inoculated and plaque-purified in MDCK cells, then passaged once more in MDCK cells at the CDC laboratory.