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A single E105K mutation far from the active site of influenza B virus neuraminidase contributes to reduced susceptibility to multiple neuraminidase-inhibitor drugs

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ABSTRACT

Drugs inhibiting the enzymatic activity of influenza virus neuraminidase (NA) are the cornerstone of therapy for influenza virus infection. The emergence of drug-resistant variants may limit the benefits of antiviral therapy. Here we report the recovery of an influenza B virus with reduced susceptibilities to NA inhibitors from a human patient with no history of antiviral drug treatment. The virus, designated B/Kochi/61/2011, was isolated by inoculating Madin–Darby canine kidney (MDCK) cells with respiratory specimens from the patient. NA inhibition assays demonstrated that the B/Kochi/61/2011 isolate showed a remarkable reduction in susceptibility to peramivir. The isolate also exhibited low to moderately reduced sensitivity to oseltamivir, laninamivir, and zanamivir. A sequence analysis of viruses propagated in MDCK cells revealed that the isolate contained a mutation (E105K) not previously associated with reduced susceptibility to NA inhibitors. However, pyrosequencing analysis showed that the NA E105K mutation was below a detectable level in the original clinical specimens, suggesting that the mutant virus may be preferably selected during propagation in MDCK cells. Analysis of the three-dimensional model of E105 and K105 NAs with peramivir suggested that the E105K mutation at the monomer–monomer interface of the NA tetramer may destabilize the tetrameric form of NA, leading to decreased susceptibility to NA inhibitors. These results have implications for understanding the mechanism of resistance against NA-inhibitor drugs.

1. Introduction

Influenza is an acute respiratory infectious disease caused by influenza A and B viruses, and is associated with significant morbidity and mortality worldwide. Two classes of antiviral drugs are currently commercially available for the prophylaxis and treatment of influenza: adamantanes and neuraminidase (NA) inhibitors. The adamantanes (amantadine and remantadine), known as M2 channel blockers, inhibit influenza A virus replication by blocking the ion-channel activity of M2 protein that is required for the release of viral RNAs into the cytoplasm of infected cells [1,2]. However, the ion-channel activity of influenza B virus BM2 protein is not affected by the drugs [3].

On the other hand, the NA inhibitors are effective against both influenza A and B viruses. During the budding process, the NA protein of influenza A and B viruses cleaves sialic acids from cellular receptors to facilitate the release of virus particles from the infected cell surface [4,5]. NA inhibitors function by competitively binding (against sialic acids) to the NA enzyme active site, thereby reducing the amount of virus released from infected cells [6–8]. While oseltamivir and zanamivir are currently available in numerous countries for clinical use, two additional NA inhibitors, peramivir and laninamivir, have been recently approved in Japan [9,10]. These inhibitors were designed to target the active site that is highly conserved among the NA proteins of influenza A and B viruses. Therefore, key amino acid changes in the catalytic or framework residues comprising the active site cause reduced affinity for NA inhibitor drugs, leading to the development of drug resistance [11,12]. The emergence of drug-resistant influenza strains could undermine the efficacy of influenza treatment in individuals and reduce the choices available for the control of epidemic and pandemic outbreaks.

A small number of influenza B viruses isolated from patients display reduced susceptibilities to oseltamivir, zanamivir, and/or peramivir due to amino acid changes within the NA active site

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(R150K, D197E, D197N, I221T, N294S, or G407S) [13–18]. Nonetheless, additional, currently unrecognized mutations may also affect the sensitivity of influenza B viruses to NA inhibitors. Consequently, the identification of novel mutations in NAs associated with drug resistance would significantly increase the ability of national surveys to detect and monitor influenza virus resistance to NA inhibitors worldwide.

Since 2007, influenza virus isolates submitted to the National Institute of Infectious Diseases (NIID) in Japan have been analyzed routinely for sensitivity to antiviral drugs [19]. Here we report the recovery of a mutant influenza B virus with a novel mutation causing decreased susceptibilities to NA inhibitors from a human patient with influenza-like illness and no history of treatment with antiviral drugs. Our study shows that a single mutation at position 105, away from the active site of NA, attributes to reduced susceptibility of influenza B viruses to oseltamivir, zanamivir, peramivir, and laninamivir.

2. Materials and methods

2.1. Cells

Madin–Darby canine kidney (MDCK; American Type Culture Collection CCL-34) cells were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum (FCS). Human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% FCS. All cells were maintained at 37 °C in 5% CO_2.

2.2. Specimens and viruses

On 12 May 2011, a 35-year-old male developed a high fever and presented to a hospital in Kochi Prefecture, Japan. A respiratory specimen was collected from the patient prior to drug treatment for influenza and submitted to Kochi Public Health and Sanitation Institute for virus isolation by inoculating onto MDCK cell cultures. The isolated viruses were then sent to the NIID and were propagated further in MDCK cells for NA inhibition assays. B/Perth/211/2001 viruses possessing either aspartic acid or glutamic acid at position 197 in the NA protein used as NA inhibitor-sensitive or resistant controls, respectively, were provided by the International Society for Influenza and other Respiratory Virus Diseases (ISIRV) [16].

2.3. Plasmid construction and recombinant viruses

Plasmid constructs for viral RNA production (pPolI)—containing the NA genes of the B/Kochi/58/2011 and B/Kochi/61/2011 viruses flanked by the human RNA polymerase I promoter and the mouse RNA polymerase I terminator—were constructed as described [20]. All constructs were sequenced to ensure that no unwanted mutations were present. Transfectant influenza viruses were generated as described previously [21]. Recombinant viruses were amplified in MDCK cells in Opti-MEM I containing 5 μg/ml of acetylated trypsin.

2.4. NA inhibitor susceptibility assays

Oseltamivir carboxylate, peramivir, zanamivir, and laninamivir were provided by F. Hoffmann-La Roche Ltd. (Basel, Switzerland), Shionogi & Co. Ltd. (Osaka, Japan), GlaxoSmithKline (Middlesex, UK), and Daiichi Sankyo Co., Ltd. (Tokyo, Japan), respectively. The NA inhibitor susceptibility assay was done using an NA-XTD Influenza Neuraminidase Assay Kit (Applied Biosystems, CA, USA) following the manufacturer's protocol. The NA-XTD kit provides a chemiluminescence-based assay with NA-XTD substrate. Chemiluminescence was detected using an LB940 plate reader (Berthhold Technologies, Bad Wildbad, Germany). Drug concentrations required for 50% inhibition of NA activity (IC_{50}) were calculated by using MikroWin 2000 software (Mikrotek Laborsysteme GmbH, Overath, Germany) as previously described [22].

2.5. Pyrosequencing

Viral RNAs were extracted from a 140-μl sample using a QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A 149-amino-acid region (spanning residues 43–191) was selected to detect mutation at residue 105 in NA. This region was subjected to reverse transcription polymerase chain reaction (RT-PCR) using a SuperScript III One-Step RT-PCR System (Invitrogen, CA, USA). Primers for RT-PCR were B-NA-172-191F (5′-TAT ATT GCT AAA ATT CTC AC-3′) and B-NA-664-645R (5′-ATT GTC AGG GCC ATC AAC TC-3′). The amplicons were ligated with RL MID adaptors (Roche Diagnostics GmbH, Mannheim, Germany) after which the products were subjected to emulsion PCR and a GS Junior 454 sequencing instrument (Roche), according to the manufacturer's instructions. Pyrosequencing analyses were performed using a GS Junior 454 sequencing instrument (Roche) and GS Amplicon Variant Analyzer (Roche).

2.6. Molecular modeling of influenza B virus tetrameric NA with peramivir

The crystal structures of influenza B virus (B/Perth/211/2001) tetrameric NA with peramivir at a resolution of 2.54 Å (PDB ID: 3K39 [12]) were taken from the RCSB Protein Data Bank [23]. Three-dimensional (3-D) models of influenza B virus tetrameric NAs (B/Kochi/58/2011 and B/Kochi/61/2011) with peramivir were constructed by the homology modeling technique using Molecular Operating Environment (MOE)-Align and MOE-Homology in the MOE version 2010.10 (Chemical Computing Group Inc., Quebec, Canada) as described [24,25]. We obtained 25 intermediate models per one homology modeling in MOE and selected the 3-D models, which were the intermediate models with the best scores according to the generalized Born/volume integral methodology [26]. The final 3-D models were thermodynamically optimized by energy minimization using an AMBER99 force field [27] combined with the generalized Born model of aqueous solvation implemented in MOE [28]. Physically unacceptable local structures of the optimized 3-D models were further refined on the basis of evaluation by a Ramachandran plot using MOE.

3. Results

3.1. Detection of an influenza B virus with reduced susceptibility to oseltamivir, peramivir, zanamivir, and laninamivir

On 12 May 2011, a respiratory specimen was taken from a patient with an influenza-like illness prior to any treatment with antiviral drugs. An influenza B virus, designated B/Kochi/61/2011, was recovered from the specimen by inoculation to MDCK cells. A chemiluminescent NA inhibition assay using NA-XTD substrate was used to characterize the susceptibilities of B/Kochi/61/2011 virus to oseltamivir, peramivir, zanamivir, and laninamivir (Table 1). The IC_{50} value of isolated viruses was expressed as fold-increase in IC_{50} compared to those of a drug-susceptible control reference virus, B/Perth/211/2001 virus [16]. B/Kochi/61/2011 showed a marked reduction in the susceptibility to peramivir (213-fold increases in IC_{50} values). The virus isolate also exhibited moderate reduced susceptibility to zanamivir (15-fold) and slightly

Table 1
NA inhibitor susceptibilities of influenza B viruses that were isolated from untreated patients in Kochi, Japan, during the 2010–2011 influenza season.[a]

Virus	Sample date	Passage history	NA change	IC_{50} values (nM) of NA inhibitors (fold differences[b])				GISAID[c] accession no.
				Oseltamivir	Peramivir	Zanamivir	Laninamivir	
B/Kochi/58/2011	25 April 2011	MDCK5	Wild-type	3.17 ± 0.19 (1.6)	0.62 ± 0.05 (2.0)	1.37 ± 0.15 (1.9)	3.05 ± 0.13 (1.6)	EPI382048
B/Kochi/61/2011	12 May 2011	MDCK5	E105K	8.28 ± 2.10 (4.2)	66.05 ± 18.50 (213.1)	10.37 ± 0.74 (14.6)	6.53 ± 1.51 (3.4)	EPI377276
B/Perth/211/2001, resistant control	Reference		D197E	18.69 ± 5.74 (9.6)	1.85 ± 0.76 (6.0)	3.10 ± 1.47 (4.4)	3.25 ± 0.62 (1.7)	
B/Perth/211/2001, sensitive control	Reference		Wild-type	1.95 ± 0.45 (1.0)	0.31 ± 0.05 (1.0)	0.71 ± 0.15 (1.0)	1.91 ± 0.40 (1.0)	

[a] IC_{50} values were determined by a chemiluminescent assay using the NA-XTD influenza neuraminidase assay kit. Average IC_{50}s and standard deviations were calculated using values from three independent assays performed in duplicates.
[b] Compared to IC_{50} values determined for sensitive control B/Perth/211/2001.
[c] GISAID, Global Initiative on Sharing Avian Influenza Data.

Table 2
NA inhibitor susceptibilities of recombinant viruses containing an E105K mutation in NA.[a]

Virus	NA change	IC_{50} values (nM) of NA inhibitors (fold differences[b])			
		Oseltamivir	Peramivir	Zanamivir	Laninamivir
rg-E105xBY73	Wild-type	2.48 ± 0.12 (1.2)	0.58 ± 0.02 (1.9)	1.13 ± 0.18 (1.6)	2.63 ± 0.32 (1.4)
rg-K105xBY73	E105K	16.66 ± 0.93 (8.3)	132.25 ± 16.32 (435.6)	29.85 ± 12.85 (42.4)	22.14 ± 9.35 (11.7)
B/Perth/211/2001, resistant control	D197E	18.20 ± 5.83 (9.0)	1.86 ± 0.80 (6.1)	3.15 ± 1.49 (4.5)	3.31 ± 0.62 (1.7)
B/Perth/211/2001, sensitive control	Wild-type	2.01 ± 0.54 (1.0)	0.30 ± 0.05 (1.0)	0.70 ± 0.15 (1.0)	1.90 ± 0.43 (1.0)

[a] IC_{50} values were determined by a chemiluminescent assay using the NA-XTD influenza neuraminidase assay kit. Average IC_{50}s and standard deviations were calculated using values from three independent assays performed in duplicates.
[b] Compared to IC_{50} values determined for sensitive control B/Perth/211/2001.

decreased susceptibility to both oseltamivir and laninamivir (4.2- and 3.4-fold, respectively). Sequence analyses of viruses propagated in MDCK cells revealed that B/Kochi/61/2011 did not possess any of the previously reported mutations associated with reduced susceptibility to NA inhibitors. When the amino acid sequence of the NA from B/Kochi/61/2011 was compared with that of an NA inhibitor-sensitive virus, B/Kochi/58/2011 (⩽2-fold; Table 1), however, we found that the two viruses had an identical amino acid sequence except for residue 105, which is a Glu in B/Kochi/58/2011 and a Lys in B/Kochi/61/2011 (i.e., E105K mutation). These results suggest that the decreased susceptibility of B/Kochi/61/2011 to NA inhibitors is caused by the E105K mutation in NA.

To determine whether the E105K mutation in the B/Kochi/61/2011 NA protein is present in clinical specimens, pyrosequencing was performed on RT-PCR products from RNA extracted from the original clinical specimen used for isolation of B/Kochi/61/2011 virus. The pyrosequencing assay showed that the E105K mutation was undetectable in the specimen viruses (<1%). Similar results were observed by conventional direct sequencing of the same clinical specimen (data not shown). These data suggest that the virus with the E105K mutation may exist with an extremely low level in the clinical specimen or occur during virus propagation in MDCK cells used for isolation.

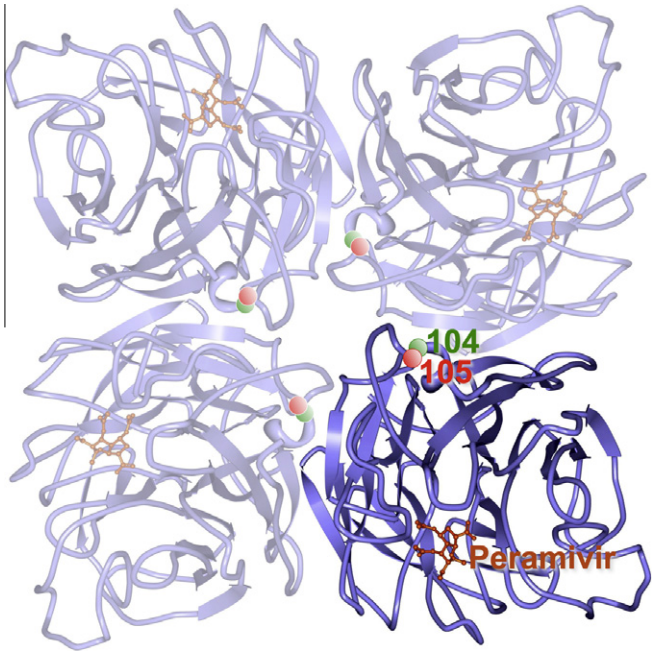

Fig. 1. Three-dimensional (3D) structure of the complex of B/Perth/211/2001 NA and peramivir [PDB ID: 3K37] [12]. Mutations known to decrease sensitivity to NA inhibitors are shown in green [31]. Mutations identified in this study are shown in red. Peramivir is shown in orange.

3.2. Identification of a mutation that confers decreased susceptibility to NA inhibitors

To confirm the significance of the identified mutation in NA for the reduced susceptibility to NA inhibitors, we generated two influenza B reassortant viruses possessing the NA gene of B/Kochi/58/2011 or B/Kochi/61/2011 viruses and the remaining genes from B/Yamagata/1/73 (BY73) virus by reverse genetics, and determined the susceptibility of the resulting viruses to NA inhibitors. A recombinant virus, whose NA gene came from B/Kochi/58/2011 with wild-type NA, designated rg-E105xBY73, was susceptible to all NA inhibitors (⩽2-fold IC_{50} increase; Table 2) when compared with B/Perth/211/2001 virus. In contrast, a recombinant virus derived from B/Kochi/61/2011 carrying the NA substitution E105K, rg-K105xBY73, exhibited 8.3-, 436-, 42-, and 12-fold reductions in susceptibility to oseltamivir, peramivir, zanamivir, and laninamivir, respectively, and reproduced the susceptibility profile of the B/Kochi/61/2011 isolate. These results clearly demonstrate that the E105K mutation in the NA protein of influenza B viruses confers the decreased susceptibility to NA inhibitors.

3.3. Molecular models of tetrameric NA proteins

In the crystallographic structure of B/Perth/211/2001 virus NA protein, E105 is not a catalytic or framework residue comprising the enzymatic active site of NA (Fig. 1). To obtain structural insights into the effect of the E105K mutation on NA inhibitor susceptibility, we constructed 3-D models of the tetrameric NA proteins from B/Kochi/58/2011 and B/Kochi/61/2011 viruses using the homology-modeling technique (as described in Section 2). When tetrameric NA models with E or K at position 105 were superimposed, no substantial difference in the main-chain backbone of the tetrameric NA molecules was observed (data not shown). Interestingly, residue 105 was located on a surface-exposed loop (amino acids 104–113; termed the 109-loop) distant from the active site where the NA inhibitor binds (Fig. 2A). Notably, E105 formed a hydrogen bond with residue 141 from the neighboring monomer (Fig. 2B). Calculations of binding energies for the hydrogen bond indicated that the E105K mutation decreased the binding energy from −493 to −481 kcal/mol in the drug-free models of NA. These results suggest that the mutation in the monomer–monomer interface region of NA may lead to a loss in the energy of interaction between monomers and thus to the instability of the NA tetramers.

4. Discussion

In the present study, we identified a previously unrecognized amino acid change (E105K) in the NA protein of influenza B virus that affects the sensitivity to NA inhibitors. This NA mutation was detected by a combination of NA inhibitor susceptibility tests and direct sequencing of the NA gene of virus propagated in MDCK cells. However, our observations made by using pyrosequencing revealed that the variant with an E105K mutation in the NA protein was not detected in the original clinical specimens. Similar evidence has been obtained for influenza A virus, where mutations at positions 136, 224, and 371 (influenza A N2 numbering) conferring reduced susceptibility to NA inhibitors were found in virus isolates cultured in MDCK cells but not in the primary clinical specimens [29,30]. These findings indicate that variants carrying mutations associated with reduced sensitivity to NA inhibitors are preferentially selected and amplified over their wild-type viruses

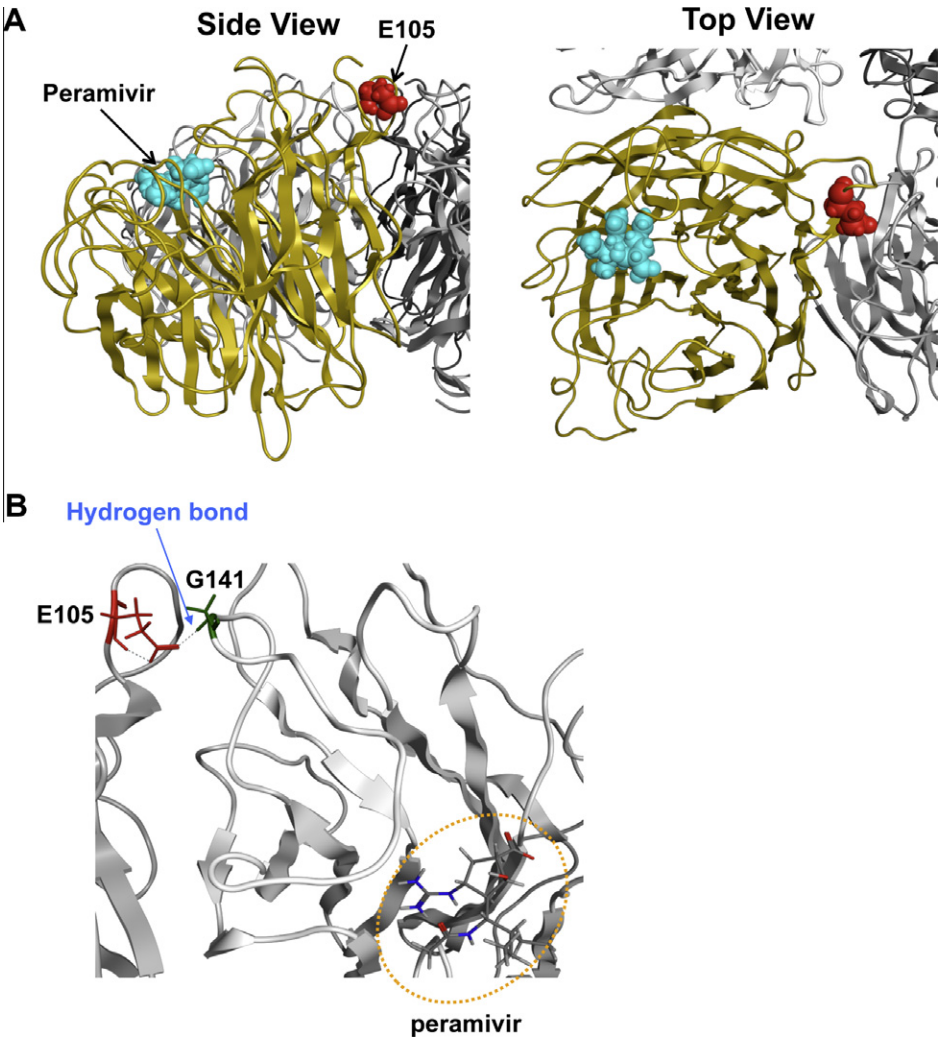

Fig. 2. Molecular models of tetrameric NA proteins with peramivir. The 3D model of the NA tetramer of the influenza B virus was constructed by homology modeling technique using Molecular Operating Environment (MOE)-Align and MOE-Homology in the MOE version 2010.10 (Chemical Computing Group Inc., Quebec, Canada). (A) Side and top views of the NA tetramer model of the B/Kochi/58/2011 virus. A monomeric NA in the tetramer with peramivir (cyan) is highlighted by a gold ribbon. An amino acid residue at position 105 is highlighted in red. (B) A close-up view of the border region of the two NA monomers in the NA tetramer model of the B/Kochi/58/2011 virus. Dotted lines indicate a hydrogen bond visualized with a MOE.

during virus propagation in MDCK cells. Detection of mutations in NA associated with an adaptation of the influenza virus for growth in cell culture could result in overestimation of the true prevalence of drug resistance in human populations. Therefore, sequencing viral genomes directly from clinical samples is critically important for the validating NA mutations identified in virus isolates cultured in vitro.

Although E105 is not a catalytic or framework residue, it is involved in forming the hydrogen bond between adjacent monomers in the NA tetramer (Fig. 2B). Importantly, the E105K substitution could weaken the interaction between monomers, suggesting that the residue has an important role in modulating stability of the tetrameric NA protein. A recent study of influenza B viruses reported that an amino acid substitution at residue 104 (equivalent to position 109 on influenza A N2) located outside the NA active site, reduces susceptibility to oseltamivir and zanamivir [31]. Interestingly, residues 104 and 105 are located in the 109-loop in the center of the NA tetramer (Fig. 1). Since a loop in a protein generally fluctuates in solution, their mutations may cause changes in interactions and fluctuations of the loop, thereby changing the stability of the tetrameric NA protein. Although we have yet to examine the role of the E105K mutation in the stability of the tetrameric NA protein, a physical change in the tetrameric NA molecule may reduce the binding affinity of the NA inhibitors. Mutations that are not located in the drug-bound cavity but cause changes in interactions with drugs at the binding site have been reported as a drug-resistant mechanism for human immunodeficiency virus type 1 protease [32–35].

On the other hand, comparison of structural models of NA tetramers with E105 or K105 does not show any substantial differences in the main-chain backbone of the NA molecules. This result does not support the potential for a mutation to critically influence folding of the NA protein so that it generates a drug-resistant structure. Instead, a more likely possibility is that the mutation primarily influences the molecular dynamics of the NA molecule. Increasing evidence indicates that fluctuation of the protein surface in solution plays a critical role in its specific functions, such as catalytic reactions of enzymes [36–40] and molecular interactions and movements [41,42]. Collectively, our 3-D models suggest that the E105K mutation of influenza B virus NA proteins occurs at a key site to modulate the structural property of NA protein interaction surfaces. These mutations may alter the stability of the NA tetramer and/or the structural property of the cavity for drug/substrate binding. Further investigations are required to define the effect of the NA protein E105K mutation on susceptibility to NA inhibitor drugs. It is noteworthy from a medical viewpoint that currently circulating influenza B viruses have the potential to acquire NA-inhibitor resistance through an E105K mutation.

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