

## Molecular Structure in Gene Mutation of Neuraminidase of Influenza Virus Type B Isolated from Swab of Patients Showing Fever Duration

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### Abstract

**Purpose** Neuraminidase inhibitors have been used for the treatment of both influenza A and B viral infections in children. Influenza viral infection occasionally shows prolonged duration of the fever despite the use of neuraminidase inhibitors, suggesting drug resistance. It has been reported that mutations of the viruses are related with the drug resistance. Here, we studied association between prolonged fever and mutations in influenza B.

**Methods** RNA was isolated from fixed nasopharyngeal swab of 206 patients with influenza in 2013/2014 season. The samples from ten patients having fever over 38°C over 48hr were analyzed for neuraminidase sequences. Structural models reflecting the gene mutation of neuraminidase of influenza B virus (BNA) were analyzed.

**Results** Patients infected with influenza B showed longer duration of fever on average than those with influenza A. Sequences of BNAs from the patients with prolonged fever were different from that of the strain for vaccine (B/Massachusetts/02/2012). Two of the sequences in the samples M2-1 and K41-1 contained mutations for potential drug resistance: S99N, T106I, K125T and S295R in the sample M2-1 and I262M, V271T, K/E272Q, E320K, D342G and M375K in the K41-1. Structural models of BNA suggested

that R295 of the M2-1 and Q272 and K375 of the K41-1 may be responsible for the drug resistance.

**Conclusions** Drug-resistant mechanism of BNA was clarified by using structural model analysis. Proper use of neuraminidase inhibitors against influenza virus infection is necessary in clinical setting.

**Key words:** Influenza virus, Type B, drug resistance, children, neuraminidase inhibitor

### Introduction

Influenza viral infection is prevalent every winter season in Japan. Influenza virus induces respiratory tract infections in various age groups, from children to elderly persons. For the treatment of influenza viral infection, neuraminidase inhibitors such as oseltamivir, zanamivir and laninamivir were developed and widely used in ambulatory settings. Neuraminidase inhibitors had the effect of shortening duration of febrile period of the patients with influenza viral infection<sup>1</sup>. On the other hand, some of the cases with influenza viral infection showed prolonged duration of the fever despite the use of oseltamivir. The percentages of these cases were much higher with influenza B viral infection than influenza A infection<sup>2,3</sup>. The precise mechanism of this phenomenon has not been clarified. In Japan, one influenza B strain with reduced drug sensitivity possessing a Gly402Ser neuraminidase substitution was isolated from a child who had received oseltamivir and seven influenza B strains with reduced sensitivity carrying other mutations were isolated from untreated children, during the 2004-2005 influenza season<sup>4</sup>. However, according to the data from patients with influenza B infection from the 5 years (2009-2013) of the international prospective Influenza Resistance Information

Study, phenotypic resistance to oseltamivir or zanamivir was not found in B/Victoria and B/Yamagata viruses<sup>5</sup>).

In the present study we collected influenza B virus genome isolated from nasopharyngeal swab of the children who had prolonged fever more than 48 hours after the treatment of neuraminidase inhibitors during the influenza B viral epidemic in 2013/2014 season. Using structural models and gene mutations of neuraminidase of influenza B virus, we analyzed reasons for prolonged fever of the patients.

## Methods

### Sampling from patients with influenza

#### 1) Isolation of swab from patients

During the influenza epidemic period from 18th February to 8th April, 2014, we collected samples from the 206 pediatric patients less than 16 year of age with influenza viral infection at four outpatient clinics in Chiba prefecture, Japan. All patients had fever and respiratory symptom and were diagnosed influenza by using rapid antigen detection kit. We gave the written consent of the study to the patients and/or their guardians. The clinicians prescribed neuraminidase inhibitor for the patient after diagnosis. Age, sex, onset date of the fever over 37.5 degree and influenza vaccination status in 2013/2014 season were investigated at the time of first visit of the clinics. The decision of additional medication except neuraminidase inhibitor for the patients depended on the clinicians. The compliance of neuraminidase inhibitor, the clinical symptom and clinical course after treatment, side effect of neuraminidase inhibitor were investigated at the time of second visit of the clinics.

#### 2) Viral typing A or B with rapid influenza antigen detection kit

Rapid influenza virus antigen detection kit (immuno-chromatography) that was used was adopted by each clinic. Nasopharyngeal swabs were used as the materials. The kit was used at the bedside and the result was informed to the patients and their guardians by clinicians. After using antigen kit, remaining materials were stocked in a refrigerator.

#### 3) Approval of Ethical Committee

This study was approved as 1749 on August 29, 2014 in Chiba University and Teirin 14-136 on Dec 4, 2014 in Teikyo University.

### Isolation of viral RNA

The swab fluid was fixed with 500 µl of a fixer (A-CLIP Institute, Chiba, Japan) and then stored at 4°C. Viral RNA was extracted from an aliquot of residual fluid from a commercial clinical diagnostic kit. Three drops of the residual fluid were put into 0.5 ml of the viral RNA retaining solution (A-CLIP Institute, Chiba, Japan). Viral RNA retaining solution was

applied to the mini column of QIAamp viral RNA mini-kit (Qiagen, Valencia, CA) as described in manufactures instruction.

### Measurement of viral typing

Viral typing was performed by M protein of influenza virus type A and hemagglutinin (HA) for type B with a real-time PCR. Precise viral typing was performed after the study period. The result of rapid antigen detection kit was confirmed by the diagnosis in PCR analysis. Type A and B subtype analysis was done basing on PCR method.

### Quantitative real-time PCR and Reverse transcription-PCR analyses

Total RNA extracted from the fixed swab stored at 4°C was treated with DNase (TURBO DNA-free; Ambion, Austin, Texas) and used as a template to synthesize complementary DNA (cDNA) with the Superscript VILO cDNA Synthesis Kit (Invitrogen). Using specific primer sets (Supplemental Table 1), the expression of each gene was quantitated with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, California) by the StepOne Real-Time PCR System (Applied Biosystems). Reaction conditions were 95°C for 15 seconds and 60°C for 1 minute, repeated for 40 cycles, with hot start at 95°C for 10 minutes. Each PCR analysis was run in duplicate for each sample.

### Gene sequences in neuraminidase region of cDNA from viral RNA:

#### Reverse transcription-PCR (RT-PCR) and sequence analysis

Isolated viral RNA neuraminidase gene was amplified by Reverse transcription-PCR (RT-PCR) using a SuperScript III One-Step RT-PCR kit (Invitrogen, Carlsbad, CA). Amplification primer sets and their covering regions were as described in Table 1 and Fig. 1, respectively.

Neuraminidase sequences of 10 viruses tested in this study were deposited into the GenBank database. Base positions of primers for neuraminidase gene are given in B/CHIBA/9/2014 (Global Initiative on Sharing Avian Influenza Data (GISAID) Isolate ID EPI\_ISL\_163854) Standard influenza virus B Yamagata and Victoria were supplied from Dr. Ogawa in Chiba Institute of Health, Chiba City.

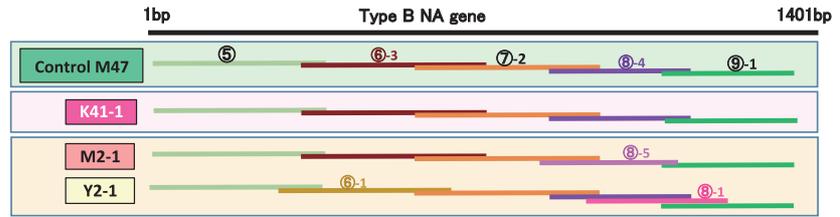
### Molecular modeling of neuraminidase region of type B influenza (BNA)

Alignment of BNA sequences was performed with Clustal W. Swiss MODEL<sup>6</sup> was implemented to model the structures of BNAs from K41-1 and M2-1 strains. Structural analysis and presentation were performed using by Swiss PDB Viewer<sup>6</sup>.

**Table 1. Primer sets for cDNA amplification**

Primer set No.	Start base of forward primer	Forward primer	End base of reverse primer	Reverse primer	Accession No
Set 5	236	AAGGGGTGACACTTCTTCTC	532	CTACTGTTGGGATTTTGCCC	CY019517.1
Set6-1	430	CATTATGCAGCCCAACCAG	769	CTGAAGCTGAGCCATCAGT	CY019517.1
Set6-3	490	GGGCAAAATCCCAACAGTAGAAA	801	ATTTCTTTTATTATTCGGCCCTCTCG	CY019517.1
Set7-2	678	CACAAGAAAGTGCTGCAA	969	TCTGGGGGTGTCCAAATAAG	CY019517.1
Set8-1	949	GTGGAGACTGATACAGCAGA	1254	CCAACCAGGTTCTTCCATTG	Non
Set8-4	864	CCATAGAATGTGCTGTAGAGA	1168	TGGGTCTCCATCATACTTTACATACAG	Non
Set8-5	832	GGAATGCACATGCGGATTTGC	1117	AGACATCGTCCGAGAGTACCA	Non
Set9-1	1100	TACTCTCGACGATGTCTAAACTAAA	1357	CCCATTAAACAGTAAATGGCTGT	Non

Fig. 1. Primer sets for BNA sequence



**Results**

**Clinical evidences**

We investigated the clinical symptoms of 206 pediatric patients with influenza virus infection and compared oseltamivir-treated, zanamivir-treated, and laninamivir-treated groups in this research period. Among 206 patients, oseltamivir-treated group, zanamivir-treated group, laninamivir-treated group was 99 patients, 83 patients, and 24 patients, respectively. Concerning the average onset of age, oseltamivir-treated group, zanamivir-treated group, laninamivir-treated group was 8.9 year of age, 9.2 year of age, and 5.3 year of age, respectively. The rate of influenza vaccination status was 45.5% in oseltamivir-treated group, 55.4% of zanamivir-treated group and 54.2% of laninamivir-treated group. The drug compliance of each neuraminidase inhibitor was good in all three groups. The duration of fever after administration of the first dose of each neuraminidase inhibitor was significantly prolonged in the patients with influenza B infection (average 43.1 hours) than in the patients with influenza A infection (average 31.8 hours), although there were no statistically significant difference in the clinical efficacy and the side effect among three groups. The number of biphasic fever episodes in patients treated with neuraminidase inhibitors was rare (two episodes of oseltamivir-treated group and one episode of zanamivir-treated group). Under the good drug compliance, all three neuraminidase inhibitors showed same efficacy for the treatment of influenza virus infection in children.

**Subtype of Type A and Type B**

Analyzed type of virus showed that 9 patients were positive with type A, including 3 patients with A/H3N2 and 6 with A/H1pdm09. In addition, 53 patients were estimated to be positive

with type B according to the results from antigen kits. Among the patients with type B, 15 patients had high fever, which was more than 38°C, for less than 48 hours, whereas 38 patients with type B had high fever for more than 48 hours. 28 of the 38 patients with prolonged fever were subject to one time sampling for real-time PCR, whereas 10 of those were subject to two times sampling. 23 of the 28 patients with one time sampling were positive with only Yamagata subtype of the B type, whereas the rest of the 28 was doubly positive with Yamagata and Victoria subtypes. 20 samples from 10 patients with two times sampling included those with 13 positive with only Yamagata subtype, 4 doubly positive and 3 negative results with real time PCR. All of the 3 negative results were from the samples at the second visits to the hospital.

**Differences in BNA sequences from that of annual vaccine**

The first swabs taken of 10 patients who had high fever over 38°C over 48hr and were subjected twice were analyzed for BNA sequences. Sequencing of three samples called K41-1, M2-1 and Y2-1 of the 10 subjects successfully completed as well as that of the M47 sample from a patient without prolonged fever. Table 2 indicates the comparison of the sequences of these samples with that of BNA of B/Massachusetts/02/2012, one of the Yamagata subtypes from which annual vaccine was produced. All the samples from the patients with prolonged and non-prolonged fever showed substitutions compared with B/Massachusetts/02/2012.

**Identification of novel mutations for drug resistance**

We identified novel mutations for drug resistance to oseltamivir and zanamivir in the K41-1 and M2-1 strains, respectively

Table 2. Amino acid substitution due to mutation of BNA gene

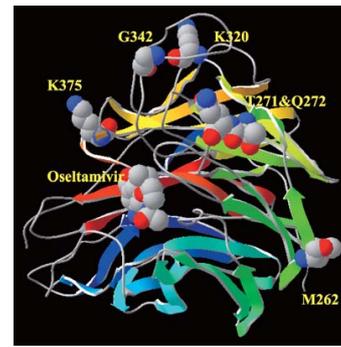
Base position	Source from Patients					AA position	Source from Patients				
	M47		K41-1	Y2-1	M2-1		M47		K41-1	Y2-1	M2-1
	Fever time: 19hr (Control) Drug:	19hr (Control) Drug:	95hr	10hr	68hr		Fever time: 19hr (Control) Drug:	19hr (Control) Drug:	95hr	10hr	68hr
296					296G/A					S99N	
317		317T/C	317T/C	317T/C		106	I106T	I106T	I106T		
374		374C/A	374C/A	374C/A		125	T125K	T125K	T125K		
443			443A/G			148		E148G			
557		557A/G		557A/G		186	K186R		K186R		
593			593G/A			198		S198N			
657			657A/T			219		K219N			
703			703G/A			235		D235N			
730			730C/T			244		P244S			
742		742G/A		742G/A		248	V248I		V248I		
786			786A/G			262		I262M			
811			811G/A			271		V271T			
812			812T/C								
814			814A/C			272		K272Q			
883		883C/A		883C/A		295	R295S		R295S		
958		958G/A		958G/A		320	E320K	E320K	E320K		
985			985A/G			329		N329D			
1018			1018G/A			340		D340N			
1025			1025A/G			342		D342G			
1027		1027A/G		1027A/G		343	K343E		K343E		
1117			1117A/G			373		K373E			
1124			1124T/A			375		M375K			
1165			1165A/G			389		T389A			
1176			1176A/T			392		E392D			
1183			1183G/A			395		A395T			
1186			1186C/T			396		L396F			

The samples, K41-1, Y2-1 and M2-1, are those from patients with prolonged fever despite the use of neuraminidase inhibitors, whereas M47 for control is from a patient without prolonged fever. Listed residues are those different from B/Massachusetts/02/2012 that is a strain of Yamagata lineage for the production of vaccine. Highlighted as blue are the residues that are same with B/wis/1/2010 that is a strain of Yamagata lineage for vaccine.

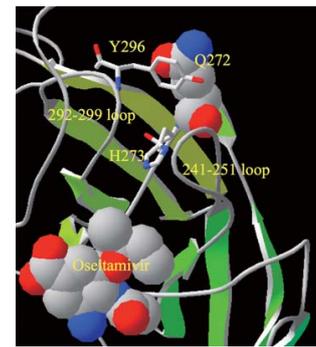
K41-1_BNA-AA Hong Kong/45/05 Memphis/20/96	-----SCPGSTFQKALLISPHRFGETKGNAPLIIREPFI 120 QAVNRSATKGVTLILLPEPEWTPYRLSCPGSTFQKALLISPHRFGETKGNAPLIIREPFI QAVNRSATKGVTLILLPEPEWTPYRLSCPGSTFQKALLISPHRFGETKGNAPLIIREPFI *****
K41-1_BNA-AA Hong Kong/45/05 Memphis/20/96	ACGPKECKHFALTHYAAQPGGYNGTRDRNKLRLHISVKLGKIPTVENSIFHMAAWSGS 180 ACGPKECKHFALTHYAAQPGGYNGTRDRNKLRLHISVKLGKIPTVENSIFHMAAWSGS ACGPKECKHFALTHYAAQPGGYNGTRDRNKLRLHISVKLGKIPTVENSIFHMAAWSGS *****
K41-1_BNA-AA Hong Kong/45/05 Memphis/20/96	ACHDGKEWTYIGVDGPDNALLKIKYGEAYDTYHSYANNILRTQESACNCGDCYLM 240 ACHDGKEWTYIGVDGPDNALLKIKYGEAYDTYHSYANNILRTQESACNCGDCYLM ACHDREWYIGVDGPDNALLKIKYGEAYDTYHSYANNILRTQESACNCGDCYLM *****
K41-1_BNA-AA Hong Kong/45/05 Memphis/20/96	TDGSASGVSECRFLKIREGRIIKEIFPTGRVHTTEECTCGFASNKTIEACARDNSYTA 300 TDGSASGVSECRFLKIREGRIIKEIFPTGRVHTTEECTCGFASNKTIEACARDNSYTA TDGSASGVSECRFLKIREGRIIKEIFPTGRVHTTEECTCGFASNKTIEACARDNSYTA *****
K41-1_BNA-AA Hong Kong/45/05 Memphis/20/96	PFVKLNVEDTAEIRLMCTETYLDTPRPDGSIITGPCESDGDKGSGGKGGFVHQRMASK 360 PFVKLNVEDTAEIRLMCTETYLDTPRPDGSIITGPCESDGDKGSGGKGGFVHQRMASK PFVKLNVEDTAEIRLMCTETYLDTPRPDGSIITGPCESDGDKGSGGKGGFVHQRMASK *****
K41-1_BNA-AA Hong Kong/45/05 Memphis/20/96	IGRWYSRTMSKTKRMGMGLYVKYDGPWDDSDALTFSGVMVSMEEPGWYSFGFEIKDKK 420 IGRWYSRTMSKTKRMGMGLYVKYDGPWDDSDALTFSGVMVSMEEPGWYSFGFEIKDKK IGRWYSRTMSKTKRMGMGLYVKYDGPWDDSDALTFSGVMVSMEEPGWYSFGFEIKDKK *****
K41-1_BNA-AA Hong Kong/45/05 Memphis/20/96	DVPCIGIEMVHDGKKTWHSAAATAYCLM----- DVPCIGIEMVHDGKKTWHSAAATAYCLMGSQGLLWDTVTGVNML DVPCIGIEMVHDGKKTWHSAAATAYCLMGSQGLLWDTVTGVNML *****

**Fig. 2. Alignment of BNA of oseltamivir-resistant and -sensitive strains**

K41-1 is an oseltamivir-resistant strain, whereas others are oseltamivir-sensitive strains of influenza B. Green and yellow shades indicate unique amino acid residues in K41-1 and the sensitive strains, respectively.



**Fig. 4. Mutation sites on K41-1 BNA**  
Model structure of BNA from K41-1 is depicted as a ribbon model. Oseltamivir molecule and mutated residues compared with oseltamivir-sensitive strains are indicated as spheres.



**Fig. 5. Enlarged view around Q272 of K41-1 BNA**  
The backbone structure of the BNA model is depicted as a ribbon model. Q272 and oseltamivir are depicted as spheres. H273 and Y296 are depicted as sticks.

M2-1_BNA-AA M47 Memphis/20/96 Hong Kong/45/05	-----LISPHRFGELKGNAPLIIREPFI 120 -----FQKALLISPHRFGELKGNAPLIIREPFI QAVNRSATKGVTLILLPEPEWTPYRLSCPGSTFQKALLISPHRFGELKGNAPLIIREPFI QAVNRSATKGVTLILLPEPEWTPYRLSCPGSTFQKALLISPHRFGELKGNAPLIIREPFI ** *****
M2-1_BNA-AA M47 Memphis/20/96 Hong Kong/45/05	ACGPKECKHFALTHYAAQPGGYNGTRDRNKLRLHISVKLGKIPTVENSIFHMAAWSGS 180 ACGPKECKHFALTHYAAQPGGYNGTRDRNKLRLHISVKLGKIPTVENSIFHMAAWSGS ACGPKECKHFALTHYAAQPGGYNGTRDRNKLRLHISVKLGKIPTVENSIFHMAAWSGS ACGPKECKHFALTHYAAQPGGYNGTRDRNKLRLHISVKLGKIPTVENSIFHMAAWSGS **** *****
M2-1_BNA-AA M47 Memphis/20/96 Hong Kong/45/05	ACHDGKEWTYIGVDGPDNALLKIKYGEAYDTYHSYANNILRTQESACNCGDCYLM 240 ACHDREWYIGVDGPDNALLKIKYGEAYDTYHSYANNILRTQESACNCGDCYLM ACHDREWYIGVDGPDNALLKIKYGEAYDTYHSYANNILRTQESACNCGDCYLM ACHDGKEWTYIGVDGPDNALLKIKYGEAYDTYHSYANNILRTQESACNCGDCYLM *****
M2-1_BNA-AA M47 Memphis/20/96 Hong Kong/45/05	TDGPASGVSECRFLKIREGRIIKEIFPTGRVHTTEECTCGFASNKTIEACARDNSYTA 300 TDGPASGVSECRFLKIREGRIIKEIFPTGRVHTTEECTCGFASNKTIEACARDNSYTA TDGSASGVSECRFLKIREGRIIKEIFPTGRVHTTEECTCGFASNKTIEACARDNSYTA TDGSASGVSECRFLKIREGRIIKEIFPTGRVHTTEECTCGFASNKTIEACARDNSYTA *** ** *****
M2-1_BNA-AA M47 Memphis/20/96 Hong Kong/45/05	PFVKLNVEDTAEIRLMCTETYLDTPRPDGSIITGPCESDGDKGSGGKGGFVHQRMASK 360 PFVKLNVEDTAEIRLMCTETYLDTPRPDGSIITGPCESDGDKGSGGKGGFVHQRMASK PFVKLNVEDTAEIRLMCTETYLDTPRPDGSIITGPCESDGDKGSGGKGGFVHQRMASK PFVKLNVEDTAEIRLMCTETYLDTPRPDGSIITGPCESDGDKGSGGKGGFVHQRMASK *****
M2-1_BNA-AA M47 Memphis/20/96 Hong Kong/45/05	IGRWYSRTMSKTKRMGMGLYVKYDGPWDDSDALTFSGVMVSMEEPGWYSFGFEIKDKK 420 IGRWYSRTMSKTKRMGMGLYVKYDGPWDDSDALTFSGVMVSMEEPGWYSFGFEIKDKK IGRWYSRTMSKTKRMGMGLYVKYDGPWDDSDALTFSGVMVSMEEPGWYSFGFEIKDKK IGRWYSRTMSKTKRMGMGLYVKYDGPWDDSDALTFSGVMVSMEEPGWYSFGFEIKDKK *****
M2-1_BNA-AA M47 Memphis/20/96 Hong Kong/45/05	DVPCIGIEMVHDGKKTWHSAAATAYCLM----- DVPCIGIEMVHDGKKTWHSAAATAYCLMGSQGLLWDTVTGVNML DVPCIGIEMVHDGKKTWHSAAATAYCLMGSQGLLWDTVTGVNML DVPCIGIEMVHDGKKTWHSAAATAYCLMGSQGLLWDTVTGVNML *****

**Fig. 3. Alignment of BNA of zanamivir-resistant and -sensitive strains**

M2-1 is a zanamivir-resistant strain, whereas others are zanamivir-sensitive strains of influenza B. Green and yellow shades indicate unique amino acid residues in M2-1 and the sensitive strains, respectively.

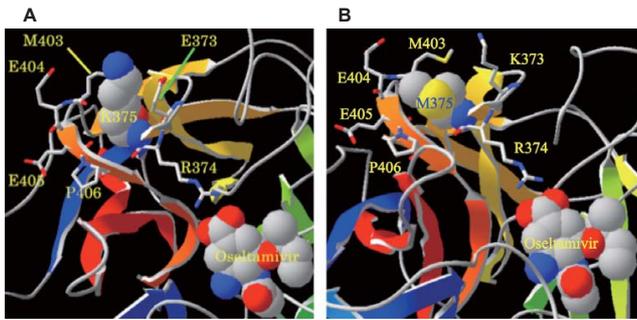
(Figs. 2 and 3). For the identification of potential mutations for drug resistance, we compared the sequences of K41-1 and M2-1 to those of drug-sensitive strains. I262M, V271T, K/E272Q, E320K, D342G and M375K are novel mutations found in K41-1. Those are potential mutations for oseltamivir-resistance. S99N, T106I, K125T and S295R are novel mutations found in M2-1, and potential mutations for zanamivir resistance.

**Modeling analysis for drug resistance**

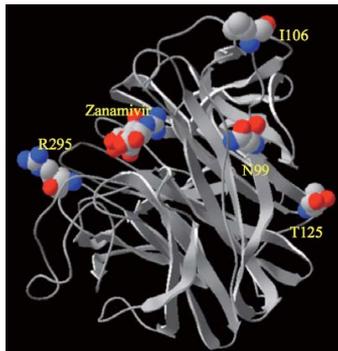
Potential sites responsible for oseltamivir resistance were mapped on a structure model of K41-1 BNA (Fig. 4). The 272nd residue is Lys or Glu in the oseltamivir-sensitive strains (Fig. 2). It has been reported that substitutions of H273 and Y296 have impacts on drug resistance<sup>7,8</sup>. Side chains of Q272, Y296 and H273 were arranged like a zipper (Fig. 5). H273, the 241-251 loop and 292-299 loop formed the binding pocket to oseltamivir. The binding pocket was scaffolded by the zipper structure of Q272, Y296 and H273. Thus, mutation at the 272nd residue may perturb the framework structure of the oseltamivir-binding pocket, which may cause the drug-resistance.

The 375th residue is Met in the oseltamivir-sensitive strains (Fig. 2). In the drug-sensitive strains, the side chain of M375 is surrounded by M403 and P406, and contributes to stabilizing the 371-373 loop (Fig. 6). In the modelled K41-1 BNA structure, the side chain of K375 interacted with E373 and was detached from M403 and P406, which may destabilize the structure of the 371-373 loop. This may cause the destabilization of the interaction between R374 and oseltamivir.

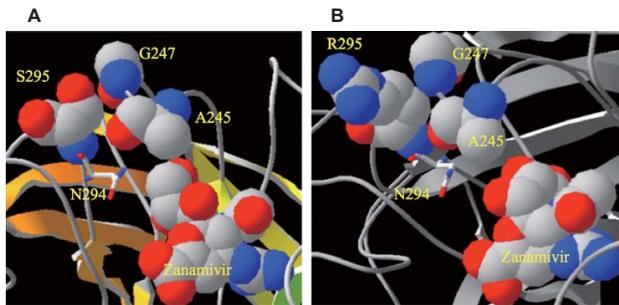
The distances between the oseltamivir-binding site and M262 and K320 are quite long. These residues are located on the surface of BNA, and have no important interaction to other residues. The 271st residue is Val or Ile in the oseltamivir-sensitive strains. Although this residue was replaced with Thr, it was unlikely that this substitution had an impact on the structure of BNA, because the structure of Val/Ile and Thr are quite similar. The 342nd residue is Asp in the oseltamivir-sensitive strains, whereas it was Gly in K41-1. It was unlikely that this substitution had an impact on the structure and function



**Fig. 6. Enlarged views around K375/M375**  
 A. View around K375 of the K41-1 strain. K375 and oseltamivir are indicated as spheres. K373, R374, M403, E404, E405 and P406 are depicted as sticks. B. View around M375 of oseltamivir-sensitive strain.



**Fig. 7. Mutation sites on BNA as to zanamivir-resistance**  
 The M2-1 BNA model is depicted as a ribbon model. Zanamivir and mutated residues in M2-1 BNA compared with zanamivir-sensitive strains are depicted as spheres.



**Fig. 8. Enlarged views around S295/R295 of BNA**  
 A View around S295 of zanamivir-sensitive strain, B Corresponding view around R295 of the M2-1 strain.

of BNA, because this residue had no side chain contact to other residues.

Potential sites responsible for zanamivir-resistance, N99, I106, T125, and R295, were mapped on a structure model of M2-1 BNA (Fig. 7). R295 was located next to N294 that composed the framework of the substrate binding pocket (Fig. 8). In zanamivir-sensitive strains, the 295th residue is Ser, which interact with G247 and A245, contributing to stabilization of the structure around N294. Replacing S295 with Arg caused loss of these interactions, which may impair the stability of the binding pocket of BNA. N99, I106, and T125 were far from the zanamivir-binding site, so were unlikely to have impacts on the inhibitor-binding.

**Discussion**

**Clinical observations**

According to the past studies, the febrile period of influenza

B viral infection was longer than of influenza A viral infection after oseltamivir treatment<sup>2,3</sup>). In the present study, the average febrile period after treatment of neuraminidase inhibitor of influenza B viral infection was significantly longer than influenza A viral infection. The rate of febrile period >48 hours after treatment of influenza B viral infection was also higher than influenza A viral infection. Except for the A/B type, the other characteristics of the subjects including age distribution, vaccination history and secondary bacterial infection did not influence prolonged duration of the fever. Neuraminidase inhibitor-resistant influenza B viruses were rare. The drug-resistant influenza B viruses were isolated from immunocompromised children with prolonged treatment of neuraminidase inhibitor<sup>9,10</sup>). However, Hatakeyama *et al.* reported that oseltamivir- and zanamivir-resistant influenza B viruses were isolated from both ordinary neuraminidase inhibitor-treated and non-treated children in Japan<sup>4</sup>). So far, the nineteen mutations of influenza B viruses related to resistance to neuraminidase inhibitors were reported<sup>7</sup>). However, the precise mechanism and clinical impact of resistance of influenza B viruses to neuraminidase inhibitors were not clarified.

**Structure change of neuraminidase gene of influenza type B isolated at clinics in Chiba Prefecture: Drug resistance analyzed in modeling of the neuraminidase with novel mutations**

We performed extensive sequence analysis of BNAs of both oseltamivir-resistant and zanamivir-resistant samples with prolonged fever. The analyzed sequences indicated numerous substitutions in comparison with BNA of the Yamagata type for the production of vaccine. In addition, the sequences showed novel mutations in both oseltamivir-resistant and zanamivir-resistant BNAs. We built structural models of mutant BNAs to analyze impacts of the mutations on drug resistance, and found that two mutations of the oseltamivir-resistant strain, K/E272Q and M375K, and one mutation of the zanamivir-resistant strain, S295R, may be responsible for drug resistance. All these mutations were located on the framework of the substrate-binding pocket of BNA, but lacked direct interaction with the bound drugs. The analysis suggested possibilities that these mutations may destabilize the structure of the framework, which may lead to weak binding of BNA to the drugs.

E119V, R292K, H274Y and N294S of neuraminidase of influenza A (ANA) were reported previously as drug resistance mutations. E119, R292, H274 and N294 of ANA correspond to E117, R292, H273 and N294 of BNA. E119 and R292 are direct binding residues to the drugs including oseltamivir and zanamivir. In the mutated protein with either E119V or R292K, direct binding between ANA and the drugs were predicted to be lost, leading to loss of inhibition by the drugs<sup>11</sup>). In contrast, H274 and N294 are framework residues, which indicate that mutations in the framework can cause drug resistance. Several possible mechanisms for the drug resistance with the H274Y and N294S mutations have been proposed based on simulation studies for ANA. According to these studies, it may be difficult to generalize the resistance mechanisms of H274Y and N294S.

The complex structure of ANA with H274Y-oseltamivir (PDB code: 3CL0) revealed that the bulkier side chain of Y274 presses out that of E276 towards the substrate-binding pocket in relative to WT<sup>12</sup>). Thus, it was proposed that such a structural

change hampers the interaction with oseltamivir. A computational simulation study proposed that infiltration of water molecules into the substrate-binding pocket causes the drug resistance of the H274Y mutant<sup>13</sup>). The other group proposed that the mutation on either H274 or N294 impairs binding to the drugs because H274 and N294 are located on the charged binding funnel<sup>14</sup>).

Another study based on simulation indicated that the mutations of H274Y and N294S shifted the locations of E276, E277 and R292 and proposed that such structural changes have impacts on interaction with oseltamivir and zanamivir<sup>15</sup>). E277 and R292 are the residues that directly interact with the drugs, whereas E276 is one of the framework residues. In the simulation of the N294S mutant by Ripoll *et al.*, a hydrogen bond between S294 and E276 was formed and the main chain carbonyl of Y347 was flipped. As a consequence, Y347 interacted with the main chain carbonyl of R292. These had an impact on the dynamics of R292 that directly bound to zanamivir and oseltamivir, which may cause drug-resistance. The other simulation study proposed that the hydrogen bonds between R118 and the drugs are weakened in the N294S mutant<sup>11</sup>).

As discussed above, several different hypotheses for the mechanism of drug-resistance by the framework mutations including H274Y and N294S of ANA have been proposed thus far. However, it is commonly presumed that the mutations have impacts on the subtle internal structure and dynamics of ANA, which cause the resistance. Thus, it is likely that the framework mutations reported here including K/E272Q, M375K and S295R may have impacts on the structure and/or dynamics of the framework.

This study has a limitation. In this study, we determined the mutation sites and the suggestive resistant mechanism from the analysis of molecular modeling of neuraminidase structure from influenza B virus RNA obtained from patient. However, to clarify the relationship to the mutation to the neuraminidase inhibitors resistance more precisely, further studies such as the determination of the rise of IC<sub>50</sub> level against neuraminidase inhibitors using the virus possessing the mutations are required. Therefore, in the future study, it is better to isolate not only the RNA but also the virus from the patient sample for the analysis of IC<sub>50</sub> level against neuraminidase inhibitors.

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### Disclosure

The authors have no financial conflict of interest.

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