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ORIGINAL ARTICLE

Mutations at the monomer–monomer interface away from the active site of influenza B virus neuraminidase reduces susceptibility to neuraminidase inhibitor drugs

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Abstract Amino acid changes in or near the active site of neuraminidase (NA) in influenza viruses reduce the susceptibility to NA inhibitor drugs. Here, we report the recovery of three influenza B viruses with reduced susceptibilities to NA inhibitors from human patients with no history of antiviral drug treatment. The three viruses were isolated by inoculating Madin–Darby canine kidney (MDCK) cells with respiratory specimens from the patients. NA inhibition assays demonstrated that two of the three isolates showed a highly reduced susceptibility to peramivir and moderately reduced susceptibility to oseltamivir, zanamivir, and laninamivir. The remaining one isolate exhibited moderately reduced sensitivity to peramivir, zanamivir, and laninamivir but was susceptible to oseltamivir. A sequence analysis of viruses propagated in MDCK cells revealed that all three isolates contained a single mutation (Q138R, P139S, or G140R) in NA not previously associated with reduced susceptibility to NA inhibitors. However, pyrosequencing analyses showed that the Q138R and G140R mutations were below a detectable level in the original clinical specimens; the P139S mutation was detected at a very low level, suggesting that the mutant viruses may be preferably selected during propagation in MDCK cells. The NA crystallographic structure showed that these mutations were located at the interface between the two monomers of the NA tetramer, away from the NA active site. In addition to amino acid substitutions around the active site of NA, these observations suggest that alterations in the monomer–monomer interface region of NA may contribute to reduced sensitivity to NA inhibitors.

Keywords Influenza B virus · Neuraminidase-inhibitor resistant · Novel substitutions in · Neuraminidase protein

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Introduction

Influenza A and B viruses are enveloped negative-strand RNA viruses, which bud from the plasma membranes of infected cells. Influenza virus particles contain neuraminidase (NA) glycoproteins, which exist as a tetramer composed of four identical subunits on the viral envelope. During viral replication, NA cleaves sialic acids from cell-surface receptors to facilitate the release of newly synthesized viral particles from infected cells [1, 2]. NA is a major target of anti-influenza virus drugs.

Neuraminidase inhibitor drugs that block the sialidase activity of NA are currently commercially available for the treatment and prophylaxis of influenza A and B viral infections. These drugs function by competitively binding (against sialic acids) to the enzymatic active site of NA, thereby preventing efficient virus release from cells [3–5]. These drugs were designed to target the active site, which is highly conserved in the NA proteins of influenza A and B viruses. Therefore, amino acid changes in and around the catalytic or framework residues that form the active site reduce the affinity for NA inhibitor drugs, thereby leading

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to the development of drug resistance [6, 7]. Recently, it was reported that influenza B viruses with a single substitution located away from the active site of NA protein exhibited decreased sensitivity to NA inhibitors [8, 9]. In this study, we report the recovery of influenza B virus variants with reduced drug sensitivity that carry a previously unrecognized substitution, which was not located in or near the active site. Our study showed that three consecutive substitutions in the subunit interface of tetrameric NA reduced the susceptibility of influenza B viruses to NA inhibitors.

Materials and methods

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. The cells were maintained at 37 °C in 5 % CO_2.

Specimens and viruses

All clinical specimens were collected from patients with influenza-like illness under the National Epidemiological Surveillance of Infectious Diseases in Japan. Boys who were 6 years, 8 years, and 1 year old visited a clinic in Kochi Prefecture, Japan in 2011 with an acute respiratory illness on March 18, April 28, and May 5, respectively. Respiratory specimens were taken from the patients before drug treatment for influenza and submitted to the Kochi Public Health and Sanitation Institute in Japan for virus isolation by inoculation with MDCK cell cultures. The isolated viruses were then sent to the National Institute of Infectious Diseases (NIID) and further propagated in MDCK cells for use in NA inhibition assays. B/Perth/211/2001 viruses with either aspartic acid or glutamic acid at position 197 in the NA protein were used as NA inhibitor-sensitive or inhibitor-resistant controls, respectively; these were provided by the International Society for Influenza and other Respiratory Virus Diseases (ISIRV) [10].

Neuraminidase inhibitor susceptibility assays

Oseltamivir carboxylate, peramivir, zanamivir, and laninamivir were provided by F. Hoffmann-La Roche (Basel, Switzerland), Shionogi (Osaka, Japan), GlaxoSmithKline (Middlesex, UK), and Daiichi Sankyo (Tokyo, Japan), respectively. The NA inhibitor susceptibility assay was performed using an NA-XTD Influenza Neuraminidase Assay Kit (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturer's protocol. The NA-XTD kit uses a chemiluminescence-based assay with a NA-XTD substrate. Chemiluminescence was detected using an LB940 plate reader (Berthhold Technologies). The drug concentrations required for 50 % inhibition of NA activity (IC_{50}) were calculated using MikroWin 2000 software (Mikrotek Laborsysteme, Overath, Germany), as described previously [11]. Criteria defining the antiviral susceptibility of viruses based on the fold change of their IC_{50} value compared to reference IC_{50} values were set by the WHO working group on surveillance of influenza antiviral susceptibility [for influenza B virus, use of normal (<5-fold increase), reduced (5- to 50-fold increase), and highly reduced (>50-fold increase) inhibition is recommended] [12].

Pyrosequencing

Viral RNAs were extracted from 140-µl samples 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 mutations in residues 138, 139, and 140 of NA. This region was subjected to reverse transcription polymerase chain reaction (RT-PCR) using a SuperScript III One-Step RT-PCR System (Invitrogen, Carlsbad, CA, USA). The primers used 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, Mannheim, Germany), and the products were subjected to emulsion PCR and sequencing with a GS Junior 454 instrument (Roche), according to the manufacturer's instructions. Pyrosequencing analyses were performed using a GS Junior 454 sequencing instrument (Roche) and a GS Amplicon Variant Analyzer (Roche).

Results and discussion

Respiratory specimens were collected from three patients before drug treatment during the 2010–2011 influenza season in Japan. Influenza B viruses (B/Kochi/41/2011, B/Kochi/59/2011, and B/Kochi/60/2011) were isolated from each sample by inoculating with MDCK cells, and their susceptibilities to the NA inhibitors oseltamivir, peramivir, zanamivir, and laninamivir (these four drugs are currently available in Japan for clinical use) were determined by a chemiluminescent NA inhibition assay using NA-XTD substrate. The IC_{50} values of the isolates were expressed as the fold increase in the IC_{50} value compared with that of a drug-susceptible reference virus, i.e., the B/Perth/211/2011 virus [10]. The NA inhibition assays