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ORIGINAL ARTICLE

A nonsynonymous SNP in human cytosolic sialidase in a small Asian population results in reduced enzyme activity: potential link with severe adverse reactions to oseltamivir

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The use of oseltamivir, widely stockpiled as one of the drugs for use in a possible avian influenza pandemic, has been reported to be associated with neuropsychiatric disorders and severe skin reactions, primarily in Japan. Here we identified a nonsynonymous SNP (single nucleotide polymorphism) in dbSNP database, R41Q, near the enzymatic active site of human cytosolic sialidase, a homologue of virus neuraminidase that is the target of oseltamivir. This SNP occurred in 9.29% of Asian population and none of European and African American population. Our structural analyses and *K*i measurements using *in vitro* sialidase assays indicated that this SNP could increase the unintended binding affinity of human sialidase to oseltamivir carboxylate, the active form of oseltamivir, thus reducing sialidase activity. In addition, this SNP itself results in an enzyme with an intrinsically lower sialidase activity, as shown by its increased *K*m and decreased *V*max values. Theoretically administration of oseltamivir to people with this SNP might further reduce their sialidase activity. We note the similarity between the reported neuropsychiatric side effects of oseltamivir and the known symptoms of human sialidase-related disorders. We propose that this Asian-enriched sialidase variation caused by the SNP, likely in homozygous form, may be associated with certain severe adverse reactions to oseltamivir.

***Keywords*:** Asia, SNP, neuraminidase inhibitor, oseltamivir, sialidase, bioinformatics

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Introduction

Influenza neuraminidase (NA) has been one of the most attractive drug targets against influenza [1-4] and numerous efforts have been made on the design of NA inhibitors [5-6]. Two effective neuraminidase inhibitors, oseltamivir phosphate (Tamiflu, Roche) and zanamivir (Relenza, Glaxo), have been marketed; and peramivir (BioCryst) has progressed to advanced clinical development. Among them Tamiflu is the only orally applicable drug so far and is currently considered to be the appropriate drug against the human influenza virus H5N1 infections [7]. Many countries have stockpiled the drug in preparation for a possible pandemic [8]. However, over a thousand adverse events have been reported to occur with its use during seasonal influenza, including a small number of rare but severe adverse events [9]. Seventy-five pediatric cases have been studied in detail, including eight fatalities, 32 neuropsychiatric events, and 12 skin/hypersensitivity events [9]. Interestingly, 69 of the 75 cases were identified in Japan, the country that

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has the highest use of oseltamivir in the world. Although whether Tamiflu directly caused these cases is still being debated, the possible severe side effects of Tamiflu should not be ignored.

Oseltamivir is processed in the liver by esterases into the active form oseltamivir carboxylate, which can inhibit the neuraminidase of influenza virus with high affinity [10-13]. Oseltamivir carboxylate resulted from two successful principles in the history of NA inhibitor design. The first is to substitute the C6 group with a more hydrophobic group that would pack well in the hydrophobic pocket created by the rotation of Glu276's carboxylate group which forms a salt bridge with Arg224. The second is to replace the C4 group of Neu5Ac2en, a transition state analogue, with a basic group that would interact with Glu119 and Glu227 of the virus neuraminidase [10-13].

Human has four sialidases that are homologues of virus neuraminidases. The structure of human cytosolic sialidase (HsNEU2) was published recently [14] and shown to be similar to that of viral neuraminidases, containing exactly the same active site residues. It seemed reasonable to investigate whether human sialidases could also bind oseltamivir carboxylate, thus potentially causing adverse reactions.

Searching the dbSNP database at NCBI [15], we found that a nonsynonymous SNP from G to A (refSNP ID rs2233385) causes Arg41 in HsNEU2 to be substituted by Gln in a small portion of Asian populations. Here we present results from structural bioinformatic analyses and *in vitro* enzymatic activity assays showing that the R41Q SNP could increase human sialidase's binding affinity to oseltamivir carboxylate, thus rendering it more sensitive to inhibition by the drug. In addition, the SNP itself results in an enzyme with an intrinsically lower sialidase activity. We suggest that this sialidase variation caused by the SNP, likely in homozygous form, may be associated with certain severe adverse reactions to Tamiflu.

Materials and Methods

Searching for the neuraminidase active site in all known human protein structures

We used the active site residues in influenza virus neuraminidase N9, Arg118, Arg371, Arg292, and Tyr406, and their sequential and spatial spacing to generate a template of the active site. We used this template to search all known human protein structures in Protein Data Bank (PDB). Eleven of the structures contain the same four residues in the correct order in sequence and less than 2.0 angstroms of Root Mean Standard Deviation (RMSD) from the template in space. Manual inspection of docking analyses using these structures with sialic acid and oseltamivir carboxylate showed that only HsNEU2 had the correct binding pocket.

Molecular simulation

We retrieved and analyzed the following structures from PDB: human sialidase HsNEU2 (PDB ID:1VCU), influenza virus neuraminidase N2 (PDB ID:2BAT), and influenza virus neuraminidase N9 complexed with oseltamivir carboxylate (PDB ID:2QWK). The structure of HsNEU2 R41Q variant was built by substituting the side chain of Arg41 to Gln. The complex structures of wild-type HsNEU2 and HsNEU2 R41Q with oseltamivir carboxylate were built by superimposing HsNEU2's active site residues Arg21, Arg304, Arg237 and Tyr334 to N9's active site residues Arg118, Arg371, Arg292 and Tyr406, respectively. The complex structures of N2 with oseltamivir carboxylate were built by the same procedure. All of these complexes were optimised in Insight II (Accelrys, San Diego, USA): after adding a two-Angstrom water layer and fixing the protein backbone atoms, we performed 500 steps of steepest descent with derivative value of 0.01 $kcal \cdot mol^{-1} \cdot A^{-1}$ and 1 000 steps of conjugate gradient under Consistent Valence Force Field (CVFF) with derivative value of 0.001 $kcal \cdot mol^{-1} \cdot A^{-1}$. We evaluated different aspects of the binding affinity between oseltamivir carboxylate and HsNEU2 in these complexes, including intermolecular energy under CVFF (van der Waals term and electrostatic term) and inhibition constant derived by Insight II. The cutoff for intermolecular energy calculation under CVFF was 100 Angstroms. The scoring function in Insight II was an empirical function taking into account the contribution of hydrogen bonds, ionic interactions, hydrophobic contacts, freezing of internal degrees of freedom and aromatic-aromatic interactions.

Expression of HsNEU2 wild-type and its R41Q variant proteins in E. coli

We expressed and purified both wild-type HsNEU2 and its R41Q variant by following the standard protocol [16-17]. The full-length coding region of *HsNEU2* was obtained by PCR amplification using an isolated human genomic library as the template. Exon1 was amplified using HsNEU2-Nt1 (5'-CGG AAT TCA TGG CGT CCC TTC CTG TCC TG-3') with a 5'-*Eco*R I restriction site and HsNEU2-Ct1 (5'-CTG AAC CTG GTG GGT GGG TGC-3'). Exon2 from Trp68 to the stop codon was amplified using HsNEU2-Nt2 (5'-TGG CAA GCT CAG GAG GTG GTG-3') and HsNEU2-Ct2 (5'-ACG CGT CGA CTC ACT GAG GCA GGT ACT-3') with a 5'-*Sal* I restriction site. The two PCR products were ligated by T4 DNA ligase and the resulting full-length fragment was subcloned into the *Eco*R I – *Sal* I sites of pET-28a expression vector. The HsNEU2 variant, R41Q, was generated by mutagenesis through PCR amplification using the overlap extension technique [18], with two PCR primers R41Q-M1 (5'-CGG AAC AGC GAG CAA GCA AGA-3') and R41Q-M2 (5'-TCT TGC TTG CTC GCT GTT CCG-3'). The resulting recombinant plasmids were verified by DNA sequencing, followed by transformation into the *E. coli* expression strain Rosetta (DE3). Both HsNEU2 wild-type and its R41Q variant proteins were purified by a two-step purification procedure using a Ni-affinity column and a gel filtration Hiload Superdex-75 column (Amersham, USA) [19]. They showed apparent molecular mass of about 45 kDa in SDS-PAGE, which was consistent with the predicted molecular weight. For both the wild-type and R41Q variant, about 3 mg of proteins in high purity were obtained from 1 L of culture for further experiments.

Sialidase assay

Sialidase activities of both recombinant wild-type HsNEU2 and its R41Q variant against the substrate 2'-(4-methylumbelliferyl)α-D-N-acetylneuraminic acid (MU-NANA) (Sigma) were determined by fluorimetric assays to measure the amount of 4-methylumbelloferone

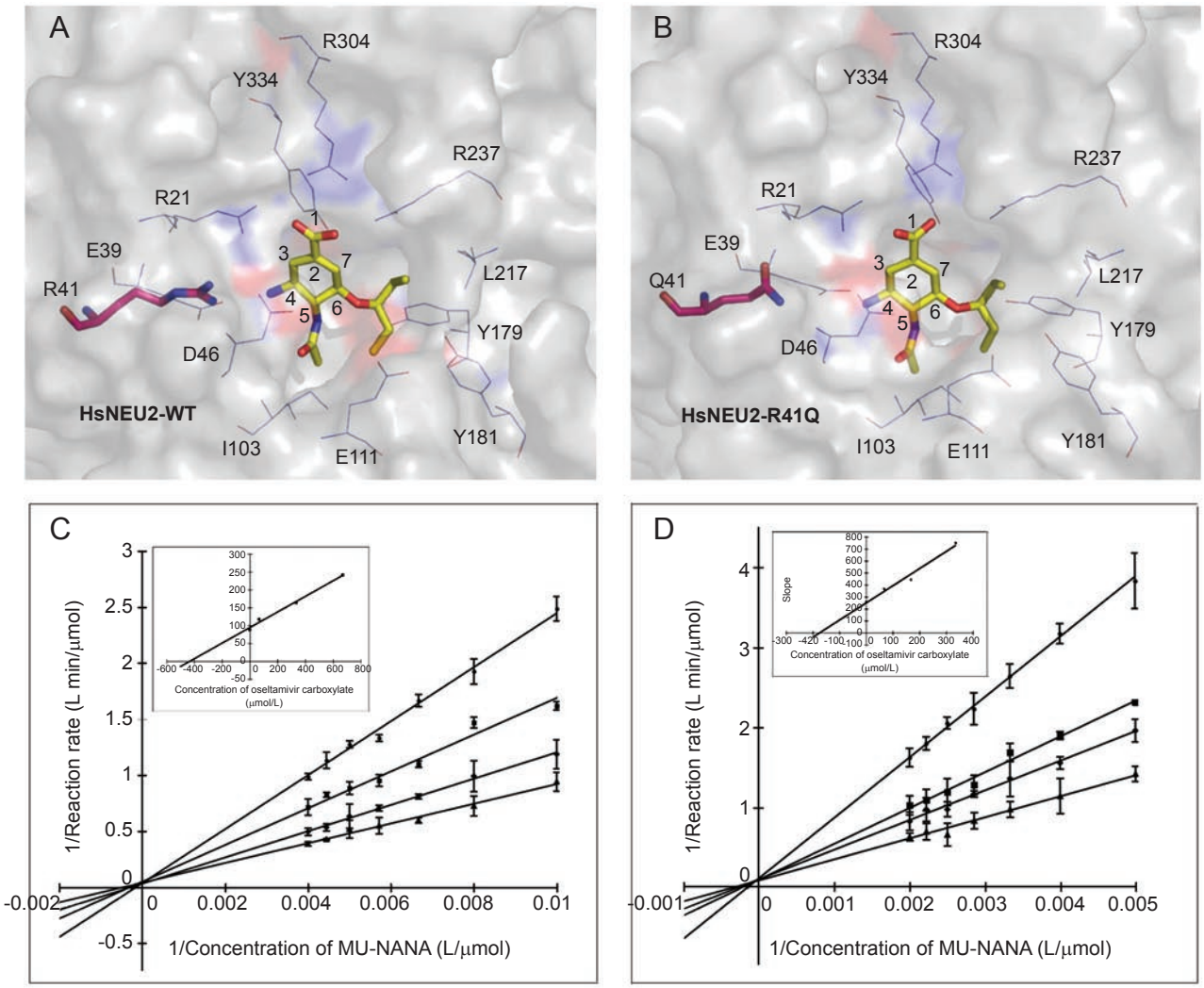

Figure 1 An SNP causing R41Q substitution in HsNEU2, occurring primarily in Asian population, could significantly reduce sialidase activity. **(A, B)** Binding of oseltamivir carboxylate to HsNEU2 wild-type (HsNEU2-WT) **(A)** and HsNEU2 R41Q variant (HsNEU2-R41Q) **(B)**. The structures of HsNEU2 and oseltamivir carboxylate were retrieved from PDB entries 1VCU and 2QWK, respectively. The complex structures were optimized by Insight II. Residues close to the active site were shown as lines while SNP-related residues (Arg in HsNEU2-WT and Gln in HsNEU2-R41Q) were hightlighted as sticks. Carbon numbering of oseltamivir carboxylate followed that of the native ligand. **(C, D)** Oseltamivir carboxylate is a competitive inhibitor for HsNEU2. The *K*i value was 0.175 mM for HsNEU2 R41Q and 0.432 mM for HsNEU2 wild-type, suggesting that HsNEU2-R41Q was more sensitive to oseltamivir carboxylate. In the absence of oseltamivir carboxylate, compared with wild-type HsNEU2, R41Q variation caused *K*m to increase from 2.136 mM to 2.795 mM, and *V*max to decrease from 24.272 μmol/L·min to 10.846 μmol/L·min. Data were presented as mean±SD (n=6 independent experiments).

Table 1 Results of sialidase assays

	*K*m (m mol/L)	*V*max (μmol/L min)	*V*max/*K*m	*K*i (m mol/L)
HsNEU2-WT	2.136	24.272	11.363	0.432
HsNEU2-R41Q	2.795	10.846	3.879	0.175
HsNEU2-R41Q/WT	1.308	0.447	0.341	0.405

cleaved by HsNEU2 from the fluorogenic substrate MU-NANA as described by Venerando *et al*., in the absence or presence of oseltamivir carboxylate, respectively [20]. The reactions were set up in 33 mM MES buffer pH 6.5 containing 4 mM $CaCl_2$. Different amounts of substrate were mixed with 1 μg of wild-type HsNEU2 or HsNEU2 R41Q in the reaction mixture with a final volume of 100 μL. After incubation at 37 °C for 15 min, 300 μL of stop buffer (0.042 M Na_2CO_3, 0.133 M glycin pH 10.0, 0.06 M NaCl) was added to stop the reaction. The fluorescence emission was measured on Synergy HT (BioTek) with excitation at 360 nm and emission at 460 nm. The calibration curve was plotted using different amounts of 4-methylumbelliferyl (4-MU) (Sigma), and 1 nmol of 4-methylumbelliferyl was equal to 6 378 fluorescence units. *K*m and *V*max were determined by the method of Lineweaver and Burk [21].

Sialidase inhibition assay

Sialidase inhibition assay by oseltamivir carboxylate obtained by saponification of the oseltamivir phosphate [22] was carried out to determine the *K*i values following the same sialidase assay procedure but with pre-incubation of wild-type HsNEU2 and HsNEU2 R41Q with oseltamivir carboxylate before adding the MU-NANA substrate. The concentration of oseltamivir carboxylate versus the reaction rate was plotted, and the *K*i values were determined by the method of Lineweaver and Burk [21].

Results

Close inspection of molecular docking of the HsNEU2 structure and oseltamivir carboxylate showed that HsNEU2 could bind oseltamivir carboxylate but the binding is weakened by the basic side chain of Arg41 in HsNEU2 that could have a repulsive effect on the basic C4 group in oseltamivir carboxylate (Figure 1A). The nonsynonymous SNP from G to A (refSNP ID rs2233385 in dbSNP database at NCBI [15]) causes Arg41 to be substituted by Gln in HsNEU2. The uncharged and shorter side chain of Gln41 would not have the same repulsive effect on the C4 basic group in oseltamivir carboxylate. On the contrary, nearby acidic residues Glu39 and Asp46 might now be able to create a negatively charged pocket to potentially form a stronger interaction with oseltamivir carboxylate. Molecular docking simulation between oseltamivir carboxylate and HsNEU2 wild-type vs. R41Q variant showed enhanced binding affinities between oseltamivir carboxylate and the R41Q variant (Figure 1B).

Sialidase assays with the MU-NANA substrate confirmed the docking prediction. The results of the *K*i measurements showed that oseltamivir carboxylate was a competitive inhibitor for HsNEU2 (Figure 1C and 1D). The *K*i constant was 0.175 mM for the HsNEU2 R41Q variant, 2.2 times lower than the *K*i constant for HsNEU2 wild-type (0.432 mM). Thus the R41Q variant was more sensitive to inhibition by oseltamivir carboxylate than the wide-type was (Figure 1C and 1D). We also compared the sialidase activity of the wide-type and variant enzymes towards the MU-NANA substrate in the absence of oseltamivir carboxylate. The *Km and V*max of wild-type HsNEU2 were 2.136 mM and 24.272 μmol/L·min, respectively, whereas they were 2.795 mM and 10.846 μmol/L·min for the R41Q variant, respectively (Figure 1C and 1D; Table 1). The *V*max/*K*m ratio was 11.363 for wild-type HsNEU2, and 3.879 for the R41Q variant. This result suggested that the activity of the R41Q variant was about three times lower than that of HsNEU2. Taken together, the HsNEU2 R41Q variant had reduced sialidase activity compared to the wild-type enzyme in the presence of oseltamivir carboxylate.

Discussion

Variations in human sialidase activity have long been implicated in serious diseases and symptoms including neuropsychiatric problems such as seizing and convulsions and skin problems [23-26]. Some of the symptoms coincide with several reported side effects of Tamiflu. People with the HsNEU2 R41Q variation might already have dampened sialidase activity, and because this variation also increases the enzyme's sensitivity to oseltamivir carboxylate, administration of oseltamivir could further reduce sialidase activity and cause symptoms similar to those in well-known sialidase-related disorders. We suspect that only homozygotes of this SNP might show adverse oseltamivir reactions because heterozygotes could presumably compensate by the other wide-type allele. Furthermore, most of the known sialidase-related disorders were discovered in people with homozygous mutant sialidase alleles, and the total number of reports of severe adverse reactions that occur with administration of Tamiflu was small.

Our hypothesis was supported by results from structural bioinformatic analyses and *in vitro* enzymatic activity assays. The difference in sialidase activity between wild-type HsNEU2 and the R41Q variant in the presence of oseltamivir carboxylate is likely to hold *in vivo* as well at least partly. Oseltamivir carboxylate is a competitive inhibitor of HsNEU2 (Figure 1C and 1D). Although the plasma concentration of oseltamivir carboxylate is known to be only up to 1.2 μM after an oral dose of 75 mg capsule twice-daily (http://www.rocheusa.com/products/tamiflu/), the concentration in local tissues and cells (in some individuals) may be much higher. Indeed there is evidence from a recent study that administration of oseltamivir could inhibit wild-type human sialidase *in vivo* [28].

We further observed that, based on the data in dbSNP, the frequency of the minor allele (A) of this SNP was 9.29% in Asian, 0.55% in Sub-Saharan African, and none in European and African American. This differential population distribution might offer a clue in explaining why most cases of severe neuropsychiatric side effects of oseltamivir

were reported in Japan and few in America or European countries [27]. However, this circumstantial evidence is confounded by the much higher per capita oseltamivir use in Japan compared to other countries. Further testing of our hypothesis would require population genetic studies to determine whether homozygotes of the minor allele (A) of this SNP were significantly enriched in cases reporting severe adverse reactions to oseltamivir. Because of the relatively low prevalence of the SNP, the population genetic tests would require a large number of cases for statistical significance. We also suggest further genotyping and enzyme assay of all four sialidases to discover any additional SNPs that may be relevant.

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