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Oseltamivir-resistant influenza viruses get by with a little help from permissive mutations

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Abstract

Influenza A viruses (IAVs) encode two critical glycoproteins, hemagglutinin and neuraminidase (NA). Hemagglutinin promotes viral docking onto cells via interactions with IAV's receptor, sialic acid and NA facilitates release of newly synthesized virions by cleaving cellular and viral sialic acid. NA inhibitors, such as oseltamivir, are widely used drugs that work by binding to the active site of NA. Although oseltamivir-resistant viruses were easily generated years ago in laboratory experiments, it was widely believed that these viruses would not be able to circulate in the human population as they did not replicate efficiently. However, oseltamivir-resistant H1N1 viruses rapidly spread during the 2007–2008 IAV season and these viruses contained precisely the same exact drug-resistance mutation identified years prior, a histidine to tyrosine substitution at NA residue 274 (H274Y). Unlike the experimentally derived NA inhibitor-resistant viruses, 2007–2008 H1N1 viruses containing H274Y replicated efficiently. Bloom *et al.* have solved this riddle by identifying permissive NA mutations that allow viruses to tolerate H274Y. Here, we discuss these important findings and speculate how these studies may facilitate early detection of drug-resistant strains in the future.

Keywords

influenza virus; neuraminidase; neuraminidase inhibitors; oseltamivir

Influenza A viruses (IAVs) must balance critical steps of the viral lifecycle to ensure proper viral fitness. IAVs initially attach to cells through specific interactions between the viral glycoprotein, hemagglutinin (HA) and cellular sialic acid. A second viral glycoprotein, neuraminidase (NA), promotes release of newly synthesized virions by enzymatically cleaving cellular and viral sialic acid. Mutations that subtly affect HA or NA functions can drastically impair viral replication. Viruses that bind to sialic acid too weakly cannot efficiently attach to cells, while viruses that bind to sialic acid too avidly are inefficiently released towards the later parts of viral replication. Similarly, viruses possessing a superactive NA do not attach to cells efficiently, while viruses that possess a crippled NA are not efficiently released. HA and NA activity are never 'turned off' during virus replication and so it is imperative that IAVs maintain the correct amount of HA and NA activity.

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Maintenance of HA and NA activity can be a difficult task since IAVs are constantly accumulating mutations in antigenic sites of these molecules, a process termed antigenic drift [1]. Indeed, many mutations in antigenic sites of HA alter viral attachment and viruses containing these mutations must acquire secondary mutations to maintain optimal viral fitness [2]. Antigenic change occurs less frequently in NA compared with HA [3], but many of these mutations most likely also have some functional effects on NA enzymatic activity.

One of the best anti-IAV therapies is based on the idea of disrupting the HA/NA balance. NA inhibitors (NAIs), such as oseltamivir, bind in the active site of NA and prevent NA enzymatic activity [4]. When viruses are grown in the presence of NAIs, they are not efficiently released from cells, they aggregate with one another and stick to other sialic acid-containing material in the airways such as mucus [5-7]. NAIs are widely prescribed for IAV infection and are very effective in limiting viral spread.

NAI-resistant viruses possessing a histidine to tyrosine substitution at NA residue 274 (H274Y, N2 numbering) were isolated from humans in a clinical trial designed to test the efficacy of oseltamivir [8]. NA residue 274 is located near the enzymatic site of NA and the H274Y substitution causes a structural change in NA that reduces oseltamivir-binding affinity [9]. Using H1N1 strains from the 1930s and 1990s, it was found that viruses possessing H274Y replicate poorly [10-12], so it was concluded that viruses possessing this particular mutation would probably not persist in the human population.

However, H1N1 viruses possessing the oseltamivir-resistant H274Y mutation appeared during the 2007–2008 IAV season and these viruses became the dominant seasonal H1N1 strain by the 2008–2009 season [13]. These viruses did not have the same replication defect seen earlier with older H1N1 viruses possessing H274Y. What changed? Why could these newer H1N1 viruses tolerate the H274Y mutation? Bloom *et al.* unequivocally answered these questions by identifying permissive mutations that arose over the last several years, which allowed H1N1 viruses to tolerate the H274Y mutation [14]. Through an elegant combination of computational and experimental approaches, it was discovered that H274Y decreases surface expression of NA in older H1N1 strains, but permissive mutations present in newer H1N1 strains negate this effect.

Methods

Computational approaches

Bloom *et al.* used a computational method to predict mutations that could potentially compensate for the H274Y mutation in NA. For this, they used a modified version of the Phylogenetic Inference of Protein Stability computer program that was originally designed for predicting stabilizing mutations. Further predictions of potential compensatory mutations that occurred in human strains were made after creating a maximum-likelihood phylogenetic tree, using a 1999 H1N1 strain and all H1N1 strains collected since 2006 (excluding 2009-pandemic H1N1 viruses).

Experimental approaches

The authors used several approaches to test the biological significance of the bioinformatically identified NA mutations. Reversegenetic approaches were used to introduce mutations into NA. For some experiments, plasmids expressing mutant NA were transfected into 293T cells and relative NA enzymatic activities were determined using a fluorogenic substrate. For other experiments, amounts of NA expressed on the surface of transfected cells were determined by flow cytometry using NA-specific monoclonal antibodies. Overall fitness of viruses possessing mutant NAs was determined by flow cytometry using viruses expressing either green fluorescent protein or mCherry.

Results

The authors began by hypothesizing that H274Y negatively affects NA folding or stability rather than enzymatic activity. To address this, they introduced the H274Y mutation into NAs from H1N1 viruses isolated from the years 1933 and 1934. Plasmids expressing these mutant NAs were transfected into 293T cells and cell surface NA activity was characterized. Indeed, NAs possessing the H274Y mutation exhibited decreased enzymatic activity and this correlated with reduced levels of NA cell surface expression. A computational method was used to identify R194G as a potential compensatory mutation that could possibly rescue the negative effect of H274Y. Mutant NAs expressing both R194G and H274Y were created and the results were crystal clear: the addition of R194G completely negated the effect seen previously with NAs possessing only H274Y. NAs expressing both mutations were expressed at high levels on the cell surface and had normal enzymatic activities. More importantly, viruses possessing only H274Y replicated poorly, while viruses possessing both H274Y and R194G replicated similar to parent viruses. These initial studies demonstrated the proof-of-principle that secondary mutations can compensate for negative effects associated with H274Y.

Similar studies were then completed using NAs from more recent H1N1 viruses. Introduction of H274Y into NAs from viruses from 1991 and 1999 led to decreased NA activity, but interestingly, the introduction of this mutation into an NA from 2006 did not have as drastic an effect. Could compensatory mutations in more modern H1N1 strains minimize the negative effects of H274Y?

In search of these types of mutations, the authors once again reached into their bag of bioinformatic tricks and generated a maximum-likelihood phylogenetic tree using a 1999 H1N1 strain and all other human H1N1 strains isolated since 2006. From this analysis they identified several potential compensatory mutations and since these mutations preceded the emergence of the H274Y mutation in the human population, they termed these 'permissive' mutations. These mutations were sequentially introduced into a 1999 NA and two of these mutations, V234M and R222Q, were found to counterbalance the negative effects of H274Y. Viruses possessing H274Y and these two permissive mutations were able to replicate to high titers both in the presence and absence of oseltamivir.

Discussion & significance

These findings are important in many ways. First, these studies show that the biochemical basis of H274Y attenuation in earlier H1N1 strains is at the level of NA surface expression rather than enzymatic activity. It may be underappreciated that overall levels of NA can affect activity as much (or even more) compared with specific mutations that modify NA enzymatic activity directly. This may explain, in part, why it has been difficult to elucidate the mechanisms by which modern seasonal H1N1 viruses are able to tolerate H274Y. Second, these studies are a wonderful example of how computational approaches can be used to identify specific mutations of biological relevance. Not only did Bloom *et al.* identify permissive mutations, they showed functional relevance by introducing these mutations into viruses. Finally, these studies have enormous implications for early detection of other drug-resistant strains in the future. Surveillance systems can be put into place to monitor the emergence of permissive mutations in pandemic H1N1 viruses, which are currently oseltamivir sensitive.

Expert commentary

Early detection of drug-resistant strains through the identification of variants possessing permissive mutations is very attractive from a public policy standpoint. For example, upon

discovering a new pandemic H1N1 strain that possessed permissive mutations for NA resistance, there could be increased production of other antivirals that target different steps of the viral replication cycle. The problem is that we do not have many approved IAV antivirals in our arsenal and the development of additional drugs is needed. With the development of each of these new classes of drugs, it is absolutely imperative that we do our best to identify mutant viruses that are able to evade these drugs. We should not dismiss any mutant virus that has low replication potential; instead we should cherish these mutants and determine the cause of their low fitness and we should experimentally identify mutations that rescue the defect. With these data we will be armed to quickly identify potential resistant viruses in the human population.

In the Bloom *et al.* study, the V234M and R222Q NA permissive mutations were found to increase NA cell surface expression of infected cells when expressed in the absence of H274Y. Such mutations would be expected to impair the overall HA/NA balance. Important questions of what other mutations 'permitted' these permissive mutations remain. Recent studies have shown that HA antigenic mutations can promote the generation of NA mutations [15] and it will be important to determine if an interplay between HA and NA was involved during the emergence of V234M and R222Q in H1N1 IAVs as they circulated in humans in the 2000s.

Although it is difficult to directly test, it will be important to try to understand if viruses possessing H274Y were directly selected based on oseltamivir resistance in the human population or whether these viruses were selected based on something else. As pointed out in the Bloom *et al.* study, initial H274Y circulation did not correlate with oseltamivir usage [16], however, it is difficult to definitively assign geographic locations with distinct selective pressures. Compensatory NA mutations arising in response to HA antigenic mutations can be located at NA residue 274 [15], supporting the hypothesis that H274Y may have arisen due to selective pressures unrelated to oseltamivir usage.

Five-year view

It will be very important to extend these studies to identify permissive mutations that may confer oseltamivir resistance of pandemic H1N1 viruses and other IAVs that circulate in the future. Additional studies focusing on how other types of permissive mutations lead to the generation of IAV strains resistant to other drugs is also essential. It will be interesting to see how the findings of Bloom *et al.* can be applied to other pathogens. As pointed out in their article, similar examples of permissive mutations have been identified during the evolution of bacterial antibiotic resistance and during immune escape of HIV [17-19]. It will be pivotal to determine if common mechanisms of compensation are utilized by diverse pathogens encountered with different selective pressure.

Key issues

- An oseltamivir resistance mutation in neuraminidase (NA), H274Y, attenuates older strains of H1N1 influenza A viruses (IAVs) by preventing normal incorporation of NA into virions.
- More recent strains of H1N1 IAVs possessing H274Y are not attenuated and normal amounts of NA are incorporated in these virions.
- Permissive mutations in more recent strains of H1N1 IAVs allow for toleration of H274Y. When introduced into older H1N1 IAVs, these permissive mutations rescue the negative effects of H274Y.

- Surveillance for permissive mutations might aid in early identification of drug-resistant strains in the future.

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