Overview

Sequencing of 2019-nCoV revealed two particularly notable features of its genome. We investigate these features and outline some examples for how the virus may have acquired them. As rumours have been circulating about this virus being engineered or otherwise created with intent, we wish to make it clear that our analyses show that such scenarios are largely incompatible with the data.

The two primary features of 2019-nCoV of interest were:

- Based on structural modeling and early biochemical experiments, 2019-nCoV appears to be optimized for binding to the human ACE2 receptor.
- The highly variable spike protein of 2019-nCoV has an optimal furin cleavage inserted at the S1 and S2 boundary via the insertion of twelve in-frame nucleotides. Additionally, this event also led to the acquisition of three O-linked glycans around the furin cleavage site.

Mutations in the receptor binding domain of 2019-nCoV

The receptor binding domain (RBD) in the spike protein of SARS-CoV and SARS-like coronaviruses is the most variable part of the virus genome. When aligned against related viruses, 2019-nCoV displays a similar level of diversity as predicted from previous studies, including to its most closely related virus - SARS-like CoV isolated from bats (RaTG13, which is ~96% identical to 2019-nCoV).

Six residues in the RBD have been described as critical for binding to the human ACE2 receptor and determining host range[1]. Using coordinates based on the Ubani strain of SARS-CoV, they are Y442, L472, N479, D480, T487, and Y491 (the corresponding residues in 2019-nCoV are L455, F486, Q493, S494, N501, and Y505). Five out of six of these residues are mutated in 2019-nCoV compared to closely related viruses, including RaTG13 (**Figure 1**). Based on modeling[1] and early biochemical experiments[2,3], 2019-nCoV seems to have an RBD that may bind with high affinity to ACE2 from human, primate, ferret, pig, and cat, as well as other species with high receptor homology. In contrast, 2019-nCoV may bind less efficiently to ACE2 in other species often associated with SARS-like viruses, including rodents, civets, and bats[1].

A phenylalanine at F486 in 2019-nCoV corresponds to L472 in the SARS-CoV Ubani strain. In tissue culture experiments the leucine at position 472 mutated to phenylalanine (L472F)[4], which has been predicted to be optimal for binding of the SARS-CoV RBD to the human ACE2 receptor[5]. However, a phenylalanine in this position is also present in several SARS-like CoVs from bats (**Figure 1**). While these analyses suggest that 2019-nCoV may be capable of binding the human ACE2 receptor with high affinity, importantly, the interaction is not predicted to be optimal[1]. Additionally, several of the key residues in the RBD of 2019-nCoV are different from those previously described to be optimal for human ACE2 receptor binding[5]. This latter point is strong evidence *against* 2019-nCoV being specifically engineered as, presumably, in such a scenario the most optimal residues would have been introduced, which is not what we observe.

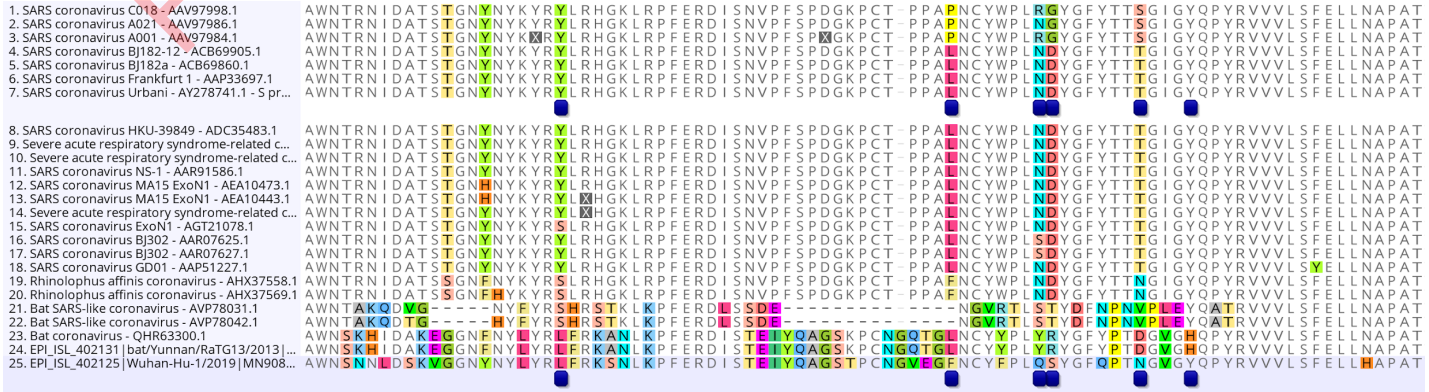

Figure 1 | Mutations in contact residues of the 2019-nCoV spike protein. The spike protein of 2019-nCoV (bottom) was

aligned against the most closely related SARS and SARS-like CoVs. Key residues in the spike protein that make contact to the ACE2 receptor have been marked with blue boxes in both 2019-nCoV and the SARS-CoV Urbani strain.

Acquisition of furin cleavage site and O-linked glycans

An interesting feature of 2019-nCoV is the acquisition of a predicted furin cleavage site in the spike protein (**Figure 2**). In addition to the furin cleavage site (RRAR), a leading P is also inserted so the fully inserted sequence becomes PRRA (**Figure 2**). The addition of a proline in this position is also predicted to create three O-linked glycans at S673, T678, and S686. The addition of a furin site has never before been observed in the lineage B betacoronaviruses and is a unique feature of 2019-nCoV. Some human betacoronaviruses, including HCoV-HKU1 (lineage A) have furin cleavage sites (typically RRKR), although not in such an optimal position.

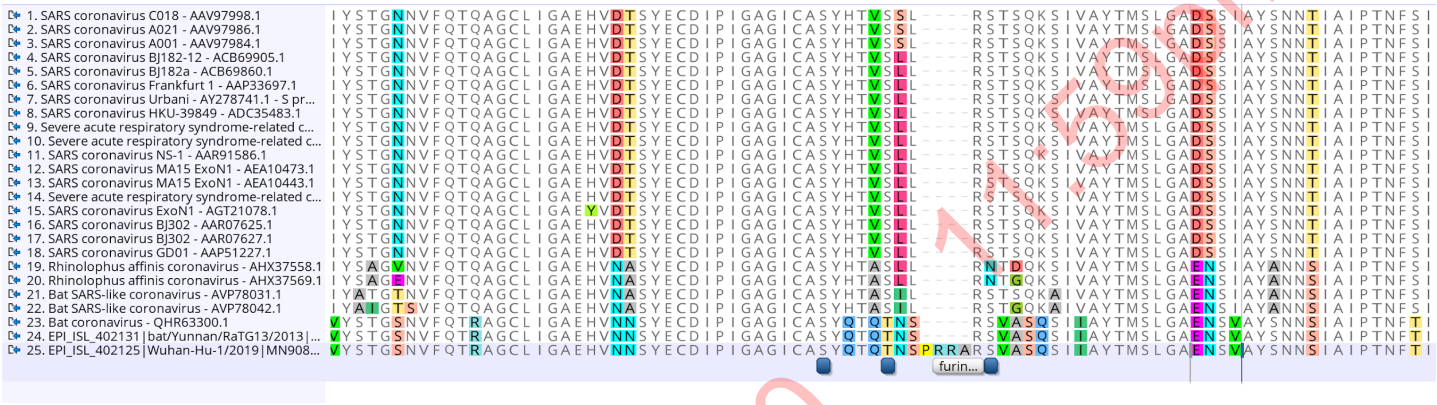

Figure 2 | Acquisition of furin cleavage site and O-linked glycans. The spike protein of 2019-nCoV (bottom) was aligned against the most closely related SARS and SARS-like CoVs. The furin cleavage site is marked in grey with the three adjacent predicted O-linked glycans in blue. Both the furin cleavage site and O-linked glycans are unique to 2019-nCoV and not previously seen in this group of viruses.

While the functional consequence - if any - of the furin cleavage site in 2019-nCoV is unknown, previous experiments with SARS-CoV have shown that it enhances cell–cell fusion but does not affect virus entry[6]. Furin cleavage sites are often acquired in condition selecting for rapid virus replication and transmission (e.g., highly dense chicken populations) and are a hallmark of highly pathogenic avian influenza virus[7–9]. The acquisition of furin cleavage sites have also been observed after repeated passage of betacoronaviruses in tissue culture (personal correspondence and NASEM call, February 3, 2020).

A potential function of the three O-linked glycans is less clear, but could create a “mucin-like domain” shielding potential epitopes or key residues on the 2019-nCoV spike protein.

Evolution of 2019-nCoV

Three main scenarios could explain how 2019-nCoV acquired the features discussed above: (1) natural selection in an animal host, (2) selection during passage, or (3) deliberate engineering. As described in the beginning, engineering (#3) can be ruled out with a high degree of confidence as the data is inconsistent with this scenario. In addition, if engineering would have been performed, one would also expect that a researcher would have used one of the several reverse genetics systems available for betacoronaviruses. However, this is not the case as the genetic data clearly shows that 2019-nCoV is not derived from any previously used virus backbone, including those recently posited by various conspiracy theories, based on a 2015 paper in *Nature Medicine*[10].

The other two scenarios are largely indistinguishable and current data are consistent with both. It is currently impossible to prove or disprove either, and it is unclear whether future data or analyses will help resolve this issue.

Selection in an animal host

Given the similarity of 2019-nCoV to bat SARS-like CoVs, particularly RaTG13, it is highly likely that bats also serve as the reservoir for this virus. However, previous human epidemics caused by betacoronaviruses have involved intermediate (possibly amplifying) hosts such as civets (SARS) and camels (MERS). It is therefore likely that an intermediate host would also exist for 2019-nCoV, although it is currently unclear what that host may be. Given the mutations in key residues of the RBD in 2019-nCoV it seems less likely that civets would be involved, although it is impossible to say with certainty at this stage.

For the virus to acquire the furin cleavage site and mutations in the spike proteins that appear to be suitable for human ACE2 receptor binding, it seems plausible that this animal host would have to have a very high population density, to allow the necessary natural selection to proceed efficiently, and an ACE2 gene that is similar to the human orthologue. Since furin cleavage sites have not been observed in this group of viruses before, it is unclear what conditions would be required for it to be acquired in the lineage leading to 2019-nCoV.

Selection during passage

Basic research involving passage of bat SARS-like coronaviruses in tissue culture and/or animal models have been ongoing in BSL-2 for many years across the world, including in Wuhan (e.g.,[11–14]). It is possible that 2019-nCoV could have acquired the RBD mutations and furin cleavage site as part of passage in tissue culture, which have been observed in previous studies with e.g., SARS-CoV[4]. However, it is less clear how the O-linked glycans - if functional - would have been acquired, as these typically suggest the involvement of an immune system, which is not present *in vitro*. In this scenario, it is also unclear how the virus would be linked to the fact that the epidemic seemed to 'take off' at a particular food market, although the exact role of this locality is currently uncertain.

Limitations and recommendations

The main limitation of what is described here is the clear ascertainment bias. We are looking for features or evolutionary aspects that could help explain how 2019-nCoV could lead to a rapidly evolving human epidemic, yet the specific features we are trying to find may be the exact features one would expect in a virus that could lead to an epidemic of the magnitude currently observed. Before 2019-nCoV 'took off' and started the current epidemic, it is plausible that many stuttering transmission chains of highly similar viruses could have entered the human population, but because they never took off they were never detected. It is extremely important to keep this in mind as any inference about the plausibility of various scenarios about the evolution and/or epidemic potential of 2019-nCoV is attempted.

To further clarify the evolutionary origins and functional features of 2019-nCoV it would be helpful to obtain additional data about the virus - both genetic and functional. This includes experimental studies of receptor binding and the role of the furin cleavage site and O-linked glycans. The identification of a potential intermediate host of 2019-nCoV as well as sequencing of very early cases, including those not connected to the market, could also help refute the passage scenario described above. Even in the light of such data, however, it is not guaranteed that data can be obtained to conclusively prove all aspects of the initial emergence of 2019-nCoV.

Background:

Bat coronavirus RaTG13 is the closest relative to nCoV-2019. Two recombinant bat viruses are close in some regions of the genomes. Pangolin virus?

Furin cleavage site rough notes about evolutionary origins:

Avian influenza example of natural and spontaneous evolution - get references and details.

There are two scenarios by which we could imagine the furin cleavage site could evolve.

1. As a human adaptation during the initial stages of the outbreak. The appearance of the mutation may have then triggered a second phase of rapid transmission. All current genome sequences are from this second phase and thus show limited diversity.

2. Adaptation to a non-human host prior to the jump to humans. This mutation is not seen in any bat coronavirus and is thus unlikely to be adaptive in those species.

Thoughts on 1: is it likely to spontaneously appear in a relatively short amount of time (and presumably small number of infections). It didn't happen in SARS with 8000 infections over 6 months. The link to the market would then be spurious - some doubt on that already. Prediction would be that the animal/environmental samples apparently found by China CDC would not have cleavage site.

Thoughts on 2: can we suggest a host where this cleavage site would likely be advantageous. Ferrets/polecats? Rodents - bamboo rats (don't know if they are popular in China)? Circulating in wild populations so limited prior human exposure until infected individual brought to the market.

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