Review

Attenuation and Degeneration of SARS-CoV-2 Despite of Adaptive Evolution

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Abstract: Evolution of SARS-CoV-2 has followed similar trends as other RNA viruses, e.g., HIV-1 and influenza A. Rapid initial diversification was followed by strong competition and rapid succession of dominant variants. Host-initiated RNA editing has been the primary mechanism for introducing mutations. A significant number of mutations were detrimental and were quickly purged. Fixed mutations are mostly diversifying mutations selected for host adaptation and immune evasion, with the latter accounting for more of the changes. However, immune evasion often comes at the cost of functionality, so that optimal functionality is still far from being accomplished. Instead, selection for antibody-escaping variants and accumulation of near-neutral mutations have led to suboptimal codon usage and reduced replicative capacity as demonstrated in non-respiratory cell lines. Beneficial adaptation of the virus includes reduced infectivity in lung tissues and increased tropism for the upper airway, resulting in shorter incubation periods, milder diseases, and more efficient transmission between people.

Key words: SARS-CoV-2, evolution, immune evasion, codon usage, attenuation, genome degradation, degeneration, COVID-19

1. Introduction

SARS-CoV-2 is one of the most intensely observed organisms in terms of evolutionary development, and COVID-19 is probably the fastest evolving disease we have documented in terms of clinical and pathological features. Reviewing SARS-CoV-2 evolution not only provides insight into future trends of the pandemic and guides COVID-19 mitigation strategies, but also sheds light on general features of the evolution of zoonotic RNA viruses. Based on what is currently available in the literature, we hereby make the following observations concerning the direction of SARS-CoV-2 evolution. First, initial explosive diversification after entering the new host species eventually gave way to one dominant lineage which has undergone an overall degeneration of genomic structure and loss of functionality. Second, adaptive mutations resulted in variants of lower virulence and higher transmissibility. The mechanisms underlying these trends will be discussed.

2. Degenerative evolution has been observed in other RNA viruses

After a zoonotic virus enters mankind, it must adapt itself to replicate in an unfamiliar host cell environment and to evade unforeseen defense mechanisms on the cellular and organismal levels. Viruses are endowed with error-prone nucleic acid polymerases which generate quick genetic variations among offspings. More than most other organisms, viruses produce large population surpluses to afford strong selection against low odds of survival. Even with quick mutations and large numbers of progenies,

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successful jump of species is rare. Some viruses, such as avian influenza viruses, may infect humans but fail to transmit from human to human.

After a zoonotic virus successfully infects a human being and is fortunate enough to gain the ability to transmit between humans, it may still be contained and shortly disappear from mankind. A prime example is severe acute respiratory syndrome coronavirus (SARS-CoV). The virus appeared in humans in late 2002 and went extinct in about a year [1,2]. Other viruses may coexist with mankind for the long term and become endemic. There is evidence that zoonotic viruses that maintain high levels of transmission among humans tend to attenuate. For example, the current human coronavirus OC43 (HCov-OC43) diverged from the bovine coronavirus around 1890 [3] and probably entered mankind as the cause of the 'Russian flu' pandemic of 1889-1895 [4,5]. The virus has attenuated ever since and is only a cause of common cold by now. This attenuation is likely the result of mutation accumulation. The pressure of host adaptation and immune evasion may have led to compromised replicative capacity.

Beside error-prone nucleic acid replication, mutations may be forced on the virus by the cellular nuclear acid editing mechanisms. *Apolipoprotein B mRNA editing enzyme, catalytic polypeptide* (APOBEC) deaminates viral cytidine to uridine. As a matter of fact, APOBEC3 is a key factor in determining the permissiveness of a cell to both DNA and RNA viruses [6,7]. Another family of nucleic acid editing enzymes is the *adenosine deaminase acting on RNA* (ADAR) which, by deaminating adenosine to inosine in double-stranded RNA, affects subsequent RNA processing, translation, and stability [8]. The p150 subunit of ADAR1, as well as APOBEC3, are upregulated by type I interferons [9,10], underlining the role of nucleic acid editing in innate antiviral immunity. Although mutations introduced via nucleic acid editing can contribute to viral adaptation, directional base substitutions are more likely disruptive to genetic information. For example, biased U>C transitions of the measles virus, presumably introduced by ADAR, suppressed translation of viral genes in infected brain tissues [11]. Interestingly, ADAR edition of viral RNA also modulates interferon production leading to a more peaceful coexistence between the virus and the host cell [12]. In addition, viral RNA rich in CpG dinucleotides are recognized by zinc-finger antiviral proteins [13,14]. This mechanism provides a selective advantage for CpG-depleted variants.

Whether accidentally introduced during genome replication or forced on by host editing enzymes, mutations accumulate in viruses faster than in cellular organisms, leading to degradation of viral genomes. During the first few months of infection by the human immunodeficiency virus (HIV), as the viral genomic sequences diversify in each patient, the overall fitness of the virus as measured by *in vitro* viral DNA production decreases with time [15]. During the early years of the HIV pandemic, the virus adapted its codon usage to that of human cells, but by the end of the 20th century, codon usage of HIV-1 showed a trend of diverging away from human codon usage patterns, indicating degeneration of genetic information [16]. Antiretroviral drugs alter codon usage patterns [17] and result in reduced fitness of HIV-1 [18,19]. Likewise, the 1918 H1N1 influenza virus demonstrated a linear degeneration of codon scores before it finally went extinct [20].

3. Adaptive mutations of SARS-CoV-2 favor upper respiratory infection

Adaptive mutations of SARS-CoV-2 are characterized by 1) convergence or homoplasy, i.e., repeated, independent emergence of the same mutations in multiple strains [21]; 2) cooccurrence of two or more mutations in fixed strains suggesting epistasis and complementarity [22]; 3) concentration of mutations in hotspots, especially in the receptor-binding domain (RBD) of the spike protein which increases affinity

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to the human angiotensin-converting enzyme 2 (ACE2) receptor [23]; 4) competition between variants resulting in rapid selective sweeps with new variants replacing old ones in a matter of months [24,25].

3.1. From the lungs to the nose

Starting with the D614G sweep early in the pandemic, all subsequent variants that gained dominant status harbor multiple mutations in the RBD resulting in increased affinity for ACE2. In addition, adaption of SARS-CoV-2 includes immune escape [26] and antagonizing the activity of interferons [27].

A key functional change in the spike protein of SARS-CoV-2 as it evolved is decreased tropism toward lung tissues and increased tropism toward airway epithelium. As early as 2020, it was found that the D614G mutant outcompeted the wild-type virus in primary human airway epithelial cells and in nasal epithelium, but not in the lungs, of infected hamsters [28,29]. The Beta, Delta, and Omicron variants all replicated to higher titers in *ex vivo* human bronchial tissues than the wild-type virus, with Omicron having the highest titers, but the opposite trend was seen when the variants were tested in lung tissues with Omicron replicating to significantly lower titers than the wild-type virus and the Delta variant [30].

The mechanism of tropism change is still unclear. Increased binding affinity toward the human ACE2 is an obvious factor to speed up replication in airway cells. A survey of single cell RNA-seq data revealed that airway cells express more ACE2 than lung cells, with nasal secretory cells showing the highest expression [31]. A study using immunohistochemistry observed similar distribution of ACE2 [32]. Local codon optimization in viral genes may also contribute to increased replication efficiency, especially at key genomic sites such as the hexanucleotides encoding the arginine dimer of the furin-cleavage site of the spike protein [33]. Higher affinity for the airway means less viruses reaching the lungs. Reduced replicative capacity in the alveolar epithelium may be a result of a documented change in its mode of entry. SARS-CoV-2 uses the transmembrane serine protease 2 (TMPRSS2) to cleave the S2' site of its spike protein to yield an S2 subunit that can induce fusion between the viral envelope and the cell membrane [34]. It has been demonstrated that the Omicron variant has lost its sensitivity to TMPRSS2 and consequently lost its ability to induce membrane fusion [35]. The variant is now more dependent on endocytosis as its mode of entry. This is demonstrated by increased sensitivity to inhibitors of endosomal proteases such as chloroquine [36].

This change in tissue tropism is reflected in the clinical features of COVID-19. Infection with the Omicron variant is characterized by upper respiratory symptoms such as rhinorrhea, sore throat, sneezing, and hoarse voice, while the absence of anosmia may reflect reduced tropism toward extra-respiratory tissues [37]. Moreover, the Omicron variant has been found to be less likely to cause severe pneumonia than the Delta variant even after adjustment for vaccination status in a multivariable analysis [38]. In patients infected with the Omicron variant, pneumonia was more strongly associated with old age, male gender, and diabetes than in patients infected with previous variants [39]. The Alpha and Beta variants, but not the Delta variant, were also less likely to cause pneumonia even after adjustment for age, sex, comorbidities, and vaccination status than the wild-type virus [40]. Morbidity and mortality of COVID-19 is associated more with immune dysregulation than with viral replication, but excessive activation of immune cells typically follows viral infection of the lungs and subsequent accumulation of macrophages and neutrophils in the lungs [41,42].

3.2. Increasing transmissibility

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Because there are less ACE2 receptors in the lungs, even SARS-CoV-2 strains that are not variants of concern replicate to lower titers in the lungs than in the bronchi, as seen in hamsters and in organoid cultures [28,43]. Increased tropism toward the upper respiratory tract means fast viral replication and more viral shedding, which, in turn, means shortened incubation periods and higher transmissibility. The incubation period of the wild-type virus among travelers from Wuhan, China, ranged from 2.1 to 11.1 days, with a mean of 6.4 days [44]. One meta-analysis of the incubation period caused by the later variants found the mean incubation period of the Alpha, Beta, Delta, and Omicron variants to be 5.00 days, 4.50 days, 4.41 days, and 3.42 days, respectively [45]. In comparison, the median incubation period of the human coronaviruses that cause common cold is 3.2 days [46].

Sungnak et al compared the transmissibility of several respiratory viruses whose receptors are distributed differently within the respiratory system and found that tropism toward the upper respiratory tract is associated with higher transmissibility [29]. Loss of replicative function in the lungs results in milder diseases, allowing the patients to be more ambulatory and to spread the virus more efficiently. Indeed, later variants of SARS-CoV-2 tend to show increased transmissibility. Using two mathematical models to calculate the effective reproduction number (*Rt*), Hasan et al demonstrated an increase in transmissibility of SARS-CoV-2 in Scandinavia as the Delta variant replaced earlier variants in 2021 [47]. The basic reproduction number (*R0*) of the wild-type virus ranged from 0.47 to 6.47, with an average of 2.69 [48]. Using a competition model, Hansen et al calculated the relative transmissibility of the Alpha, Delta, and Omicron variants compared with the wild-type virus and found that the *Rt* of the three variants were 1.51, 3.28, and 10.33 times of the wild-type virus [49].

Enhanced transmissibility is obviously the most important factor in terms of selective advantage against other variants. Theoretically, there is an optimum configuration of the spike protein which interacts with the human ACE2 and proteases with maximum efficiency, and there is an ideal mode of entry which results in maximum tropism toward the nasal epithelium and minimum tropism toward the alveolar epithelium. By *in vitro* evolution and testing, Zahradník et al produced an ideal model, IBD-62, with an ACE2-binding affinity that is 1000-fold higher than that of the wild-type virus [50]. IBD-62 contains a combination of S477N, Q498R, and N501Y mutations, which were all found later in the Omicron variant [51]. As a matter of fact, most mutations that could increase the binding affinity between the RBD and ACE2 had already occurred even before Omicron [52]. The *R0* of the Omicron variant is already on par with the most transmissible respiratory virus known to date, i.e, the measles virus [53]. Can the transmissibility be further improved?

4. Degenerative evolution of SARS-CoV-2

The virus still has room to improve, but nature may not be able to accomplish it. While some gain-of-function mutations are realized, they tend to be out-numbered by functionally destructive mutations.

The most dominant mechanism that drives mutations in the genome of SARS-CoV-2 is RNA editing. The most common mutation seen in the latter variants is C>U transition, presumably induced by the APOBEC enzymes [20,54]. When APOBEC deaminates cytosine in the antigenome (the replication intermediate, or the negative strand), it results in G>A transition in the viral genome. ADAR induces A>G transition when it deaminates adenine in the genome and induces U>C transition when it works on the antigenome. Giorgio et al studied editing of SARS-CoV-2 RNA in the transcriptome of bronchoalveolar lavage samples of infected patients and found high levels of A>G and U>C transitions indicative of ADAR action. The counts of A>G and U>C transitions are roughly equal, consistent with the fact that ADAR acts

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on dsRNA [55]. They also found significant C>U transitions which are more abundant than G>A transitions, indicating APOBEC editing and its preference for the positive strand. In comparison, substitution patterns characteristic of replication errors introduced by the viral RNA-dependent RNA polymerase (C>A, U>C, G>U, A>C, and U>G) are much rarer in the transcriptome. Kim et al co-expressed APOBEC enzymes and SARS-CoV-2 RNA segments in HEK293T cells and proved that APOBEC3A, APOBEC1, and APOBEC3G can all edit SARS-CoV-2 RNA [56]. One signature mutation caused by APOBEC3A in the 5'UTR appeared early in 2020 and has been fixed in dominant variants including the Delta and Omicron variants. One signature mutation pattern of APOBEC1 contributed to the H655Y mutation on the spike protein which is associated with the loss of TMPRSS2 usage. APOBEC3A also significantly increased viral yield in Caco-2 cells infected with SARS-CoV-2. These findings by Kim et al indicate that the virus can take advantage of the host cell RNA editing enzymes for adaptive evolution. But as expected, most mutations turn out to be destructive and subject to purifying selection [57-59], whether they are imposed on the virus by the host or due to errors during viral replication.

4.1. Darwinian mechanisms that drive degeneration of genetic information

4.1.1. Natural selection for immune evasion sacrifices functionality of genes

SARS-CoV-2 has accumulated far more nonsynonymous mutations than synonymous mutations, indicating positive selection [60-62]. Amino acid substitutions presumably led to rapid diversification early in the pandemic as the virus adapted to the new host species, and immune evasion has been a driving force since 2020 [63]. Most mutations concentrate on the spike protein, which is the main target of antibodies.

The Omicron variant (BA.1) has 15 mutations in the RBD, far more than the one to four found in any other major variants. Six of the 15 mutations are known to increase affinity toward ACE2, while nine are known to decrease affinity [51]. The overall affinity of the spike protein of the Omicron variant as measured experimentally is on par with that of the wild-type virus [51,64]. The effects of destructive mutations are balanced by constructive mutations, but the destructive mutations allow for evasion of pre-existing neutralizing antibodies in the population [51]. The idea that most mutations in the RBD decreases affinity to ACE2 is corroborated by a study by Li et al early in the pandemic. Using vesicular stomatitis virus pseudotyped with the spike protein of SARS-CoV-2, the group measured the infectivity of 51 variants with mutations in the RBD. They found 13 mutations showing decreased infectivity while only three showed increased infectivity. Some mutations that decreased infectivity enabled the spike protein to evade antibodies [65]. The majority of the RBD mutations did not yield in a measurable phenotypic difference in binding affinity.

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Theoretically, there is a limited number of epitopes that could be altered without seriously crippling viral replication. Therefore, the rate at which the virus accumulates immunity-escaping mutations must slow down with time. Neher compared the synonymous and nonsynonymous mutation rates of the viral clades that arose from 2019 to 2022. Synonymous mutation rates remained constant at about five to eight base changes per genome per year for all clades, but nonsynonymous mutation rates dropped from the highest of 16.41 per genome per year (clade 19B++) to the lowest of 2.81 per genome per year (clade 22A) [58]. Figure 1 is plotted using Neher's data, demonstrating a negative correlation between intra-clade mutation rates and time. At some point, additional mutations will be so detrimental that it is hard for them to gain dominance over preexisting variants and be fixed.

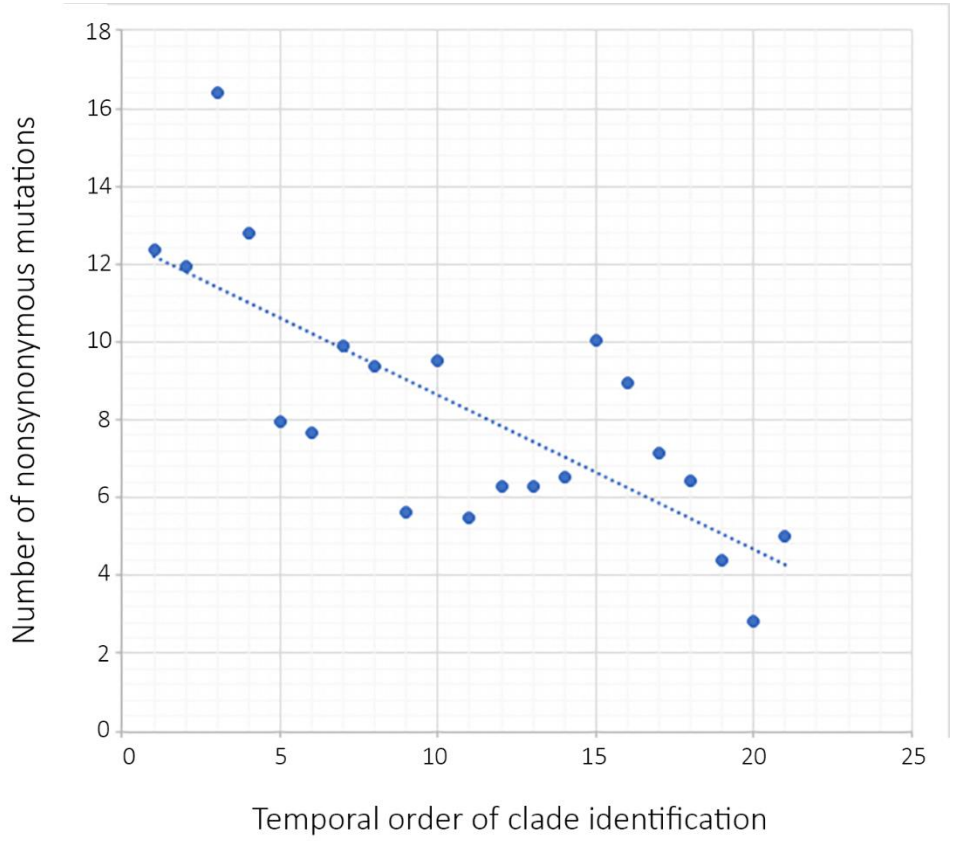

Figure 1. Decline of intra-clade mutation rate from early clades to late clades

Therefore, even if the ideal genome ever appears by mutation and recombination, it will soon degenerate again because the virus needs to continue mutating to evade a practically infinite inventory of antibodies and antiviral T lymphocytes in the human population.

4.1.2. Accumulation of non-selectable mutations

Even though nonsynonymous mutations concentrate in the spike protein, they also accumulate in other genes throughout the viral genome. Unlike the surface protein and the RNA polymerase, accessory proteins demonstrate little restraint on amino acid substitutions [58,66]. Accumulation of mutations in proteins that are loosely selected may contribute to genetic degradation in the long term.

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Synonymous mutations affect codon usage adaptation and translation efficiency [67,68]. While synonymous mutations that promote adaptation to human codon usage show a tendency to increase, most synonymous mutations, mainly those induced by APOBEC enzymes, are oblivious to natural selection [67]. Since C to U transitions are by far the most abundant base substitutions in the mutational spectrum of SARS-CoV-2 [69-71], uracil accumulates steadily in the viral genome while the number of cytosine declines with time [72]. Since intramolecular base-pairing forms secondary structures which can stabilize the genome, and AU bonding is less tight than GC bonding, C>U transitions also destabilizes the viral genome, which may facilitate nucleotide deletions, leading to shorter genomes in all major variants (Table 1) [72]. SARS-CoV-2 still has higher cytosine count and lower uracil count than any of the four human coronaviruses [73], will it continue to evolve toward the nucleotide compositions of the current human coronaviruses, and phenotypically become more like them?

Table 1. Nucleotide deletions in major variants of concern compared to early clade consensus

	Alpha	Beta	Gamma	Delta	Omicron
Genome length	29,750	29,751	29,764	29,756	29,742
Deletions	19	18	5	13	27

4.2. Phenotypic loss due to genetic degradation

4.2.1. Codon degeneration and reduced translation efficiency

Both nonsynonymous and synonymous mutations may deoptimize codon usage. Early in the pandemic, during 2020, Posani et al found a general trend of codon deoptimization among most genes of SARS-CoV-2. The ENC (effective number of codons) plot indicated natural selection being a driving force of codon deoptimization, especially for genes of the spike protein and the RNA-dependent RNA polymerase [74]. Mogro et al discovered a downward trend of codon adaptation index (CAI) of SARS-CoV-2 in multiple human tissues, and surprisingly, the Omicron variant (BA.1) had a higher CAI than other variants of concern, although lower than the earliest isolates [75]. Wu et al experimentally demonstrated higher protein expression when codon usage was restored to optimum, and the group also found a sudden increase in CAI when Omicron came to the scene [76]. The high CAI of Omicron may shed light on the mysterious origin of the variant [77]. If the variant was generated in a chronically infected, immunodeficient person, the nucleic acid editing enzymes may still cause codon degeneration even without the need for immune escape, although it may be slower. On the other hand, we know that bat coronaviruses have higher CpG content, higher percentage of C, and lower percentage of U [78]. The high CAI of Omicron favors the “reverse zoonosis” hypothesis. Others proposed that the variant was a recombinant between an ancestral lineage and a hypermutated virus [66], but how the ancestral lineage was spared from codon degeneration is still a question.

Nonetheless, among humans, the future of the Omicron variant is most likely codon deoptimization and further attenuation.

4.2.2. Reduced replication capacity in non-respiratory cell lines

Through adaptation, newer SARS-CoV-2 variants have lost replication efficiency in lung cells and gained efficiency to replicate in airway cells. Comparison of the overall genomic functionality of early and late variants should, therefore, be conducted in cells that none of them has been exposed to, e.g., the

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monkey kidney cell line, Vero. Using Vero E6 cells, Mautner et al showed that the B.1.1 strain (not a variant of concern) replicated the fastest while Omicron was the slowest [79]. The difference between the Delta variant and the Omicron variant was greater when they were compared in Vero E6 cells expressing TMPRSS2, because Omicron lost its ability to use TMPRSS2 for cell entry [35]. Omicron also showed severely weakened ability to cause cytopathic effect, including cell fusion, in Vero cells.

Caco-2 is a human colon epithelial carcinoma cell line. When the infectivity of SARS-CoV-2 variants were compared in Caco-2, Omicron replication was drastically slower than any other variant and produced very low titers of progeny virus in the end [79]. This may be a clue to explain the narrower tissue specificity of Omicron in comparison with earlier variants. The pathology of Omicron infection is more localized to the respiratory system, resulting in less symptoms of the digestive and nervous systems [37,80,81]. Interestingly, in the airway epithelial carcinoma cell line Calu-3, the disadvantage of Omicron was less dramatic, presumably due to its adaptation to airway cells [79].

5. Conclusion

Like other zoonotic RNA viruses, SARS-CoV-2 is evolving toward attenuation and genetic degradation. The most important cause of mutation is APOBEC editing, and the most important force for mutation accumulation is immune evasion. Immune evasion and accumulation of near-neutral mutations both compromise the functionality of viral genes, leading to codon deoptimization and reduced replication capacity. The direction of SARS-CoV-2 evolution is toward production of a virus that limits its pathology to the upper respiratory tract, i.e., a fifth human coronavirus that causes common cold.

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