Circulating SARS-CoV-2 variants B.1.1.7, 501Y.V2, and P.1 have gained ability to utilize rat and mouse Ace2 and altered in vitro sensitivity to neutralizing antibodies and ACE2-Ig

Weitong Yao[1,2], Yifei Wang[1], Danting Ma[1], Xiaojuan Tang[1,2], Haimin Wang[1], Chao Li[1], Hua Lin[3], Yujun Li[1]*, Guocai Zhong[1,2]*

Affiliations:

[1] Shenzhen Bay Laboratory, Shenzhen 518132, China

[2] School of Chemical Biology and Biotechnology, Peking University Shenzhen Graduate School, Shenzhen 518055, China

[3] Biomedical Research Center of South China, Fujian Normal University, Fuzhou 350117, China

* Corresponding author. Email: liyj@szbl.ac.cn; zhonggc@szbl.ac.cn

Running title: Receptor usage and neutralization sensitivity of SARS-CoV-2 variants

Keywords: SARS-CoV-2; Spike mutation; mouse Ace2; rat Ace2; ACE2-Ig; neutralizing antibody

Abstract

Spontaneous and selection-pressure-driven evolution of SARS-CoV-2 has started to pose more challenges to controlling the pandemic. Here, we first investigated cross-species receptor usage of multiple SARS-CoV-2 variants that emerged during the pandemic. We found that, in contrast to an early isolate WHU01, the circulating variants B.1.1.7/501Y.V1, B.1.351/501Y.V2, and P.1/501Y.V3 were able to use rat and mouse Ace2 orthologs as entry receptors, suggesting that rats and mice might be able to harbor and spread these variants. We then evaluated in vitro sensitivity of these variants to three therapeutic antibodies in clinics (etesevimab/LY-CoV016, casirivimab/REGN10933, and imdevimab/REGN10987) and an ACE2-Ig variant we developed recently. We found that all the tested SARS-CoV-2 variants showed reduced sensitivity to at least one of the tested antibodies but slightly increased sensitivity to the ACE2-Ig protein. These data demonstrate that the ACE2-Ig is a good drug candidate against SARS-CoV-2 variants that emerge over the course of the pandemic.

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the etiological agent of the ongoing coronavirus disease 2019 (COVID-19) pandemic, has already caused about 100 million confirmed infections and over 2 million documented deaths across 224 countries, according to World Health Organization's online updates (https://www.who.int/emergencies/diseases/novel-coronavirus-2019). The pandemic has triggered unprecedentedly extensive worldwide efforts to develop countermeasures against COVID-19, and a number of encouraging progresses have been achieved in developing prophylactic vaccines and antibody therapeutics[1-9]. So far, there are at least six prophylactic COVID-19 vaccines that have been approved by different countries for clinical use. These include two mRNA vaccines (Pfizer-BioNTech, US; Moderna, US)[1,2], two inactivated vaccines (Sinopharm, China; Sinovac, China)[3-5], and two adenoviral vectored vaccines (Sputnik V, Russia; AstraZeneca-Oxford, UK)[6,7]. There are also some convalescent patient-derived antibodies that have been approved for clinical use, such as Regeneron's antibody cocktail consisting of casirivimab (REGN10933) and imdevimab (REGN10987)[8].

SARS-CoV-2 is a betacoronavirus that has broad host ranges[10-13]. We recently found that SARS-CoV-2 can use human ACE2 and a wide range of animal-Ace2 orthologs, but not rat or mouse Ace2, for cell entry [11]. However, a single amino-acid change within the Spike receptor-binding domain (RBD; Q498H, Q498Y, or N501Y) could be sufficient to confer SARS-CoV-2 the ability to utilize mouse Ace2[ref.14-17]. SARS-CoV-2 is a single-stranded RNA virus with moderate mutation and recombination frequencies[18,19]. Spontaneous and selection-pressure-driven mutations, especially those within the Spike RBD region, might alter cross-species receptor usage by SARS-CoV-2, or compromise the efficacy of neutralizing antibodies and vaccines based on the Spike sequence of early SARS-CoV-2 strains, posing additional challenges to controlling the pandemic. Therefore, in this study, we investigated cross-species

receptor usage, as well as neutralization sensitivity, of multiple SARS-CoV-2 variants that emerged during the pandemic.

Results

Spike RBD is the major determinant of viral receptor usage and neutralization sensitivity, we thus primarily focused on RBD mutations found in different variants, especially the Variants of Concern (VOC) that emerged from the UK (VOC202012/01, also known as B.1.1.7 or 501Y.V1), South Africa (VOC202012/02, also known as 501Y.V2 or B.1.351), and Brazil (VOC202101/02, also known as P.1 or 501Y.V3), respectively. These include an N439K mutation from the circulating variant B.1.141[ref.20], an N501Y mutation from B.1.1.7/501Y.V1 [21,22], E484K and N501Y mutations from B.1.351/501Y.V2[23] and P.1/501Y.V3 [24], and a Y453F mutation from two mink-associated variants that emerged in Netherlands and Denmark mink farms[25-27] (Figure 1). We further included a Spike Y453F-Q498H double mutant that has not been detected in patient samples yet, but could evolve from the early isolate WHU01 through adopting only two nucleotide mutations, or from the mink-associated Y453F variant through adopting a single nucleotide mutation. To evaluate Spike protein-mediated viral entry of the above-mentioned variants, we constructed Spike expression plasmids for these variants and produced luciferase reporter retroviruses pseudotyped with one of these different Spike variants. As rats and mice are rodent species that have huge population size and could have close contacts with human and domestic animal populations, it's important to monitor SARS-CoV-2's ability to utilize rat and mouse Ace2 as receptor. We therefore used the Spike variant-pseudotyped reporter viruses to infect 293T cells transfected with an expression plasmid of human, rat, or mouse ACE2 ortholog. Parallel infection experiments using 293T cells transfected with an empty vector plasmid were included as controls. Consistent with our previous report, the early strain WHU01 only infected 293T cells expressing human ACE2, but

not rat or mouse Ace2 (Figure 2A). Though the variant carrying Δ(69-70)-N439K mutations and the mink-associated variant carrying Δ(69-70)-Y453F mutations did so too (Figures 2B and C), the mink-associated variant that carries Δ(69-70)-Y453F-F486L-N501T-M1229I mutations also infected 293T cells expressing mouse Ace2, albeit with much lower efficiency than infecting cells expressing human ACE2 (Figure 2D). It is of note that, in addition to the Y453F-Q498H variant, all the variants that carry an N501Y mutation efficiently utilized all the three Ace2 orthologs (Figures 2E-H). The variant that carries K417N-E484K-N501Y mutations utilized rat Ace2 even more efficiently than using human ACE2 (Figure 2G). These data suggest that the circulating SARS-CoV-2 variants B.1.1.7/501Y.V1, B.1.351/501Y.V2, and P.1/501Y.V3 may be able to efficiently infect mice and rats, adding one more concerning factor to these VOC strains.

We then performed neutralization assays to evaluate sensitivity of these SARS-CoV-2 variants to three therapeutic antibodies in clinics (etesevimab/LY-CoV016, casirivimab/REGN10933, and imdevimab/REGN10987)[8,9] and an ACE2-Ig variant we developed recently[11]. When tested against the early strain WHU01, the Regeneron antibody casirivimab neutralized the virus with the best potency, about 3-fold more potent than our ACE2-Ig, and about 20-fold more potent than the monoclonal antibody etesevimab (Figure 3A and Table 1). When tested against the variants, all the antibodies in general showed decreased sensitivity to at least one variant (Figures 3B-H). Specifically, variants carrying an N501Y or N501T mutation were found to be highly resistant to etesevimab (Figures 3D-G). Variants carrying a Y453F mutation were found to be highly resistant to casirivimab (Figures 3C and D). It is of note that the Δ(69-70)-Y453F-F486L-N501T-M1229I mutations carried by mink-associated variants and the K417N-E484K-N501Y mutations carried by the South Africa VOC strain B.1.351/501Y.V2 made the virus highly resistant to both etesevimab and casirivimab, adding another concerning factor to these two variants (Figures 3D and G). In addition, the Spike Y453F-Q498H double

mutation made the virus partially resistant to etesevimab and highly resistant to both of the Regeneron antibodies, casirivimab and imdevimab (Figure 3H). Consistent with these neutralization data, structural data analysis revealed contact of etesevimab with RBD residues K417 and N501, casirivimab with RBD residues Y453, E484, and F486, and imdevimab with RBD residues N439 and Q498 (Figures 3I-K). On the other hand, when tested against the variants, ACE2-Ig protein did not lose efficacy at all. Rather, it seems that ACE2-Ig gains potency along with the course of the pandemic, consistent with the neutralizing mechanism of this protein (Figures 3A-G and Table 1).

Discussion

The Spike protein is the major host-range determinant and neutralizing antigen of SARS-CoV-2. It has been undergoing a number of mutations since the outbreak of the COVID-19 epidemic in the end of 2019[ref.10,20-25,27-29]. With more and more SARS-CoV-2 variants being identified to carry diverse Spike mutations within the RBD region, especially the Variants of Concern initially identified in the UK (B.1.1.7 lineage)[21,22], South Africa (501Y.V2 lineage)[23], and Brazil (P.1 lineage)[24], it is critical to understand whether the host range and neutralization sensitivity of these variants have changed. In this study, we first investigated cross-species receptor usage of an early SARS-CoV-2 isolate and multiple SARS-CoV-2 variants that emerged during the pandemic (Figure 2). A mink-associated variant that carries Δ(69-70)-Y453F-F486L-N501T-M1229I mutations was found also able to utilize mouse Ace2. In addition, all the variants that carry an N501Y mutation, a shared feature of the UK, South Africa, and Brazil VOC strains, were found able to efficiently use rat and mouse Ace2 orthologs for entry (Figures 2E-G), consistent with the identification of N501Y as the mouse-susceptibility determinant of a mouse-adapted SARS-CoV-2 strain[16]. Moreover, the K417N-E484K-N501Y mutations found in the South Africa variant 501Y.V2 even enable the virus to

utilize rat Ace2 more efficiently than using human ACE2. These data suggest that rats and mice may be able to harbor and spread these variants. Rats and mice are vaccine-inaccessible rodent species that have large population size and could have close contacts with both human and domestic animal populations. It is possible that the N501Y-associated circulating variants might have emerged as an adaptation to rats before being passed back to humans. It is therefore critical to closely monitor cross-species transmission and evolution of these SARS-CoV-2 variants.

We also evaluated sensitivity of these variants to three therapeutic antibodies in clinics (etesevimab/LY-CoV016, casirivimab/REGN10933, and imdevimab/REGN10987) and an ACE2-Ig variant we developed recently (Figure 3 and Table 1). We found that all the tested Spike mutations were able to confer resistance to at least one of the three tested antibodies. A mink-associated strain and the South Africa VOC strain 501Y.V2 are highly resistant to both etesevimab and casirivimab. The South Africa VOC strain 501Y.V2 and the Brazil VOC strain P.1/501Y.V3 are identical within the RBD region except that 501Y.V2 RBD adopted a K417N mutation and P.1/501Y.V3 RBD adopted a K417T mutation, it's therefore likely that the Brazil VOC strain P.1/501Y.V3 is also highly resistant to both etesevimab and casirivimab. More importantly, two simple amino-acid mutations in the Spike, Y453F-Q498H, made the virus partially resistant to etesevimab and highly resistant to both casirivimab and imdevimab, the two monoclonal antibodies constituting Regeneron's FDA-approved antibody cocktail. The Y453F-Q498H double mutant has not been detected in patient samples yet. However, it could easily evolve from the early isolate WHU01 through adopting only two nucleotide mutations, or evolve from mink-associated Y453F variant or mouse-adapted Q498H variant through adopting a single nucleotide mutation. Potential cross-species transmission of SARS-CoV-2 variants, such as the South Africa VOC strain 501Y.V2, from humans to rats or mice, to domestic animals, and then back to humans, might accelerate the emergence of new variants.

Immune pressures, such as clinical applications of non-broadly neutralizing antibodies or vaccines, or patients' immune reactions to SARS-CoV-2 infections, are also prone to drive more and more immune-evasion mutations. These mutation-driving factors altogether will likely accelerate the loss of efficacy of the therapeutic antibodies and possibly the vaccines in clinics, posing additional challenges to the ongoing devastating pandemic. Indeed, during preparation of this manuscript, multiple preprint reports have shown that the 501Y.V2 variant significantly reduced the potency of vaccine-induced neutralization antibodies[30-32]. It is therefore urgent to develop broadly anti-SARS-CoV-2 therapeutics or vaccines.

Soluble ACE2 protein, which neutralizes SARS-CoV-2 infection through competitive blocking the interaction between viral Spike protein and cell-surface receptor ACE2, represents a good candidate that will be hardly escaped by SARS-CoV-2 variants. It could also be a good alternative anti-SARS-CoV-2 agent to the populations who are reluctant to take, not responsive to, or don't have access to any prophylactic vaccines. Recently we have developed a panel of ACE2-Ig variants that potently neutralize SARS-CoV-2 early isolate at picomolar range, and have demonstrated that the protein is broadly effective against four distinct coronaviruses, suggesting that the protein could be used to protect from SARS-CoV-2 and some other SARS-CoV-2-like viruses that might spillover into humans in the future[11]. In this study, we demonstrated that the ACE2-Ig protein is broadly effective against all the tested SARS-CoV-2 variants, including the ones simultaneously resistant to two or three neutralizing antibodies. Perhaps more importantly, it seems that SARS-CoV-2 evolution tend to reduce sensitivity to neutralizing antibodies while increasing sensitivity to ACE2-Ig (Figure 3 and Table 1). These data suggest that ACE2 is still likely an essential receptor for SARS-CoV-2, and that ACE2-Ig could be used to treat and prevent infection of any SARS-CoV-2 variant that emerges over the course of the pandemic. Animal studies to test and optimize pharmacokinetics, as well as *in vivo* anti-SARS-CoV-2 efficacy, of the ACE2-Ig protein are ongoing.

Methods

Methods and associated references are provided below.

Acknowlegements

We thank Dr. Yu J. Cao (School of Chemical Biology and Biotechnology, Peking University Shenzhen Graduate School, Shenzhen, China) for generously providing the 293F cells used in this study for the production of ACE2-Ig protein and SARS-CoV-2 antibodies. We thank Dr. Michael D. Alpert (Emmune, Inc., USA) for sharing useful comments on this manuscript.

This work was supported by Guangdong Provincial Department of Science & Technology COVID-19 Contingency Funds (2020B1111340063, G.Z.), Shenzhen Bay Laboratory Startup Funds (21230041, G.Z.), and Major Program of Shenzhen Bay Laboratory (S201101001-2, G.Z.).

Contributions

G.Z. conceived and designed this study. W.Y., Y.W., D.M., X.T., H.W., and Y.L. generated all experimental materials. W.Y. and Y.L. performed all experiments, acquired and analyzed all data. C.L. and H.L. contributed key resources. G.Z. wrote the manuscript.

Competing interests

Shenzhen Bay Laboratory has filed a PCT patent application for ACE2-Ig variants.

References

1 Polack, F. P. *et al.* Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. *N Engl J Med* **383**, 2603-2615, (2020).

2 Baden, L. R. *et al.* Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine. *N Engl J Med*, (2020).

3 Wang, H. *et al.* Development of an Inactivated Vaccine Candidate, BBIBP-CorV, with Potent Protection against SARS-CoV-2. *Cell* **182**, 713-721 e719, (2020).

4 Xia, S. *et al.* Safety and immunogenicity of an inactivated SARS-CoV-2 vaccine, BBIBP-CorV: a randomised, double-blind, placebo-controlled, phase 1/2 trial. *Lancet Infect Dis* **21**, 39-51, (2021).

5 Gao, Q. *et al.* Development of an inactivated vaccine candidate for SARS-CoV-2. *Science* **369**, 77-81, (2020).

6 Logunov, D. Y. *et al.* Safety and immunogenicity of an rAd26 and rAd5 vector-based heterologous prime-boost COVID-19 vaccine in two formulations: two open, non-randomised phase 1/2 studies from Russia. *Lancet* **396**, 887-897, (2020).

7 Voysey, M. *et al.* Safety and efficacy of the ChAdOx1 nCoV-19 vaccine (AZD1222) against SARS-CoV-2: an interim analysis of four randomised controlled trials in Brazil, South Africa, and the UK. *Lancet* **397**, 99-111, (2021).

8 Baum, A. *et al.* REGN-COV2 antibodies prevent and treat SARS-CoV-2 infection in rhesus macaques and hamsters. *Science* **370**, 1110-1115, (2020).

9 Shi, R. *et al.* A human neutralizing antibody targets the receptor-binding site of SARS-CoV-2. *Nature* **584**, 120-124, (2020).

10 Zhou, P. *et al.* A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* **579**, 270-273, (2020).

11 Li, Y. *et al.* SARS-CoV-2 and Three Related Coronaviruses Utilize Multiple ACE2 Orthologs and Are Potently Blocked by an Improved ACE2-Ig. *J Virol* **94**, (2020).

12 Shi, J. *et al.* Susceptibility of ferrets, cats, dogs, and other domesticated animals to SARS-coronavirus 2. *Science* **368**, 1016-1020, (2020).

13 Cui, J., Li, F. & Shi, Z. L. Origin and evolution of pathogenic coronaviruses. *Nat Rev Microbiol* **17**, 181-192, (2019).

14 Wang, J. *et al.* Mouse-adapted SARS-CoV-2 replicates efficiently in the upper and lower respiratory tract of BALB/c and C57BL/6J mice. *Protein Cell* **11**, 776-782, (2020).

15 Dinnon, K. H., 3rd *et al.* A mouse-adapted model of SARS-CoV-2 to test COVID-19 countermeasures. *Nature* **586**, 560-566, (2020).

16 Gu, H. *et al.* Adaptation of SARS-CoV-2 in BALB/c mice for testing vaccine efficacy. *Science* **369**, 1603-1607, (2020).

17 Li, Y. *et al.* Single amino-acid change in mouse Ace2 or viral spike enables SARS-CoV-2 to utilize mouse Ace2. (2021. manuscript submitted).

18 Kaushal, N. *et al.* Mutational Frequencies of SARS-CoV-2 Genome during the Beginning Months of the Outbreak in USA. *Pathogens* **9**, (2020).

19 Gribble, J. *et al.* The coronavirus proofreading exoribonuclease mediates extensive viral recombination. *PLoS Pathog* **17**, e1009226, (2021).

20 Thomson, E. C. *et al.* The circulating SARS-CoV-2 spike variant N439K maintains fitness while evading antibody-mediated immunity. *bioRxiv*, (2020).

21 Davies, N. G. *et al.* Estimated transmissibility and severity of novel SARS-CoV-2 Variant of Concern 202012/01 in England. *medRxiv*, (2020).

22 Laydon, D. J. *et al.* Transmission of SARS-CoV-2 Lineage B.1.1.7 in England: Insights from linking epidemiological and genetic data. *MedRxiv*, (2020).

23 Tegally, H. *et al.* Emergence and rapid spread of a new severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) lineage with multiple spike mutations in South Africa. *medRxiv*, (2020).

24 Diseases, J. N. I. o. I. Brief report: New Variant Strain of SARS-CoV-2 Identified in Travelers from Brazil. doi:https://www.niid.go.jp/niid/images/epi/corona/covid19-33-en-210112, (2021).

25 Oude Munnink, B. B. *et al.* Transmission of SARS-CoV-2 on mink farms between humans and mink and back to humans. *Science* **371**, 172-177, (2021).

26 Welkers, M. R. A., Han, A. X., Reusken, C. B. E. M. & Eggink, D. Possible host-adaptation of SARS-CoV-2 due to improved ACE2 receptor binding in mink. *Virus evolution*, (2020).

27 Elaswad, A., Fawzy, M., Basiouni, S. & Shehata, A. A. Mutational spectra of SARS-CoV-2 isolated from animals. *PeerJ* **8**, e10609, (2020).

28 Kemp, S. A. *et al.* Neutralising antibodies drive Spike mediated SARS-CoV-2 evasion. *MedRxiv*, (2020).

29 Li, Q. *et al.* The Impact of Mutations in SARS-CoV-2 Spike on Viral Infectivity and Antigenicity. *Cell* **182**, 1284-1294 e1289, (2020).

30 Wu, K. *et al.* mRNA-1273 vaccine induces neutralizing antibodies against spike mutants from global SARS-CoV-2 variants. *bioRxiv*, (2021).

31 Wang, Z. *et al.* mRNA vaccine-elicited antibodies to SARS-CoV-2 and circulating variants. *bioRxiv*, (2021).

32 Rees-Spear, C. *et al.* The impact of Spike mutations on SARS-1 CoV-2 neutralization. *bioRxiv*, (2021).

Figures and legends

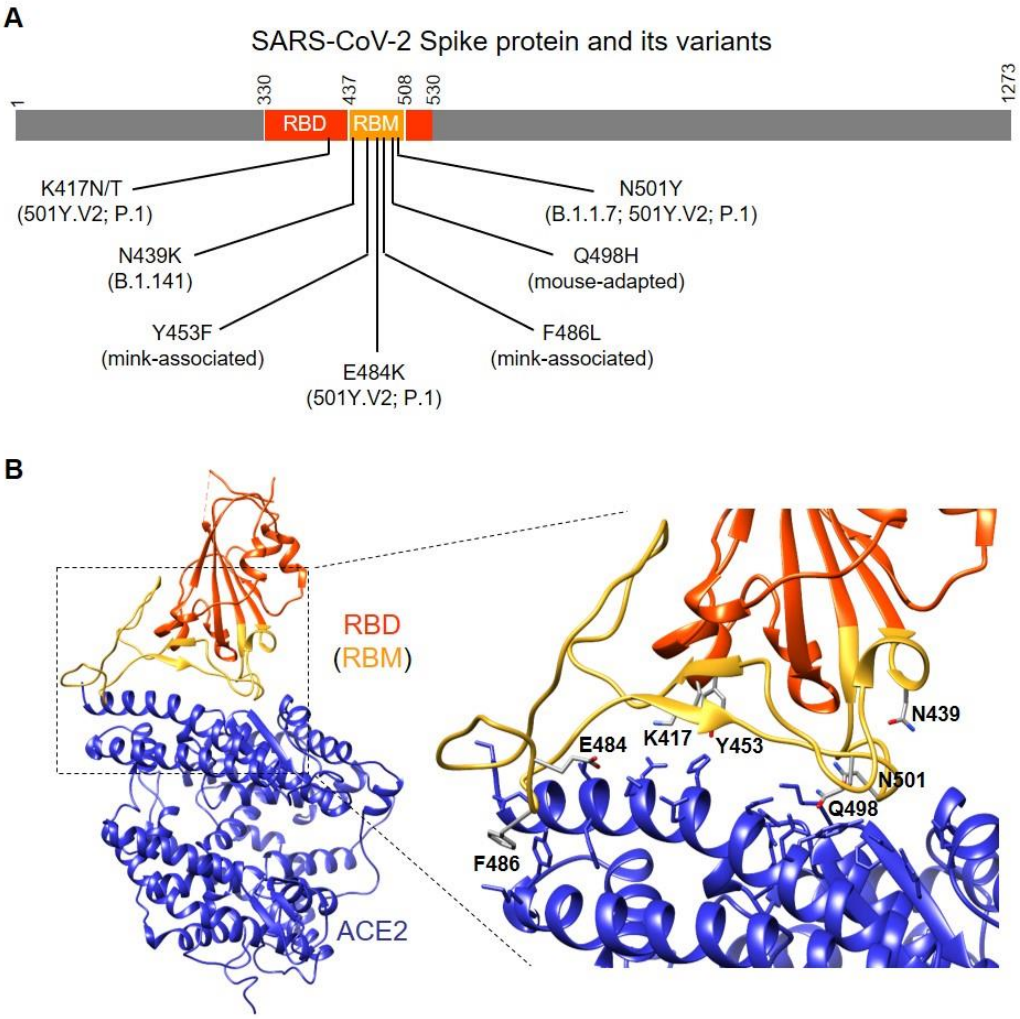

Figure 1. SARS-CoV-2 Spike variants. (**A**) SARS-CoV-2 Spike amino acid numbers are indicated. The receptor binding domain (RBD) is in red and the receptor binding motif (RBM) is in yellow. Spike mutations investigated in this study are indicated, with the associated SARS-CoV-2 variant number shown in parentheses. (**B**) Interactions between SARS-CoV-2 RBD (red) and ACE2 (PDB code 6M0J). The ACE2 residues in less than 4 Å from RBD atoms are shown. SARS-CoV-2 variant-associated RBD mutations that were investigated in following study are shown and labelled, including residues K417N, N439K, Y453F, E484K, F486L, Q498H, and N501Y.

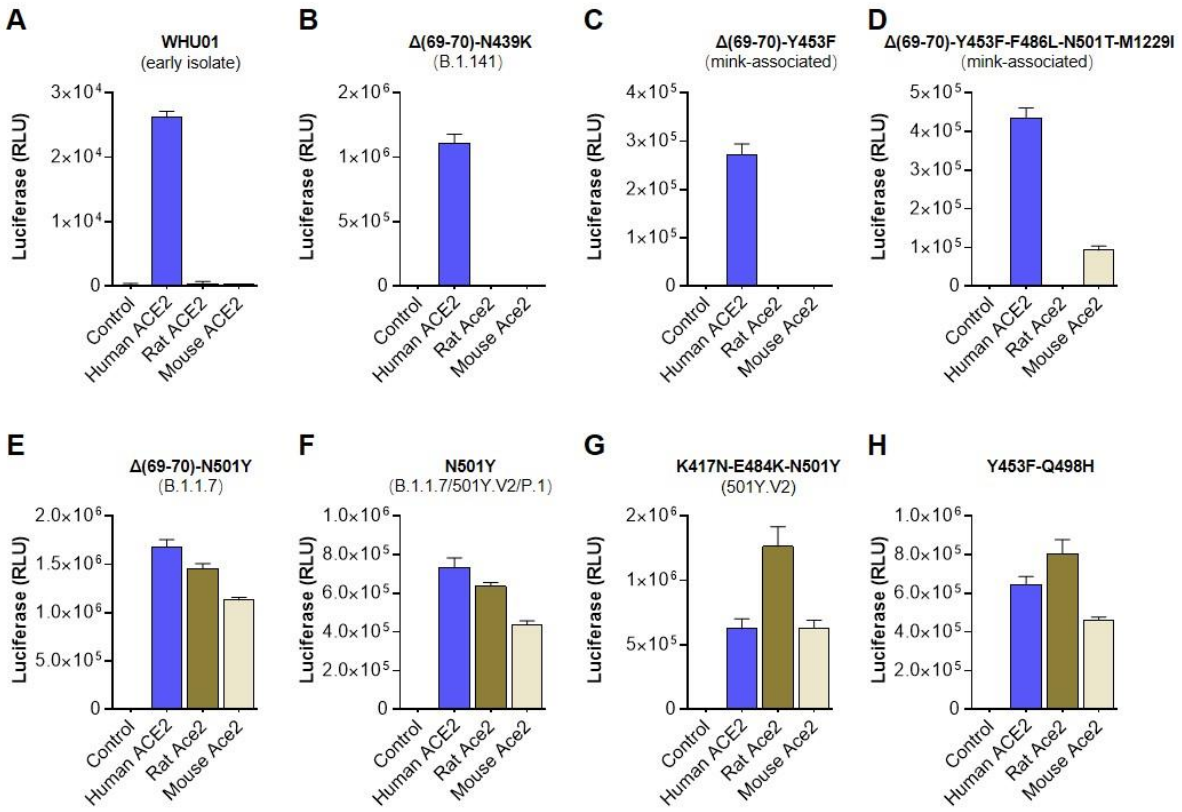

Figure 2. Cross-species receptor usage of multiple SARS-CoV-2 variants that emerged during the pandemic. 293T cells in 96-well plates were transfected with 60 ng of a vector control plasmid, or a plasmid expressing an ACE2 ortholog of human, rat, or mouse. Cells were then infected with reporter retroviruses pseudotyped with the indicated SARS-CoV-2 Spike variants. ACE2 ortholog-mediated viral entry was measured by luciferase reporter expression at 48 hours post infection. Data shown are representative of two or three experiments independently performed by two different people with similar results, and data points represent mean ± s.d. of three biological replicates.

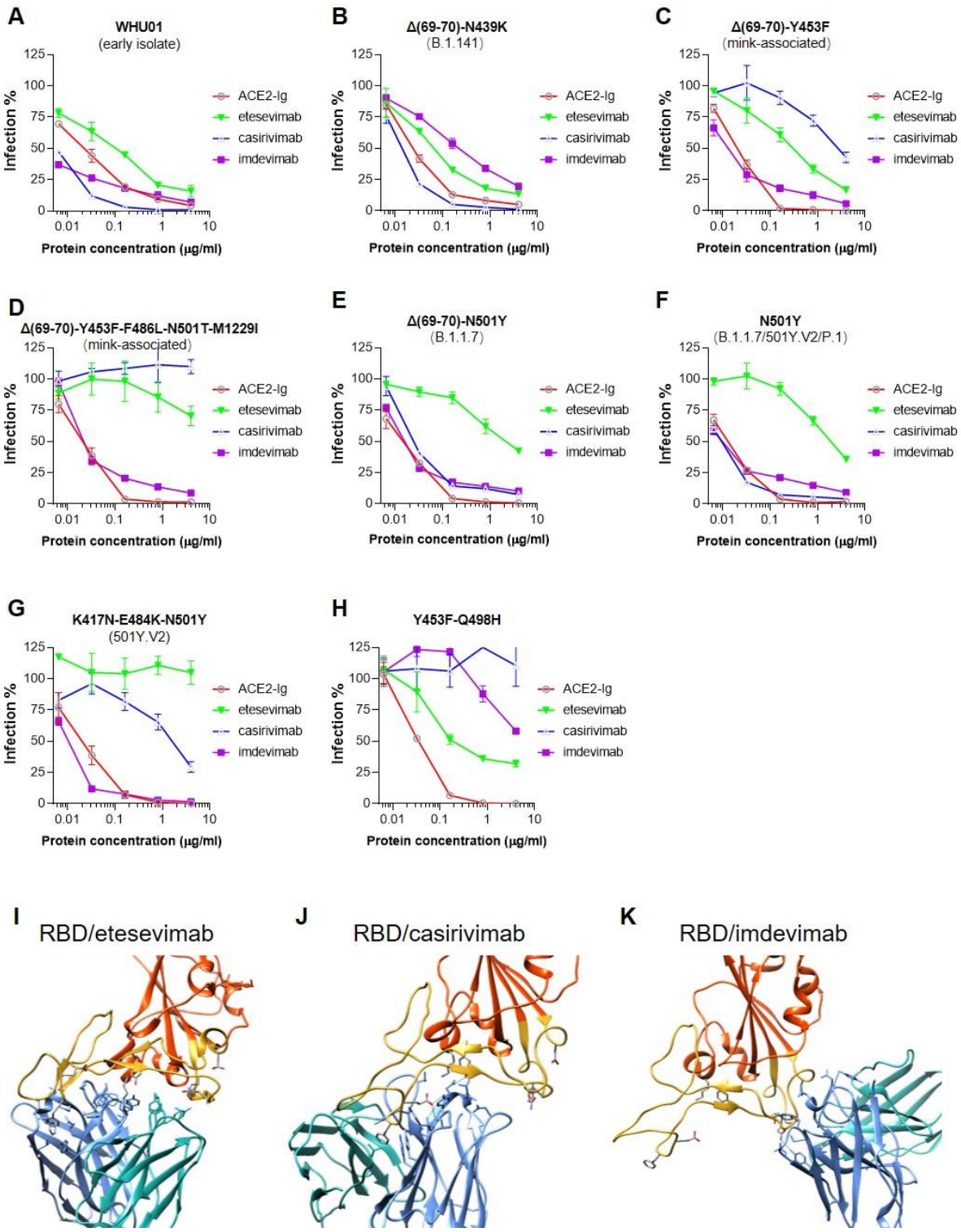

Figure 3. Sensitivity of SARS-CoV-2 variants to neutralizing antibodies and ACE2-Ig. (**A-H**) HeLa-hACE2 cells in 96-well plates were infected with SARS-CoV-2 Spike variant-pseudotyped retroviruses in the presence of an ACE2-Ig variant we developed recently or a clinical-stage anti-SARS-CoV-2 antibody (etesevimab, casirivimab, or imdevimab) at the

indicated concentrations. Etesevimab was originally named as CB6[ref.9], casirivimab as REGN10933[ref.8], and imdevimab as REGN10987[ref.8]. Viral entry was measured by luciferase reporter expression at 48 hours post infection. Luminescence values observed at each concentration were divided by the values observed at concentration zero to calculate percentage-of-infection (Infection%) values. Data shown are representative of two experiments independently performed by two different people with similar results, and data points represent mean ± s.d. of three biological replicates. Estimated IC50 and IC90 values for each protein are summarized in Table 1. (**I-K**) Interactions between SARS-CoV-2 RBD and monoclonal antibody etesevimab (**I**; PDB code 7C01), casirivimab (**J**; PDB code 6XDG), and imdevimab (**K**; PDB code 6XDG). The RBD is shown in red and yellow, and antibodies are shown in blue and green. The antibody residues in less than 4 Å from RBD atoms and SARS-CoV-2 variant-associated RBD mutations are shown. SARS-CoV-2 variant-associated RBD mutations that were investigated in this study are shown, including residues K417N, N439K, Y453F, E484K, F486L, Q498H, and N501Y, as shown in Figure 1B.

Table 1. IC50 (in parentheses) and IC90 values of the tested proteins (μg/mL)

	WHU01	Δ(69-70)-N439K	Δ(69-70)-Y453F	Δ(69-70)-Y453F-F486L-N501T-M1229I	Δ(69-70)-N501Y	N501Y	K417N-E484K-N501Y	Y453F-Q498H
etesevimab	>4.0 (0.1)	>4.0 (0.065)	>4.0 (0.3)	>>4.0 (>4.0)	>4.0 (2.2)	>4.0 (2.0)	n.r.	>4.0 (0.18)
casirivimab	0.045 (<0.0064)	0.1 (0.015)	>4.0 (1.6)	n.r.	1.5 (0.25)	0.11 (0.009)	>4.0 (1.7)	n.r.
imdevimab	1.5 (<0.0064)	>4.0 (0.22)	1.5 (0.015)	2.5 (0.02)	3.5 (0.16)	3 (0.1)	0.06 (0.01)	>>4.0 (>4.0)
ACE2-Ig	0.7 (0.022)	0.45 (0.025)	0.12 (0.02)	0.12 (0.02)	0.12 (0.015)	0.11 (0.013)	0.15 (0.02)	0.15 (0.035)

IC90 (μg/mL)
[0.32, 0.16]
[0.16, 0.8]
[0.8, 4.0]
>4.0
>>4.0 (IC50 >4.0)
n.r. (non-responsive)

Materials and methods

Cells

293T and HeLa cells were kindly provided by Stem Cell Bank, Chinese Academy of Sciences, confirmed mycoplasma-free by the provider, and maintained in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) at 37 °C in a 5% CO_2-humidified incubator. Growth medium was supplemented with 2 mM Glutamax-I (Gibco, Cat. No. 35050061), 100 μM non-essential amino acids (Gibco, Cat. No. 11140050), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Cat. No. 15140122), and 10% FBS (Gibco, Cat. No. 10099141C). HeLa-based stable cells expressing human ACE2 (HeLa-hACE2) were maintained under the same culture condition as HeLa, except that 3 μg/mL of puromycin was added to the growth medium. 293F cells for the production of ACE2-Ig protein and SARS-CoV-2 antibodies were generously provided by Dr. Yu J. Cao (School of Chemical Biology and Biotechnology, Peking University Shenzhen Graduate School) and maintained in SMM 293-TII serum-free medium (Sino Biological, Cat. No. M293TII) at 37 °C, 8% CO_2, in a shaker incubator at 125 rpm.

Plasmids

DNA fragments encoding Spike proteins of SARS-CoV-2 WHU01 (GenBank: MN988668.1) was synthesized by the Beijing Genomic Institute (BGI, China) and then cloned into pcDNA3.1(+) plasmid between EcoRI and XhoI restriction sites. Plasmids encoding SARS-CoV-2 Spike variants were generated according the in-fusion cloning protocol. To facilitate SARS-CoV-2 pseudovirus production, Spike sequences for WHU01 and all the variants investigated in this study all contain a furin-cleavage site mutation (ΔPRRA). We had shown in our previous study that the ΔPRRA mutation does not affect SARS-CoV-2 cross-species receptor usage or neutralization sensitivity[11]. Plasmids encoding the ACE2-Ig variant were generated in our previous study[11]. DNA fragments encoding heavy and light chains of SARS-

CoV-2 antibodies (etesevimab, casirivimab, and imdevimab) were synthesized by Sangon Biotech (Shanghai, China) and then cloned into a pCAGGS plasmid. The retroviral reporter plasmids encoding a Gaussia luciferase reporter gene were constructed by cloning the reporter genes into the pQCXIP plasmid (Clontech). DNA fragments encoding C-terminally S-tagged ACE2 orthologs were synthesized in pUC57 backbone plasmid by Sangon Biotech (Shanghai, China). These fragments were then cloned into pQCXIP plasmid (Clontech) between SbfI and NotI restriction sites.

Production and Purification of ACE2-Ig protein and SARS-CoV-2 antibodies

293F cells at the density of 6×10^5 cells/mL were seeded into 100 mL SMM 293-TII serum-free medium (Sino Biological, Cat. No. M293TII) one day before transfection. Cells were then transfected with 100 µg plasmid in complex with 250 µg PEI MAX 4000 (Polysciences, Inc, Cat. No. 24765-1). Cell culture supernatants were collected at 48 to 72 hours post transfection. Human IgG1 Fc-containing proteins were purified using Protein A Sepharose CL-4B (GE Healthcare, Cat. No. 17-0780-01), eluted with 0.1 M citric acid at pH 4.5 and neutralized with 1 M Tris-HCl at pH 9.0. Buffers were then exchanged to PBS and proteins were concentrated by 30 kDa cut-off Amicon Ultra-15 Centrifugal Filter Units (Millipore, Cat. No. UFC903096).

Production of reporter retroviruses pseudotyped with SARS-CoV-2 Spike variants

MLV retroviral vector-based SARS-CoV-2 Spike pseudotypes were produced according to our previous study[11], with minor changes. In brief, 293T cells were seeded at 30% density in 150 mm dish at 12-15 hours before transfection. Cells were then transfected with 67.5 µg of polyethylenimine (PEI) Max 40,000 (Polysciences, Inc, Cat. No. 24765-1) in complex with 3.15 µg of plasmid encoding a Spike variant, 15.75 µg of plasmid encoding murine leukemia virus (MLV) Gag and Pol proteins, and 15.75 µg of a pQCXIP-based luciferase reporter plasmid. Eight hours after transfection, cell culture medium was refreshed and changed to

growth medium containing 2% FBS (Gibco, Cat. No. 10099141C) and 25 mM HEPES (Gibco, Cat. No. 15630080). Cell culture supernatants were collected at 36-48 hours post transfection, spun down at 3000×g for 10 min, and filtered through 0.45 μm filter units to remove cell debris. SARS-CoV-2 Spike-pseudotyped viruses were then concentrated 10 times at 2000×g using 100 kDa cut-off Amicon Ultra-15 Centrifugal Filter Units (Millipore. Cat. No. UFC910024).

SARS-CoV-2 pseudovirus infection of 293T cells expressing ACE2 orthologs

Pseudovirus infection assay was performed according to our previous study[11]. In brief, 293T cells were seeded at 20% density in poly-lysine pre-coated 96-well plates 12-15 hours before transfection. Cells in each well were then transfected with 0.2 μL of lipofectamine 2000 (Life Technologies, Cat. No. 11668019) in complex with 60 ng of a vector control plasmid or a plasmid encoding an ACE2 ortholog. Cell culture medium was refreshed at 12 hours post transfection. Additional 18 hours later, cells in each well were infected with 10 μL of SARS-CoV-2 pseudovirus (10×concentrated) diluted in 100 μL of culture medium containing 2% FBS (Gibco, Cat. No. 10099141C). Culture medium was refreshed at 2 hours post pseudovirus infection and the medium was refreshed every 12 hours. Cell culture supernatants were collected and subjected to a Gaussia luciferase assay at 48 hours post infection.

Gaussia luciferase luminescence flash assay

To measure Gaussia luciferase expression, 20 μL of cell culture supernatant of each sample and 100 μL of assay buffer containing 4 μM coelenterazine native (Biosynth Carbosynth, Cat. No. C-7001) were added to one well of a 96-well black opaque assay plate (Corning, Cat. No. 3915), and measured with Centro LB 960 microplate luminometer (Berthold Technologies) for 0.1 second/well.

SARS-CoV-2 pseudovirus neutralization assay

Pseudovirus neutralization experiments were performed following our previous study[11]. In brief, SARS-CoV-2 Spike variant-pseudotyped luciferase reporter viruses were pre-diluted in DMEM (2% FBS, heat-inactivated) containing titrated amounts of the ACE2-Ig or one of the three anti-SARS-CoV-2 antibodies. Virus-inhibitor mixtures were incubated at 37 °C for 30min, then added to HeLa-hACE2 cells in 96-well plates and incubated overnight at 37 °C. Virus-inhibitor-containing supernatant was then removed and changed with 150 μL of fresh DMEM (2% FBS) and incubated at 37 °C. Cell culture supernatants were collected for Gaussia luciferase assay at 48 h post infection.

Data collection and analysis

All the experiments were repeated at least twice with two different people. GraphPad Prism 6.0 software was used for figure preparation and statistical analyses.

Statistical analysis

Data expressed as mean values ± s.d. Statistical analyses were performed using two-sided two-sample Student's t-test using GraphPad Prism 6.0 software when applicable. Differences were considered significant at $P < 0.01$.

Data availability

The study did not generate unique datasets or code. Our research resources, including methods, plasmids, and protocols, are available upon reasonable request to qualified academic investigators for noncommercial research purposes. All reagents developed in this study, such as vector plasmids, as well as detailed methods, will be made available upon written request.