The D614G mutation of SARS-CoV-2 spike protein enhances viral infectivity and decreases neutralization sensitivity to individual convalescent sera

Running Title: D614G mutant spike increases SARS-CoV-2 infectivity

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Abstract

Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The spike protein that mediates SARS-CoV-2 entry into host cells, is one of the major targets for vaccines and therapeutics. Thus, insights into the sequence variations of S protein are key to understanding the infection and antigenicity of SARS-CoV-2. Here, we observed a dominant mutational variant at the 614 position of S protein (aspartate to glycine, D614G mutation). Using pseudovirus-based assay, we found that S-D614 and S-G614 protein pseudotyped viruses share a common receptor, human angiotensin-converting enzyme 2 (ACE2), which could be blocked by recombinant ACE2 with the fused Fc region of human IgG1. However, S-D614 and S-G614 protein demonstrated functional differences. First, S-G614 protein could be cleaved by serine protease elastase-2 more efficiently. Second, S-G614 pseudovirus infected 293T-ACE2 cells significantly more efficiently than the S-D614 pseudovirus, Moreover, 93% (38/41) sera from convalescent COVID-19 patients could neutralize both S-D614 and S-G614 pseudotyped viruses with comparable efficiencies, but about 7% (3/41) convalescent sera showed decreased neutralizing activity against S-G614 pseudovirus. These findings have important implications for SARS-CoV-2 transmission and immune interventions.

Keywords: antiviral therapeutics, coronavirus, D614G mutation, neutralizing antibodies, pseudovirus, SARS-CoV-2, spike protein
Introduction

SARS-CoV-2 is a novel coronavirus reported in 2019 that caused the recent outbreak of coronavirus disease-2019 (COVID-19)\(^1\). By June 17, 2020, the World Health Organization (WHO) reported that 8.06 million people worldwide had been infected with SARS-CoV-2, and 440,290 individuals died of COVID-19. This pandemic had a significant adverse impact on international social and economic activities. The RNA genome of SARS-CoV-2 was rapidly sequenced to facilitate diagnostic testing, molecular epidemiologic source tracking, and development of vaccines and therapeutic strategies\(^2\). Coronaviruses are enveloped, positive-stranded RNA viruses that contain the largest known RNA genomes to date. The mutation rate for RNA viruses is extremely high, which may contribute to its transmission and virulence. The only significant variation in the SARS-CoV-2 spike (S) protein is a non-synonymous D614G (Aspartate (D) to Glycine (G)) mutation\(^3\). Primary data showed that S-G614 is a more pathogenic strain of SARS-CoV-2 with high transmission efficiency\(^3\), however whether D614G conversion in S protein affect the viral entry and infectivity in cell model is still unclear.

The S protein of coronavirus, the major determinant of host and tissue tropism, is a major target for vaccines, neutralizing antibodies, and viral entry inhibitors\(^4,5\). Similar to SARS-CoV, the cellular receptor of SARS-CoV-2 is angiotensin-converting enzyme 2 (ACE2); however, the SARS-CoV-2 S protein has
a 10- to 20-fold higher affinity for ACE2 than the corresponding S protein of SARS-CoV\textsuperscript{6,7}. Coronaviruses use two distinct pathways for cell entry:
protease-mediated cell-surface pathway and endosomal pathway\textsuperscript{8}. The S proteins of several coronaviruses are cleaved by host proteases into S1 subunit for receptor binding and S2 subunit for membrane fusion at the entry step of infection. Several cellular proteases, including furin, transmembrane protease serine 2 (TMPRSS2) and cathepsin (Cat)B/L, are critical for priming the SARS-CoV-2 S protein to enhance ACE2-mediated viral entry\textsuperscript{4}. Recently, Chandrika Bhattacharyya \textit{et al} reported that a novel serine protease (elastase-2) cleavage site was introduced in S-G614 protein of SARS-CoV-2 variant\textsuperscript{9}. However, it is unknown whether S-G614 protein can be processed and activated by elastase-2 in cell model. The S protein plays a key role in the evolution of coronavirus to evade the host immune system. It is still uncertain whether D614G mutation affect the antigenic properties of the S protein. Whether elastase-2 inhibitors and convalescent serum samples of COVID-19 can block the infection of SARS-CoV-2 D614G variant remains unclear.

In this study, we analyzed the S gene sequences of SARS-CoV-2 submitted to the Global Initiative on Sharing All Influenza Data (GISAID) database. We showed the expression and cleavage of S-D614 and S-G614 protein in cell lines. Using a luciferase (Luc)-expressing lentiviral pseudotype system, we established a quantitative pseudovirus-based assay for the evaluation of SARS-CoV-2 cell entry mediated by the viral S protein variants. We also compared the neutralizing
sensitivity of the S-D614 and S-G614 protein pseudovirus to convalescent sera from COVID-19 patients. Our study provide further insight into the transmission and immune interventions of this newly emerged virus.

Methods

Plasmids. The codon-optimized gene encoding SARS-CoV-2 S protein (GenBank: QHD43416) with C terminal 19 amino acids deletion was synthesized by Sino Biological Inc. (Beijing, China), and cloned into the Kpn I and Xba I restriction sites of pCMV3 vector (pCMV3-SARS-CoV-2-S-C19del, denoted as pS-D614). The D614G mutant S expressing plasmid (denoted as pS-G614) was constructed by site-directed mutagenesis, with pS-D614 plasmid as a template. The HIV-1 NL4-3 ΔEnv Vpr luciferase reporter vector (pNL4-3.Luc.R-E-) constructed by N. Landau10, was provided by Prof. Cheguo Cai from Wuhan University (Wuhan, China). The vesicular stomatitis virus G (VSV-G)-expressing plasmid pMD2.G was provided by Prof. Ding Xue from the Tsinghua University (Beijing, China). The expression plasmid for human ACE2 and ELANE (elastase-2) were obtained from Genecopoeia (Guangzhou, China).

Cell lines. HEK293T cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM; Hyclone, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 mg/mL of
streptomycin, and 100 unit/mL of penicillin at 37 °C in 5% CO₂. HEK293T cells
transfected with human ACE2 (293T-ACE2) were cultured under the same
conditions with the addition of G418 (0.5 mg/mL) to the medium.

Antibodies and inhibitors. The anti-RBD (receptor-binding domain) monoclonal
antibody against the SARS-CoV-2 S protein was kindly provided by Prof. Aishun Jin
from Chongqing Medical University. Recombinant human ACE2 linked to the Fc
domain of human IgG1 (ACE2-Ig, Sino Biological Inc.). Camostat mesylate (Tokyo
Chemical Industry, Tokyo, Japan) and aloxistatin (E-64d; MedChemExpress,
Monmouth Junction, NJ, USA) were dissolved in dimethyl sulfoxide (DMSO) at a
stock concentration of 50 mM.

Serum samples. A total of 41 convalescent COVID-19 patient sera (at 2-4 weeks
after symptom onset) were collected from three designated hospitals in Chongqing
in the period from February 5 to February 10, 2020 (Supplementary Table 1). All
sera were tested positive using MCLIA kits supplied by Bioscience Co. (Tianjin,
China)¹¹. Patient sera were incubated at 56 °C for 30 min to inactivate the
complement prior to experiments.

SARS-CoV-2 genome analysis
The online Nextstrain analysis tool (https://nextstrain.org/ncov) was used to track
D614G mutation in SARS-CoV-2 genomes. Global of 2834 genomes sampled
between Dec 20, 2019 and Jun 12, 2020 were visualized using the ‘rectangular’ layout. The mutations were labeled in branch.

All complete SARS-CoV-2 S gene sequences were downloaded from NCBI website (https://www.ncbi.nlm.nih.gov/sars-cov-2/) on Jun 1, 2020. We obtained 4701 S coding sequences from NCBI, after excluded partial and frameshift sequences, 4649 completed S sequences were used to further analysis. All S nucleotide sequences were translated to amino acid sequences. The nucleotide and amino acid sequences of S were aligned with multiple sequence alignment software MUSCLE separately. The ‘Wuhan-Hu-1’ strain (NC_045512) was used to be the reference sequence, and the mutations were extracted using private PERL scripts.

**Western blot analysis of SARS-CoV-2 S protein expression.** To analyze S protein expression in cells, S-D614 and S-G614 expressing plasmids were transfected into HEK293T cells respectively. Total protein was extracted from cells using RIPA Lysis Buffer (CWbiotech, Beijing, China) containing 1 mM phenyl methyl sulfonyl fluoride (PMSF; Beyotime, Shanghai, China). Equal amounts of protein samples were electrophoretically separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The immunoblots were probed with the indicated antibodies. Protein bands were visualized using SuperSignal™ West Pico Chemiluminescent Substrate kits (Bio-Rad, Hercules, CA, USA) and quantified by densitometry using ImageJ.
Production and titration of SARS-CoV-2 S pseudoviruses. SARS-CoV-2 pseudotyped viruses were produced as previously described with some modifications. Briefly, $5 \times 10^6$ HEK293T cells were co-transfected with $6 \mu g$ pNL4-3.Luc.R-E- and $6 \mu g$ recombinant SARS-CoV-2 S plasmids (pS-D614, or pS-G614) using the Lipofectamine 3000 transfection reagent (Invitrogen) according to the manufacturer’s instructions. The cells were transferred to fresh DMEM 12 h later. The S-D614 and S-G614 protein pseudotyped viruses in supernatants were harvested 48 h after transfection, centrifuged, filtered through a 0.45 μm filter and stored at −80°C. The pMD2.G was co-transfected with the pNL4-3.Luc.R-E- plasmid to package the VSV-G pseudovirus.

The titers of the pseudoviruses were calculated by determining the number of viral RNA genomes per milliliter of viral stock solution using real-time (RT)-qPCR with primers and a probe that target LTR. Sense primer:

5'-TGTGTGCCCCGTCTGTGTGTG-3', anti-sense primer:

5'-GAGTCCTGCGTGACAGAGC-3', probe:

5'-FAM-CAGTGGCGCCGAACAGGGA-BHQ1-3'. Briefly, viral RNAs were extracted with Trizol (Invitrogen, Rockville, MD) and treated with RNase-free DNase (Promega, Madison, WI, USA) and re-purified using mini columns. Then RNA was amplified using the TaqMan One-Step RT-PCR master mix reagents (Applied...
Biosystems, ThermoFisher). The known quantity of pNL4-3.Luc.R-E- vector was used to generate standard curves. The S-D614 and S-G614 protein pseudotyped viruses were adjusted to the same titer (copies/ml) for the following experiments.

**SARS-CoV-2 spike-mediated pseudovirus entry assay.** To detect S variants mediated viral entry, 293T-ACE2 cells (2×10^4) grown in 96-well plates were respectively infected with same amount of S-D614 or S-G614 pseudovirus (3.8×10^4 copies in 50 μL). Cells were transferred to fresh DMEM medium 8 h post-infection, and relative luminescence units (RLU) were measured 24-72 h post-infection by Luciferase Assay Reagent (Promega, Madison, WI, USA) according to the manufacturer's protocol.

**Neutralization and inhibition assays.** The 293T-ACE2 cells (2×10^4 cells/well) were seeded in 96-well plates. For the neutralization assay, 50 μL pseudoviruses, which is equivalent to 3.8×10^4 vector genomes, were incubated with serial dilutions of sera sample from patients and human normal serum as a negative control for 1 h at 37 °C, then added to the 96-well 293T-ACE2 cells with 3 replicates. For the inhibition assay, the cells were pretreated with the protease inhibitors (camostat mesylate or E-64d) 2 h before infection. After 12 h incubation, the medium was replaced with fresh cell culture medium. Luciferase activity was measured 72 h after infection and the percent neutralization was calculated using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA). Percentages of RLU
reduction (inhibition rate) were calculated as: 1 - (RLU of sample sera - control wells)/( RLU from mock control sera - control wells)) X100%. The titers of neutralizing antibodies were calculated as 50% inhibitory dose (ID$_{50}$).

**Statistical analyses.** Statistical analyses of the data were performed by using GraphPad Prism version 6.0 software. Statistical significance was determined using ANOVA for multiple comparisons. Student's t-tests were applied to compare the two groups. Differences with $P$-values < 0.05 were deemed statistically significant.

**Ethical approval.** The study was approved by the Ethics Commission of Chongqing Medical University (ref. no. 2020003). Written informed consent was waived by the Ethics Commission of the designated hospital for emerging infectious diseases.

**Results**

**D614G mutation of SARS-CoV-2 spike protein was globally distributed**

The spike (S) protein of SARS-CoV-2, containing 1273 amino acids, forms a trimeric spike on the virion surface that plays essential roles in viral entry. We analyzed the SARS-CoV-2 S protein amino acid sequence from viral genomic sequences in GISAID database. In line with prior reports, we found a globally distributed S protein mutation, D614 to G614, which represented in 64.6% of all the
analyzed sequences (Fig.1 and Table 1). Among the top 10 most abundant non-synonymous mutations observed in S protein, the relative abundance of the D614G mutant (the clade G) was the highest around the world, indicating that G614 strain may be selectively advantageous. Since the S protein is critical to coronavirus infection, we sought to explore the impact of the most prevalent D614G mutation on the S protein expression and function.

D614G mutation enhanced the cleavage of S protein variant by proteases

We predicted potential cleavage sites of proteases in S protein variants by PROSPER\textsuperscript{15}, and found a novel serine protease (elastase-2) cleavage site at 615-616 residues on S1-S2 junction region of S-G614 protein (Fig. 2a, Supplementary Table 2). To evaluate the expression and cleavage of SARS-CoV-2 S protein in the human cell line, the codon-optimized S expressing plasmids (pS-D614 and pS-G614) were transfected into HEK293T cells, respectively. Immunoblot analysis of whole cell lysates revealed that both S-D614 and S-G614 proteins showed two major protein bands (unprocessed S and cleaved S1 subunit), when reacted with the monoclonal antibody targeting the RBD on the SARS-CoV-2 spike (Fig. 2b). However, the pS-G614-transfected cells showed stronger S1 signal than pS-D614-transfected cells, indicating that D614G mutation altered cleavability of S protein by the cellular protease. Moreover, the elastase-2 inhibitor sivelestat sodium significantly decreased the S1 signal of S-D614 protein (Fig. 2b). These data indicated that the D614G mutation of SARS-CoV-2 S facilitates its cleavage by
host serine protease elastase-2. The coronavirus S protein must be cleaved by host proteases to enable membrane fusion, which is critical for viral entry. Next, we sought to explore the impact of D614G mutation on virus entry.

**Evaluating viral entry efficacies between S-D614 and S-G614 pseudotyped lentiviral particles**

Lentiviral vectors can be pseudotyped with various heterologous viral glycoproteins that modulate cellular tropism and cell-entry properties\(^{16}\). Due to the highly pathogenic nature of SARS-CoV-2, infectious SARS-CoV-2 must be handled in a biosafety level 3 (BSL-3) facility. We generated a pseudotyped SARS-CoV-2 based on the viral S protein using a lentiviral system, which introduced a Luc reporter gene for quantification of coronavirus S-mediated entry. pNL4-3.Luc.R-E- was co-transfected with pS-D614, pS-G614, respectively to package the SARS-CoV-2 S pseudotyped single-round Luc virus in HEK293T cells.

The titers of S-D614 and S-G614 protein pseudotyped viruses were determined by RT-qPCR expressed as the number of viral RNA genomes per milliliter, and then adjusted to the same concentration (3.8x10^4 copies in 50 μL) for the following experiments. The virus infectivity was determined by a Luc assay expressed in RLU. HEK293T cells expressing human ACE2 (293T-ACE2) was used test the correlation between ACE2 expression and pseudovirus susceptibility. VSV-G pseudovirus was used as a control. As shown in Fig. 3a, both HEK293T and 293T-ACE2 cells could be effectively transduced by VSV-G pseudovirus. However,
the entry of S-D614 and S-G614 pseudovirus is highly dependent on its cellular receptor ACE2 expression. The 293T-ACE2 cells showed an approximately 250-fold and 530-fold increase in Luc activity compared to HEK293T cells, when transduced by S-D614 and S-G614 pseudoviruses, respectively (Fig. 3a). We next detected the inhibitory ability of ACE2-Ig, a fusion protein consisting of the extracellular domain (Met 1-Ser 740) of human ACE2 linked to the Fc region of human IgG1 at the C-terminus\(^\text{17}\). Both S-D614 and S-G614 pseudoviruses were potently inhibited by ACE2-Ig, and the IC\(_{50}\) (the concentration causing 50% inhibition of pseudovirus infection) was 0.13 and 0.15 \(\mu\)g/mL, respectively (Fig. 3b).

To further compare the viral entry efficiency mediated by S variants, we detected the Luc activity at different time post-infection. With the G614 Spike variant, the increase in viral transduction over the D614 variant was 2.2-fold at 48 h post-infection. The highest transduction efficiency (approximately 2.5 \(\times\) 10\(^4\) RLU) was observed 72 h post-infection with S-G614 pseudovirus, which was approximately 2.4-fold higher than S-D614 pseudovirus (Fig. 3c). These data suggested that the D614G mutation in S protein significantly promotes virus entry into ACE2-expressing cells, and ACE2-Ig efficiently blocks both wild-type and mutant S pseudotype virus infection. We next explored the mechanism with which S-G614 increased pseudovirus infectivity.

**Protease inhibitors blocked the infection of S-G614 pseudovirus**

The proteolytic activation of S protein is required for coronavirus infectivity, and
protease-mediated cell-surface pathway is critical for SARS-CoV-2 entry. Since we observed that S-G614 could be more efficiently cleaved by host protease when exogenously expressed in 293T cells, we assumed that host proteases may be involved in the enhancement of S-G614 virus entry. Two clinically proven protease inhibitors, camostat mesylate and E-64d, which block host TMPRSS2 and CatB/L, respectively, were tested on S-D614 and S-G614 protein pseudotyped lentiviral particles. As shown in Fig. 3a, in 293T-ACE2 cells lacking TMPRSS2, the serine protease inhibitor camostat mesylate did not inhibit S-D614 or S-G614 pseudovirus infection. While the cysteine proteases inhibitor E-64d significantly blocked these two pseudoviruses entry, and the IC$_{50}$ was 0.37 μM for S-D614 pseudovirus and 0.24 μM for S-G614 pseudovirus (Fig. 3b). As expected, these protease inhibitors had no impact on VSV-G pseudovirus infection. Together, these results suggested that S-mediated viral entry into TMPRSS2 deficient 293T-ACE2 cells is endosomal cysteine proteases CatB/L dependent, therefore S-D614 and S-G614 pseudovirus show similar sensitivity to the CatB/L inhibitor E-64d in 293T-ACE2 cells.

Neutralization effect of convalescent COVID-19 patient sera against S-D614 and S-G614 pseudoviruses

Neutralizing antibodies are important for prevention and possibly recovery from viral infections, however, as viruses mutate during replication and spread, host neutralizing antibodies generated in the earlier phase of the infection may not be as effective later on$^{18,19}$. To test whether D614G mutations could affect the
neutralization sensitivity of the virus, the neutralization activity of serum samples from convalescent patients with COVID-19 against SARS-CoV-2 S-D614 and S-G614 pseudoviruses were evaluated. To perform the neutralization assay, 50 μl pseudovirus (3.8x10⁴ copies) was incubated with serial diluted serum. As shown in Fig.4a, the inhibition rate of serum from convalescent COVID-19 patients was analyzed at a single dilution of 1:1000. Among the 41 tested sera, 38 of them showed neutralizing activities against both S-D614 and S-G614 pseudoviruses with comparable efficiencies. Notably, 3 serum samples (patients 1#, 7#, 40#) decreased inhibition rate against the S-G614 pseudovirus. The serum from patient 1# failed to neutralize the S-G614 pseudovirus though it neutralized about 30% of the S-D614 pseudovirus at 1:1000 dilution. Then the inhibition curves and ID₅₀ for serum samples from 5 convalescent patient and a health donor were analyzed. Sera from patients 17# and 39#, which were able to neutralize both two pseudotype viruses to similar extents, showed similar ID₅₀ values (Fig.4b). However, sera from patients 1#, 7# and 40# showed high neutralizing activity against S-D614 pseudovirus with ID₅₀ ranging from 894 to 1337, but decreased neutralizing activity against S-G614 pseudovirus with ID₅₀ ranging from 216 to 367, indicating 3.6 to 4.7-fold reduction in neutralizing titers (Fig.4b). These data indicated that D614G mutation changes the antigenicity of S protein, thereby decreasing neutralization sensitivity to individual convalescent sera.

**Discussion**
Pseudovirus-based assays have been widely used for the study of cellular tropism, receptor recognition, viral inhibitors, and evaluation of neutralizing antibodies without the requirement of BSL-3 laboratories. The present study generated a pseudotyped SARS-CoV-2 based on the viral S protein using a lentiviral system, which incorporated a Luc reporter gene for the easy quantification of coronavirus S-mediated entry. We study the major mutation in S protein at position 614, and found that serine protease elastase-2 participate in the proteolytic activation of the S-G614 protein, thereby enhancing viral entry into 293T-ACE2 cells. In addition, we found that the S-G614 pseudovirus was more resistant to neutralizing antisera than S-G614 pseudovirus.

In this study, we found that the entry efficiency of S-G614 pseudotyped virus was about 2.4 times higher than that of the S-D614 pseudovirus when normalized input virus doses, suggesting that D614G mutation promotes the infectivity of SARS-CoV-2 and enhances viral transmissibility. Since pseudovirus was a single-round of infection assay, this seemingly small increase in entry activity could cause a large difference in viral infectivity in vivo. Hangping Yao et al reported that a patient-derived viral isolate ZJU-1, which harbors the D614G mutation, has a viral load 19 times higher than isolate ZJU-8 (harboring the S-D614) when infecting Vero-E6 cells. However, the ZJU-1 isolate contains other two non-synonymous mutations in ORF1a and envelope (E) gene. Our results also provide some explanation for the association of the D614G mutation in S protein of SARS-CoV-2
with increased entry efficiency. The S-G614 protein contains a novel serine protease cleavage site, so it could be cleaved by host elastase-2 more efficiently. Previously studies on SARS-CoV demonstrated that the protease-mediated cell surface entry facilitated a 100- to 1,000-fold higher efficient infection than did the endosomal pathway in the absence of proteases\textsuperscript{21}.

The elastase-2, also known as neutrophil elastase, plays an important role in degenerative and inflammatory diseases. Sivelestat, approved to treat acute respiratory distress syndrome (ARDS) in Japan and South Korea, has a beneficial effect on the pulmonary function of ARDS patients with systemic inflammatory response syndrome\textsuperscript{22}. About 10–15% of patients with COVID-19 progresses to ARDS\textsuperscript{23}. Since sivelestat may not only mitigate the damage of neutrophil elastase to lung connective tissue, but also limit the virus spreading capabilities by inhibiting S protein processing, Mahmoud M. A. Mohamed \textit{et al} advocated the use of sivelestat to alleviate neutrophil-induced damage in high-risk COVID-19 patients\textsuperscript{24}. Our results showed that the S-G614 protein could be cleaved more efficiently, which may be due to a novel elastase-2 cleavage site on the S1-S2 junction region of S-G614 protein, so it might be an effective option for treatment of COVID-19 caused by SARS-CoV-2 harboring the D614G mutation.

Takahiko Koyama \textit{et al} reported that D614G is located in one of the predicted B-cell epitopes of SARS-CoV-2 S protein, and this is a highly immunodominant region.
and may affect effectiveness of vaccine with wild-type S protein\textsuperscript{18}. D614 is conserved in S protein of SARS-CoV in 2003. Previous studies in SARS-CoV suggested that the peptide $S_{597-625}$ is a major immunodominant peptide in humans and elicits a long-term B-cell memory response after natural infection with SARS-CoV\textsuperscript{25}. Regions between amino acids 614 and 621 of SARS-CoV-2 S protein were also identified as a B cell epitope by different methods, and the change of D614G may affect the antigenicity of this region\textsuperscript{26}. Here, we observed that 7\% (3/41) convalescent sera showed markedly different neutralization activities between S-G614 and S-G61 protein pseudotyped viruses, indicating that D614G mutation reduces the neutralizing antibody sensitivity. Whether these patients were at high risk of reinfection of S-G614 variant should be explored in further studies. It will also be important to determine the breadth of neutralizing capacity of vaccine-induced neutralizing antibodies.

Very recently, several groups also reported that the S-G614 enhances viral infectivity based on pseudovirus assays\textsuperscript{27-29}, but due to the small sample size, they found no effect on neutralization sensitivity of the virus\textsuperscript{27,28}. Given the evolving nature of the SARS-CoV-2 RNA genome, antibody treatment and vaccine design might require further considerations to accommodate the D614G and other mutations that may affect the immunogenicity of the virus.

Our study has some limitations. First, the C-terminal 19 amino acids of S protein
were lacking in order to improve the packaging efficiency of SARS-CoV-2 S protein pseudotyped virus, and this pseudovirus only recapitulates viral entry events; therefore, additional assays with authentic SARS-CoV-2 are required. Second, we only tested neutralizing antibodies against the S protein. However, previous studies on SARS-CoV indicated that only a small fraction of memory B cells specific for SARS-CoV antigens are directed against neutralizing epitopes present on the S protein\textsuperscript{30}. Third, in addition to D614G, further studies on other mutations in the S protein are needed to evaluate their impact on SARS-CoV-2 infection, pathogenicity and immunogenicity. Further studies are needed to determine the impact of these mutations on the severity of COVID-19.

In summary, we established a SARS-CoV-2 spike-mediated pseudovirus entry assay and studied the cell entry of S-D614 and S-G614 pseudotyped viruses. Our study provided evidence that D614G mutation introduces an additional elastase-2 cut site in S protein, thereby promoting its cleavage and viral cell entry, resulting in more transmissible SARS-CoV-2. Importantly, the D614G mutation reduced the sensitivity of the virus to serum neutralizing antibody in individual convalescent COVID-19 patients. Our study will help for understanding the SARS-CoV-2 transmission and the design of vaccines and therapeutic interventions against COVID-19.

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**Author contributions**

A-L.H., N.T., and K.W. conceived the project and supervised the study. J.H., C-L.H., Q-Z.G. and G-J.Z. performed most experiments. J.H. and K.W. performed serum neutralization assay. H-J.D. and L-Y. H. performed SARS-CoV-2 genome analysis. Q-X.L., J.C. and X-X. C. collected the serum samples. J.H. and Q-Z.G. contributed to the statistical analysis. K.W. and N.T. wrote the manuscript. All the authors analyzed the final data, reviewed and approved the final version.

**Competing interests**

The authors declare no competing interests.
Data availability

The data supporting the findings of this study are available from the authors upon reasonable request.

References:


17. Lei, C. et al. Neutralization of SARS-CoV-2 spike pseudotyped virus by recombinant


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Top 10 abundant non-synonymous mutations observed in S protein of SARS-CoV-2.
**Figures and figure legends**

**Figure 1**

![Diagram of SARS-CoV-2 phylogenetic analysis](image)

**Fig. 1.** Phylogenetic analysis of SARS-CoV-2 sequences from GISAID.

Prevalence of S-D614G genomes over time produced by the Nextstrain analysis tool using GISAID dataset (n = 2,834 genomes samples from January 2020 to May 2020).
Fig. 2 Detection of S-D614 and S-G614 protein expression and cleavage. a, Additional serine protease (elastase-2) cleavage site in S1-S2 junction was found in S-614G protein of SARS-CoV-2. b, Detection of S protein expression in HKE293T cells by Western blot using the anti-RBD monoclonal antibody. Cells were transfected with pS-D614, pS-G614 plasmid or with an empty vector, and incubated with sivelestat sodium or not. To compare the S1 and S ratio, integrated density of S1/S was quantitatively analyzed using ImageJ software. Significant differences were analyzed by one-way ANOVA. **P < 0.01.
**Fig. 3**

**A**

<table>
<thead>
<tr>
<th>Pseudovirus</th>
<th>293T</th>
<th>293T-ACE2</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSV-G</td>
<td>10^7</td>
<td>10^6</td>
</tr>
<tr>
<td>S-D614</td>
<td>10^5</td>
<td>10^6</td>
</tr>
<tr>
<td>S-G614</td>
<td>10^4</td>
<td>10^5</td>
</tr>
</tbody>
</table>

**B**

Neutralization (%) vs. ACE2-Ig(μg/ml)

**C**

<table>
<thead>
<tr>
<th>Time (hpi)</th>
<th>Pseudovirus infection (RLU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hpi</td>
<td>S-D614: 600 ± 20, S-G614: 600 ± 20 (ns)</td>
</tr>
<tr>
<td>48 hpi</td>
<td>S-D614: 3000 ± 100, S-G614: 3000 ± 100 (**)</td>
</tr>
<tr>
<td>72 hpi</td>
<td>S-D614: 1000 ± 50, S-G614: 1000 ± 50 (**)</td>
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**Fig. 3** The S-G614 protein pseudotyped virus showed increased infectivity. **a,** HEK293T and 293T-ACE2 cells were infected with lentiviruses pseudotyped with vesicular stomatitis virus G (VSV-G) and SARS-CoV-2 S protein variants. Virus titers were quantified by RT-qPCR and adjusted to 3.8x10^4 copies in 50 μL to normalize input virus doses. The relative luminescence units (RLU) detected 72 h post-infection (hpi). **b,** Inhibition of pseudovirus entry by ACE2-Ig. Pseudovirions were pre-incubated with ACE2-Ig and added to 293T-ACE2 cells, RLU were detected 72 hpi. **c,** Viral entry efficiency mediated by S variants. The RLU were
detected 24-72 hpi. The data are presented as the means ± standard deviations (SDs) of three independent biological replicates. Significant differences were analyzed by one-way ANOVA. **$P < 0.01$, ***$P < 0.001$. 
**Fig. 4 Detection of protease inhibitors against SARS-CoV-2 pseudovirus**

**a-b,** The TMPRSS2 and CatB/L inhibitors were evaluated by pseudovirus cell entry assay. 293T-ACE2 cells were pre-incubated with camostat mesylate (a) or E-64d (b), and subsequently inoculated with pseudovirions. The VSV-G pseudovirus was used as the control. RLU were detected at 72 h.
post-pseudovirus inoculation. Cell viability was examined by the methyl thiazolyl tetrazolium (MTT) assay. The data are presented as the means ± SDs of three independent biological replicates. Statistical significance was tested by two-way ANOVA with Dunnett posttest. **$P < 0.01$. 
Fig. 5 Detection of neutralizing antibodies in convalescent sera against S-D614 and S-G614 protein pseudotyped viruses. a, The inhibition rate of sera
from 41 convalescent COVID-19 patients against S-D614 and S-G614 pseudoviruses. Serum sample from healthy individual was tested as a negative control. Convalescent sera were collected at 2-4 weeks post symptom onset from confirmed case patients (#1 - #41). Sera was analyzed at a single dilution of 1:1000. RLU were detected at 72 hpi. Compared the RLU of serum neutralized sample to control and calculated the inhibition rate. The data are presented as the mean percentages of inhibition ± SDs of three independent biological replicates. 

b, The inhibition curves for serum samples from 5 convalescent patient and a health donor. The initial dilution was 1:40, followed by 4-fold serial dilution. Neutralization titers were calculated as 50% inhibitory dose (ID50), expressed as the serum dilution at which RLU were reduced by 50% compared with virus control wells after subtraction of background RLU in cell control wells. Representative data are shown as percent neutralization (mean ± SDs of three independent biological replicates).