SARS-CoV-2 gained the unique spike protein S1-N-Terminal Domain from the promising pangolins mixing vessel: A novel clue for the pandemic outbreak

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SARS-CoV-2 gained the unique spike protein S1-N-Terminal Domain from the promising pangolins mixing vessel: A novel clue for the pandemic outbreak

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Abstract

The clue behind the emergence of SARS-CoV-2 remains a huge debate among the virologist, policymakers, and general public, while the gain-of-function hypothesizes mostly focused on the furin cleavage site and receptor-binding domain (RBD) of the spike (S) protein. Here, we report that the SARS-CoV-2 gained the novel human-specific spike protein S1-N-Terminal Domain (S1-NTD) (Type-I) which is present only in SARS-CoV-2. Interestingly, SARS-CoV-2-rB-CoV showed unique bat-specific Type-II and Type-III-S1-NTD, which is not present in other SARS coronaviruses, including SARS-CoV-2 variants. We also found widespread recombination and selection pressure in the S1-NTDs of the bat viruses. In addition, the Pangolin/GX/2018 and Pangolin/Guangdong/2019 lineages showed close identity (73-79%) with the Type-I-S1-NTD and Type-II-S1-NTD respectively. This justifies that the pangolin is the mixing vessel (intermediate host) to exchange the bat-specific Type-II-S1-NTD in the SARS-CoV-2-rB-CoV into Type-I-like-S1-NTD in pangolin through recombination. Furthermore, the pangolin virus with Type-I-like-S1-NTD jumped into humans which then transformed into SARS-CoV-2 with Type-I-S1-NTD by host selection pressure. Remarkably, we characterized the bat/Cambodia virus as a recombinant SARS-CoV-2/RatG13 with the S1-NTD of bat-SARS-like viruses; while only bat/RatG13 with Type-I-S1-NTD established a huge pandemic outbreak. Additionally, recent SARS-CoV-2 S1-NTD specific neutralizing antibody-based studies support the role of S1-NTD in the post-attachment of the virus; fusion, virus dissemination, and cell-cell fusion thereby prevent the onset of infection; and most of the SARS-CoV-2 variants with increased transmissibility were linked to the S1-NTD mutations. Collectively, our results strongly suggest that the gain of Type-I-S1-NTD in the SARS-CoV-2 is the reason for the pandemic outbreak.

Introduction

The sudden emergence of highly adaptable and transmissible SARS-CoV-2 in the human population in December 2019, in Wuhan, China^{1,2}, raised the question about its origin. However, pangolins were considered an intermediate host between humans and bat²⁻⁵, due to the occurrence of almost identical spike protein receptor-binding domain (S1-RBD) in pangolin-CoVs and SARS-CoV-2⁵⁻⁷. However, all SARS-CoV-2 related bat and pangolin viruses except RatG13 showed much lower sequence identity in the spike gene^{3-5,8}, which is reported to be critical in determining the virus entry and tissue tropism, host-range, human-human infection, and host immune responses⁹⁻¹⁵. The SARS-CoV-2 spike protein is divided into a signal peptide, S1, and S2 subunits. The S1 subunit is further divided into two independent receptor binding domains such as sugar receptor binding S1-NTD (14-305 amino acid residues) and protein receptor binding S1-RBD (319-541 amino acid residues)^{10,14,16,17}. Further, it is widely accepted that the coronaviruses obtained the S1-NTD from host galectins through gene capture, and then gained the S1-RBD through gene duplication from the S1-NTD^{13,14}. Due to the presence of the S1-RBD in the tip of the spike head whereas S1-NTD is perhaps positioned underneath the S1-RBD^{14,18,19}; S1-RBD has more likelihood to expose to the host immune system and evolve at a faster rate than S1-NTD¹⁴. Further, the presence of extensive N-linked glycan shielding in the S1-NTD is expected to play a role in the immune evasion^{9,20-22}. Therefore, the less diversified S1-NTDs

are considered a more reliable domain for the coronaviruses to bind the sugar receptors thereby facilitating the S1-RBDs to search their high-affinity protein receptors¹⁴. Remarkably, the level of diversity and interaction of these domains with the host receptor is a critical determinant of the coronavirus host range, cross-species infection, tissue tropism, and host immune responses¹³⁻¹⁵. However, the genetic diversity and evolutionary origin of the S1-RBD were highly characterized in the SARS-CoV-2^{3-5,16,23,24}. On the other hand, the genetic diversity and evolutionary origin of the SARS-CoV-2 S1-NTDs are not yet fully established.

We present evidence for the emergence of the SARS-COV-2 pandemic outbreak due to the gain of novel Type-I-S1-NTD in its spike protein. Further, we report the existence of bat-specific different types of S1-NTD in the SARS-COV-2-rB-CoVs and report the different recombinant SARS-COV-2-rB-CoVs especially in the regions of S1-NTD and S1-RBD. Finally, we report the evidence to suggest pangolins as a mixing vessel to generate the human-specific Type-I-S1-NTDs from bat-specific Type-II-S1-NTDs.

Genetic diversity in SARS-CoV-2 related bat coronavirus

Our analysis revealed the presence of two clades of SARS-CoV-2 related bat coronavirus (SARS-CoV-2-rB-CoV) at the complete genome level (**Fig. 1a**). The Clade-I viruses (RaTG13; RShSTT182h, RmYN02; RacCS203 and RpYN06) exposed the nucleotide diversity between 4-10% with SARS-CoV-2 and within the clade-I viruses. The clade II viruses (bat/PrC31, bat-SL-CoVZC45, and bat-SL-CoVZXC21) have a diversity of 10-13% with SARS-CoV-2 and clade-I viruses (**Extended Data Fig. 1a**). The Pangolin/Guangdong/2019 lineage showed a close relationship with the clade-II SARS-CoV-2-rB-CoV (**Extended Data Fig. 1a & 1b**). Remarkably, Pangolin/GX/2018 lineage possessed evolutionary intermediate genetic diversity between SARS-CoV-1 and SARS-CoV-2 (**Extended Data Fig. 1a & 1b**). Next, at the Spike complete gene level, we observed genetic diversity of 25-35% among the SARS-CoV-2-rB-CoV. However, genetic diversity between the SARS-CoV-2-rB-CoV and SARS-CoV-1-rB-CoV (SARS-CoV-1 related-bat-CoVs) is in the range of 20-26% (**Extended Data Fig. 1c & 1d**). These results indicate that the genetic diversity in the S gene is a random diversity without specific to certain lineage, perhaps due to the genetic recombination events in this gene.

Genomic region's specific genetic diversity in SARS-CoV-2

Next, we observed that the SARS-CoV-2 displayed four hypervariable regions in comparison to SARS-CoV-1 and one hyper-variable region in comparison to SL-CoVZXC21 and bat-SL-CoVZC45 in SimPlot analysis (**Fig. 1b**). However, we are unable to detect any significant recombination between SARS-CoV-2 and SARS-CoV-1 in BootScan method of analysis (**Extended Data Fig. 1e**). Further, in our NCBI BLAST analysis, clade-II SARS-CoV-2-rB-CoV and SARS-CoV-1 showed different region's specific high and low nucleotide sequence diversities compared to Wuhan-Hu-1 (**Extended Data Fig. 1f**); owing to the widespread recombination events in this virus^{1,6,25,26}. According to our SimPlot and NCBI BLAST analysis, we divided the SARS-CoV-2 genome sequences into 11 different regions. The genomic portions of regions 1, 2, 4, 5, 8, 10, and 11 displayed low genetic diversity among

the SARS-CoV-2, SARS-CoV-2-rB-CoV, and SARS-CoVs (**Extended Data Fig. 2a-d; Extended Data Fig. 2g-h; Extended Data Fig. 3a-b; Extended Data Fig. 3e-f; Extended Data Fig. 4a-d**).

On the other hand, regions with substantial genetic diversity, such as region 3 and region 9 (protein 8) revealed 3-10% genetic diversity among SARS-CoV-2 and SARS-CoV-2-rB-CoV (clade-I and clade-II), and over 40% genetic diversity against SARS-CoV-1-rB-CoVs (**Extended Data Fig. 2e-f; Extended Data Fig. 3g-h**). This indicates that these regions are the novel signature sequences of SARS-CoV-2 and SARS-CoV-2-rB-CoVs. Further, the high diversity portion of region 7 (which belongs to the S1-RBD) on the other hand, has revealed two distinct clusters (**Extended Data Fig. 3c-d**). The SARS-CoV-2, RaTG13, RShSTT182, RShSTT200, Pangolin/GX/2018, and Pangolin/Guangdong/2019 formed a cluster-I. Surprisingly, the SARS-CoV-2-rB-CoVs of bat-SL-CoVZXC21, bat-SL-CoVZC45, RpYN06, bat/PrC31, bat/RacCS203, RmYN02 formed cluster-II with SARS-CoV-1-rB-CoV. This indicates the widespread recombination in the region.

Surprisingly, the most diversified section of region 6 (which contains the S1-NTD) had a maximum diversity of 90% among the viruses used in this study (**Extended Data Fig. 5a-b**). The SARS-CoV-2 and SARS-CoV-2-rB-CoVs established three distinct clades (Type-I to III-S1-NTD) in this region (**Fig. 1c-e, Supplementary Fig. 1a-b**). The SARS-CoV-2 and RaTG13 formed Type-I-S1-NTD with intra-type nucleotide diversity of 6% (1.5% amino acid (aa) diversity) and inter-type variability of 44-85% (37-92% aa diversity) (**Fig. 1d; Extended Data Fig. 5a**). Next, Pangolin/GX/2018 formed Type-I-like-S1-NTD and inter-type variability of 27-90% (27-28% diversity with Type-I-S1-NTD) (**Fig. 1d; Extended Data Fig. 5a**). Similarly, Type-II-S1-NTD was established by SL-CoVZXC21, bat-SL-CoVZC45, RpYN06, and Bat/PrC31, with intra-type diversity of 2-25% (1-16% aa diversity) and 44-83% (40-92% aa diversity) inter-type variability (**Fig. 1d; Extended Data Fig. 5a**). Conversely, Type-III-S1-NTD was generated by RmYN02 and Bat coronavirus RacCS203, with intra-type diversity of 6% and inter-type diversity of 68-91% (**Fig. 1d; Extended Data Fig. 5a**). Interestingly, SARS-CoV-2-rB-CoVs of RShSTT200 and RShSTT182 revealed significant genetic diversity of 69-75% with all these three types (SARS-CoV-2 and SARS-CoV-2-rB-CoVs) of S1-NTDs but had a tighter link with S1-NTDs of highly varied SARS-CoV-1-rB-CoVs (**Fig. 1d; Extended Data Fig. 5a**). According to our findings, none of the human viral isolates (SARS-CoV-2 variants/lineages) formed an evolutionary intermediate between SARS-CoV-2 and bat/RaTG13 in this region (**Supplementary Fig1a-b**), which indicates that all variants/lineages of SARS-CoV-2 originated from the base of NC_045512.2/Wuhan-Hu-1 Type-I-S1-NTD.

Moreover, Pangolin/GX/2018 lineage is the closest in this region to SARS-CoV-2 through Type-I-like-S1-NTD with 27% and 12% nucleotide and amino acid diversity, respectively (**Fig. 1d; Extended Data Fig. 5a**). But, Pangolin/Guangdong/2019 lineage is there in this region with 21-24% and 15%, nucleotide, and amino acid diversity respectively, with Type-II-S1-NTDs of SARS-CoV-2-rB-CoVs (**Fig. 1d; Extended Data Fig. 5a**). These findings imply that only Type-I-S1-NTD (region 6) is found in the human population in the ongoing pandemic outbreak, whereas Type-II and III-S1-NTD were found only in the bat population.

Pangolin virus lineages contain both SARS-CoV-2 human-like (Type-I-like-S1-NTD) and bat-like (TypeII-like-S1-NTD) viruses, indicating that pangolins could be used as a mixing vessel (intermediate host) for human and bat viruses to generate human-specific Type-I-S1-NTD which represent the ongoing outbreak.

Recombinant events in the SARS-CoV-2-rB-CoVs

In our recombination analysis, we confirmed that the bat/Cambodia/RShSTT200 and bat/Cambodia/RShSTT182 are the recombinant SARS-CoV-2 with S1-NTD of SARS-like bat coronavirus (**Fig.2a-b; Extended Data Fig. 6a-b; Supplementary Fig. 2a-b**). Similarly, our analysis also confirmed the bat/China/RpYN06 which has unique Type-II-S1-NTD is a recombinant SARS-CoV-2 with S1-RBD of SARS-like bat coronaviruses (**Extended Data Fig. 6c-d; Supplementary Fig. 2c**). Next, we observed that the Bat/RacCS203 (which displays Type-III-S1-NTD) is a recombinant SARS-COV-2 with S1-RBD of SARS-like bat coronaviruses (**Extended Data Fig. 6e-f; Supplementary Fig. 2d**). Further, we characterized the Bat/Yunnan/RmYN02 (which exhibits Type-III-S1-NTD) as a recombinant SARS-CoV-2 with S1-RBD of SARS-like bat coronaviruses; further, it holds unique protein-8 (**Extended Data Fig. 6g-h; Supplementary Fig. 2e**). Interestingly, the clade-II of SARS-CoV-2-rB-CoVs at the complete genome level which includes bat coronavirus isolate PrC31/China/2018, bat-SL-CoVZC45, bat-SL-CoVZXC21(which exhibit Type-II-S1-NTD) are the recombinant SARS-CoV-2 with some part of pp1ab and S1-RBD of SARS-like bat coronaviruses (**Fig. 2c; Extended Data Fig. 7a-e; Supplementary Fig. 2f-h**). More importantly, Pangolin/Guangdong/2019 lineage which has high sequence identity with SARS-CoV-2 S1-RBD, were displayed Type-II-S1-NTD in its genome, while Pangolin/GX/2018 lineage displayed the Type-I-like-S1-NTD and SARS-CoV-2-like-S1-RBD (**Fig 1d; Fig. 2d; Extended Data Fig. 3c-d; Extended Data Fig. 5a**). Collectively, these results indicate the presence of widespread recombination in the SARS-COV-2-rB-CoVs especially in the regions of S1-NTD and S1-RBD.

Selection pressure in the S1-NTDs

The S1-NTDs nucleotide sequence-based effective number of codon usage (ENc) suggest that the SARS-CoV-2 and bat-SARS viruses have moderate and minor codon use bias respectively (**Fig 3a**). Following that, in ENc-GC3s plot analysis^{27,28}, we observed that a vast majority of the points fall below the expected curve in the ENc-GC3s plot analysis for the S1-NTD (region 6) of SARS-CoV-2, SARS-CoV-1, pangolin SARS coronavirus, and bat SARS viruses (**Fig 3b**), indicating the strong presence of selection pressure, rather than mutation pressure. Next, our neutrality plot analysis revealed that the S1-NTD (region 6) of SARS-CoV-2 had a slope of 0.09669 ($Y=0.09669 \cdot X+37.28$, $R^2=0.04538$; $p=0.0093$) (**Fig 3c**), indicating that the mutation pressure and natural selections were 9.7% and 90.3%, respectively. Similarly, we observed the substantial selection pressure, rather than mutation pressure, in the S1-NTD (region 6) of SARS-Cov-2-rBat-CoVs, Pangolin-CoVs, SARS coronavirus, and bat SARS viruses (**Extended Data Fig. 8a-g**). We also ran a Parity rule 2 bias study and found that the uneven T3>A3 and C3>G3 numbers in the S1-NTD (region 6) of SARS-Cov-2 and SARS-Cov-2-rBat sequences (**Fig. 3d**) suggesting the existence of

mutation and selection pressure. Taken altogether, these results indicate that the presence of strong selection pressure in this region which shaping the virus evolution.

Discussions

In this work, we report that the SARS-CoV-2 gained the novel S1-NTD in its genome. Most of the studies focused on the rapid spread of the SARS-CoV-2 due to its S1-RBD diversity to binds with the human ACE2 receptor and the presence of a specific furin cleavage site (FCS)^{4,29-35}. However, the furin cleavage site is not only present in SARS-CoV-2, but also present in MERS-CoV, HKU1, and HCoV-OC43^{4,32,34,36}, and the SARS-CoV-2 without FCS also can infect target cells efficiently in the presence of trypsin or human airway trypsin-like protease (HAT)³⁷. Further, the S1-RBD for the human ACE2 is also present in SARS-CoV-1; however, SARS-CoV-2 has improved binding to ACE2 receptor than SARS-CoV-1^{38,39}. Moreover, SARS-CoV-2 has been shown to infect efficiently 293T and Vero cells without human ACE2 transfection^{12,40}. Therefore, it could be inferred that the SARS-CoV-2-S1-RBD and FCS might be the cause for the severity of the infection and in addition, some other factors also contribute to the increased transmissibility of infection in humans which is the hallmark for the SARS-CoV-2 in comparison to SARS-CoV-1 and MERS-CoV.

In comparison to other coronaviruses, SARS-CoV-2 has a large flat, non-sunken sialic acid-binding domain with extended and divergent loop sections of the SARS-CoV-2 S1-NTD is expected to improve viral transmission and infectivity by facilitating sialic acid-binding ability⁴¹⁻⁴⁵. Furthermore, glycosylated cell surface proteins and lipids are in all types of cells and play a role in immunity and cell-cell communication^{13,14,46-48}. Perhaps, SARS-CoV-2 can first attach to any type of cells with the S1-NTD to the cell surface sugar receptor. Further, allow the S1-RBD to search for the specific receptor for the virus entry into the cell thereby dramatically increases the transmissibility of infection. The SARS-CoV-2 S1-NTD specific mAb treatment showed the role of S1-NTD in the receptor post-attachment virus entry (without affecting the binding of the S1-RBD with the ACE2), and cell-cell fusion thereby completely abolish the clinical onset of the infection^{12,49}. In addition, most of the highly transmissible variants of SARS-CoV-2 such as Mink Cluster 5, B.1.1.7, B.1.351, P.1, B.1.617.1, B.1.617.2, B.1.526, B.1.525, B.1.177, B.1.427 and B.1.429 were linked to the mutations/deletions in the S1-NTDs in natural infection^{12,49-56} and also in the experimental condition⁴⁹. Furthermore, the Cambodia bat viruses (RShSTT200 and RShSTT182) which are the recombinant of SARS-CoV-2/RatG13 with S1-NTD of SARS-like bat coronavirus were not detected in the ongoing pandemics, while bat/RatG13 (with Type-1-S1-NTD) established pandemic, which indicates the significance of the novel Type-I-S1-NTD in the SARS-CoV-2 genome for its rapid pandemic spread.

The pseudotyped virus of S protein RatG13 has a poor ability to infect many cell types, including bat and human cells^{23,40}. The RaTG13 is the only one bat virus RNA sequence having the Type-I-S1-NTD remains unknown. It could be due to (i) Reverse zoonosis of human-specific Type-I-S1-NTD to bats, as SARS-CoV-2 is known to cause reverse zoonosis⁵⁷⁻⁶³; or (ii) Contamination of human virus to bat sample during sample collection, transport, storage, and sample processing, and this theory might be disproved by isolation of

the virus and experimental infection; or (iii) The RaTG13 virus may spontaneously evolve in bats; however, the RaTG13 (with Type-I-S1-NTD) and Pangolin/GX/2018 lineage (with Type-I-like-S1-NTD) S gene pseudotyped viruses fail to infect bat cells⁴⁰; whereas, Pangolin/Guangdong/2019 lineage (with TypeII-like-S1-NTD) efficiently infect the bat cells⁴⁰. Therefore, it can be inferred that the Type-I-S1-NTD is the most potential for enhancing human adaptation.

Importantly, we produced evidence of extensive recombination events in bat viruses, particularly in the S1-NTD and S1-RBD areas, and we also confirmed the existence of selection pressure to evolve the S1-NTD region in this study. In light of this, we hypothesize that SARS-CoV-2-rB-CoVs with Type-II-S1-NTD probably jumped to pangolins and then recombination between Type-II-S1-NTD (SARS-CoV-2-rB-CoV) and Type-I-like-S1-NTD (which shares 73% identity with Type-I-S1-NTD) may have occurred, resulting in the emergence of SARS-CoV-2 related pangolin virus with Type-I-like-S1-NTD. Furthermore, jumping of this virus into the human, and evolved into SARS-CoV-2 with Type-I-S1-NTD due to the host selection pressure. Our results suggest that the pangolins are the promising mixing vessel for bat and human SARS coronaviruses (**Fig. 4**), as pigs are the mixing vessel for influenza viruses⁶⁴⁻⁶⁷.

In conclusion, SARS-CoV-2 gained the novel S1-NTD; probably originated from bat through pangolin mixing vessels by recombination. The identification of the potent host receptor for the different types of S1-NTDs to determine its role in viral spread, host range, and severity of infection needs to be studied. Further, structure-based studies on the importance of mutations in the Type-I-S1-NTD and monitoring of mutations in this domain are warranted, to predict the future direction of the current pandemic; to design novel antiviral molecules, development of vaccines, and neutralizing antibodies to prevent and control the ongoing and future outbreaks.

Materials and Method

Genetic diversity analysis

a. Phylogenetic analysis

The NCBI and GISAID databases were used to find the SARS-CoV-2 and similar bat coronavirus sequences. For the phylogenetic study, MEGA7 was utilized, with the Neighbor-Joining method being used to infer the evolutionary history, bootstrap tests (1000 replicates), the Maximum Composite Likelihood method being used to compute the evolutionary distances, and gamma distribution (shape parameter = 5) being employed to model the rate variation among sites. Differences in composition bias among sequences were assessed for evolutionary comparisons, and all ambiguous sites were ignored in the analysis for each sequence pair.

b. Net Between Group Mean Distance (NBGMD) analysis

The NBGMD was calculated using the MEGA7 through the Kimura 2-parameter model, with a gamma distribution (shape parameter=5) used as a model to measure rate variation among

sites, a bootstrap test (1000 replicates) used to estimate the Standard error and all ambiguous sites were removed from the analysis for each sequence pair. Standard error estimates were displayed above the diagonal.

Recombination analysis

a. SimPlot analysis

The percent identity between the query and reference sequences was determined using the SimPlot 3.5.1 programme⁶⁸. The nucleotide sequences were first aligned in MEGA7 before being exported to SimPlot 3.5.1 for additional analysis. To measure the identity between the query and reference sequences, we employed the 500 base pair of the window at a 50 base pair step, using the Kimura two-parameter model.

b. Recombination Detection Program (RDP) analysis

RDP4⁶⁹ was used to detect potential recombination events in SARS-CoV-2 and its related viruses. MEGA7 was used to align the nucleotides, which were subsequently exported to RDP4 for additional processing. The analysis was conducted with default parameter values for the BOOTSCAN, GENECONV, Chimaera, RDP, MaxChi, SISCAN, and 3seq methods, and a minimum of four or more approaches was assessed for probable recombination using a Bonferroni corrected p-value cut-off (0.05). The BOOTSCAN method-based images are displayed in the figures.

Determining the selection and mutation pressure

a. Effective Number of Codons (ENc)

One of the approaches for determining the codon usage bias is the effective number of codons used from 61 codons for the 20 amino acids, which might range from 20 to 61. ENc values less than 35 suggest strong codon bias, while ENc values greater than 50 imply broad random codon usage^{27,70}. ENc values were calculated using an online server in this investigation (<http://ppuigbo.me/programs/CAIcal/>)⁷¹.

b. ENc-GC3s plot

The ENc values are plotted against the third position of GC3s of codon values in this study to establish the key variables affecting the codon usage bias, such as selection or mutation pressure²⁸. The expected curve was determined by estimating the expected ENc values for each GC3S as recommended in previous publications^{27,28}. The ENc and GC3s for every gene were obtained from an online CAI analysis server (<http://ppuigbo.me/programs/CAIcal/>)⁷¹. When codon bias is just driven by mutation pressure, the genes will lie on or near the expected curve, however, when codon bias is influenced by selection and other variables, the genes will fall well below the expected curve.^{27,28}.

c. Neutrality plot analysis

The GC12 values of codons are plotted against the GC3 values in a neutrality plot to determine the degree of influence of mutation pressure and natural selection on codon usage patterns. The GC12 and GC3 values for S1-NTD (region 6) nucleotide sequences were retrieved from the online CAI analysis server (<http://ppuigbo.me/programs/CAIcal/>)⁷¹.

d. Parity Rule 2 (PR2)-bias plot

To measure the mutation pressure and natural selection affecting the codon use bias, the AT bias $[A3/(A3+ T3)]$ is plotted against the GC bias $[G3/(G3 + C3)]$ ²⁸. Using the ACUA Software⁷², the A3, T3, G3, and C3 values of nucleotide sequences of S1-NTD (region 6) were calculated

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Author contributions

PAD performed most of the bioinformatics experiments. KN assisted PAD for most of the bioinformatics works. PAD and KN wrote the first draft of the manuscript. PAD conceived the study, designed experiments, and wrote the final version of the manuscript.

Conflict of interest: None.

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Figure 1

Fig. 1: The S1-NTD based genetic diversity in SARS-CoV-2. a, The Phylogenetic tree using the complete genome's nucleotide sequences of the SARS-CoV-2 and related viruses. **b,** Similarity plot based on the complete genomes nucleotide sequences of the SARS-CoV-2, RatG13, bat-SL-CoVZC45, bat-SL-CoVZXC21, and SARS-CoV GZ02; SARS-CoV-2 used as a reference sequence. **c & d,** The region 6 (21578-22560nt) specific phylogenetic tree and net between-group mean distance respectively for SARS-CoV-2 related viruses. **e,** The region 6 specific amino acid-based phylogenetic tree for SARS-CoV-2 related viruses.

Figure 2

Fig. 2: Recombinant events in the SARS-CoV-2-rB-CoVs. **a**, Similarity plot based on the complete genomes nucleotide sequences of the SARS-CoV-2, Cambodia bat virus RShSTT182 and Bat coronavirus Cp/Yunnan2011; SARS-CoV-2 used as a reference sequence. **b**, The recombination analysis based on BOOTSCAN using complete genomes nucleotide sequences of the SARS-CoV-2, Cambodia bat virus RShSTT182 and Bat coronavirus Cp/Yunnan2011; SARS-CoV-2 used as a reference sequence. **c**, Similarity plot based on the complete genomes nucleotide sequences of the SARS-CoV-2, bat coronavirus PrC31, and Bat SARS coronavirus Rp3; SARS-CoV-2 used as a reference sequence. **d**, Similarity plot based on the complete genomes nucleotide sequences of the SARS-CoV-2, RpYN06, RatG13 Pangolin/GX/2018, and Pangolin/Guangdong/2019; SARS-CoV-2 used as a reference sequence.

Figure 3

Fig. 3: Selection pressure in the S1-NTDs. a, ENc values; **b**, ENc plotted against GC3s; **c**, Neutrality plot analysis of the GC12 and that of the GC3; and **d**, Parity Rule 2 (PR2)-bias plot analysis of the SARS-CoV-2, SARS-Cov-2-rB-CoVs, Pangolin-CoVs, SARS-CoVs, and bat SARS viruses.

Figure 4

Fig.4: A proposed schematic representation of the origin of the Type-I-S1-NTD in SARS-CoV-2. **a**, The schematic figure explains the classification and the recombination of the SARS-Cov-2-rB-CoVs. **b**, The schematic figure explains the origin of the SARS-CoV-2 specific Type-I-S1-NTD from the bat-specific Type-II-S1-NTD through the pangolin mixing vessel. The red color represents the SARS-CoV-2 related genomic regions; the green color represents the SARS-CoVs (SARS-Cov-2-rB-CoVs) related genomic regions; The purple color represents the Type-I-like-S1-NTDs; the blue color represents the Type-II-S1-NTDs, and the yellow color represents the pangolin virus.

Extended Data Fig. 1

Extended Data Fig. 1: The genetic diversity in the SARS-CoV-2 and related viruses. a, The net between-group mean distance of the SARS-CoV-2 and related viruses using complete genomes nucleotide sequences. **b,** Similarity plot based on the complete genomes nucleotide sequences of the SARS-CoV-2 and related viruses. **c,** The Phylogenetic tree using the complete Spike gene nucleotide sequences of the SARS-CoV-2 and related viruses. **d,** The net between-group mean distance of the SARS-CoV-2 and related viruses using complete Spike gene nucleotide sequences. **e,** The BootScan results are based on the complete genomes nucleotide sequences of the SARS-CoV-2, bat-SL-CoVZXC21, bat-SL-CoVZC45, RaTG13, and SARS-CoV GZ02. The NC_045512.2/Wuhan-Hu-1 virus has been used as a reference sequences query. **f,** NCBI public database BLAST analyses of the complete genome of Wuhan seafood market pneumonia virus isolate Wuhan-Hu-1 (NC_045512.2), showed the different region-specific nucleotide diversity with the other SARS-CoVs and bat SARSr-CoVs.

Extended Data Fig. 2

Extended Data Fig. 2: The genetic relationship of SARS-CoV-2 and related viruses in the region 1, 2, 3, and 4. a & b, The region 1 (5'UTR) specific phylogenetic tree and net between-group mean distance respectively for SARS-CoV-2 related viruses. c & d, The region 2 (266 to 1917nt) specific phylogenetic tree and net between-group mean distance respectively for SARS-CoV-2 related viruses. e & f, The region 3 (1924-3956nt) specific phylogenetic tree and net between-group mean distance respectively for SARS-CoV-2 related

viruses. **g & h**, The region 4 (3956-20576nt) specific phylogenetic tree and net between-group mean distance respectively for SARS-CoV-2 related viruses.

Extended Data Fig. 3

Extended Data Fig. 3: The genetic relationship of SARS-CoV-2 and related viruses in the region 5, 7, 8, and 9. a & b, The region 5 (20557-21557nt) specific phylogenetic tree and net between-group mean distance respectively for SARS-CoV-2 related viruses. **c & d**,

The region 7 (22561-23161nt) specific phylogenetic tree and net between-group mean distance respectively for SARS-CoV-2 related viruses. **e & f**, The region 8 (23221-27909nt) specific phylogenetic tree and net between-group mean distance respectively for SARS-CoV-2 related viruses. **g & h**, The region 9 (27910 -28257nt) specific phylogenetic tree and net between-group mean distance respectively for SARS-CoV-2 related viruses.

Extended Data Fig. 4

Extended Data Fig. 4: The genetic relationship of SARS-CoV-2 and related viruses in the region 10 and 11. a & b, The region 10 (28258 -29674nt) specific phylogenetic tree and net between-group mean distance respectively for SARS-CoV-2 related viruses. **c & d**, The region 11 (3'UTR) specific phylogenetic tree and net between-group mean distance respectively for SARS-CoV-2 related viruses.

Extended Data Fig. 5

Extended Data Fig. 5: The Amino acid diversity in the S1-NTD (region 6). **a**, The region 6 amino acid-specific net between-group mean distance respectively for SARS-CoV-2 related viruses. **b**, The amino acid diversity in the different SARS viruses.

Extended Data Fig. 6

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Extended Data Fig.6: Recombination in the clade-I SARS-CoV-2-rB-CoV. **a & b**, The recombination analysis based on Similarity plot (a) and BOOTSCAN (b) using the complete genomes nucleotide sequences of the SARS-CoV-2, Cambodia bat virus RShSTT200 and Bat coronavirus Cp/Yunnan2011; SARS-CoV-2 used as a reference sequence. **c & d**, The recombination analysis based on Similarity plot (c) and BOOTSCAN (d) using the complete genomes nucleotide sequences of the SARS-CoV-2, RsYN06, and Bat SARS coronavirus Rp3; SARS-CoV-2 used as a reference sequence. **e & f**, The recombination analysis based on Similarity plot (e) and BOOTSCAN (f) using the complete genomes nucleotide sequences of the SARS-CoV-2, RacCS203, and Bat SARS coronavirus HKU3-12; SARS-CoV-2 used as a reference sequence. **g & h**, The recombination analysis based on Similarity plot (g) and BOOTSCAN (h) using the complete genomes nucleotide sequences of the SARS-CoV-2, RmYN02, and Bat SARS coronavirus Rp3; SARS-CoV-2 used as a reference sequence.

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Extended Data Fig. 7

Extended Data Fig. 7: Recombination in the clade-II SARS-CoV-2-rB-CoV. **a & b**, The recombination analysis based on Similarity plot (a) and BOOTSCAN (b) using the complete genomes nucleotide sequences of the SARS-CoV-2, bat-SL-CoVZXC21 and Bat SARS coronavirus HKU3-1; SARS-CoV-2 used as reference sequence. **c & d**, The recombination analysis based on Similarity plot (c) and BOOTSCAN (d) using the complete genomes nucleotide sequences of the SARS-CoV-2, bat-SL-CoVZC45, and Bat SARS coronavirus HKU3-8; SARS-CoV-2 used as a reference sequence. **e**, The recombination analysis based on BOOTSCAN using the complete genomes nucleotide sequences of the SARS-CoV-2, bat coronavirus PrC31, and Bat SARS coronavirus Rp3.

Extended Data Fig. 8

Extended Data Fig. 8: The Neutrality plot analysis in the S1-NTD (region 6). **a**, SARS-Cov-2-rBat; **b**, Pangolin/GX/2018 lineage; **c**, Pangolin/Guangdong/2019 lineage; **d**, SARS-CoV-1; **e**, SARS-Cov-1-rBat; **f**, Bat-SARS viruses; **g**, Other diversified bat Bat-SARS viruses.

Supplementary Figure 1

Supplementary Fig.1: Phylogenetic relationship S1-NTD (region 6). a, The region 6 (21578-22560nt) specific phylogenetic tree for SARS-CoV-2 related viruses. b, The region 6 specific amino acid-based phylogenetic tree for SARS-CoV-2 related viruses.

Supplementary Figure 2

118

Supplementary Fig.1: The Similarity plot analysis of SARS-CoV-2-rB-CoV. **a**, Similarity plot based on the complete genomes nucleotide sequences of the SARS-CoV-2, Cambodia bat virus RShSTT182 and Bat coronavirus Cp/Yunnan2011; Cambodia bat virus RShSTT182 used as a reference sequence. **b**, Similarity plot based on the complete genomes nucleotide sequences of the SARS-CoV-2, Cambodia bat virus RShSTT200 and Bat coronavirus Cp/Yunnan2011; Cambodia bat virus RShSTT200 used as a reference sequence. **c**, Similarity plot based on the complete genomes nucleotide sequences of the SARS-CoV-2, RsYN06, and Bat SARS coronavirus Rp3; RsYN06 used as a reference sequence. **d**, Similarity plot based on the complete genomes nucleotide sequences of the SARS-CoV-2, RacCS203, and Bat SARS coronavirus HKU3-12; RacCS203 used as a reference sequence. **e**, Similarity plot based on the complete genomes nucleotide sequences of the SARS-CoV-2,

RmYN02, and Bat SARS coronavirus Rp3; RmYN02 used as a reference sequence. **f**, Similarity plot based on the complete genomes nucleotide sequences of the SARS-CoV-2, bat coronavirus PrC31, and Bat SARS coronavirus Rp3; bat coronavirus PrC31 used as a reference sequence. **g**, Similarity plot based on the complete genomes nucleotide sequences of the SARS-CoV-2, bat-SL-CoVZXC21, and Bat SARS coronavirus HKU3-1; bat-SL-CoVZXC21 used as a reference sequence. **h**, Similarity plot based on the complete genomes nucleotide sequences of the SARS-CoV-2, bat-SL-CoVZC45, and Bat SARS coronavirus HKU3-8; bat-SL-CoVZC45 used as a reference sequence.