COVID-19: CRISPR/Cas-like System of nsp3 Promotes the Mutant Recombination and Drug Resistance

Wenzhong Liu [1,2,*], Hualan Li[2]

[1] School of Computer Science and Engineering, Sichuan University of Science & Engineering, Zigong, 643002, China;
[2] School of Life Science and Food Engineering, Yibin University, Yibin, 644000, China;
[*] Correspondence.Wenzhong Liu, liuwz@suse.edu.cn.

Abstract

Patients with novel coronavirus pneumonia usually suffer from bacterial and fungal infections, and the drug resistance problem caused by the pandemic is becoming more and more serious. Simultaneously, the SARS-COV-2 virus has a rapid mutation phenomenon, and somegene coding regions by mutation and recombination may be related to the drug resistance of the virus. Therefore, studying the relationship between the co-infection of bacteria and fungi and the evolution of SARS-COV-2 has important guiding significance for preventing a pandemic. We found that the SARS-COV-2 virus's nsp3 protein had a CRISPR/Cas 9 (II-B)-like function by searching for conserved domains. The system could target and edit the negative-strand RNA of SARS-COV-2. We speculated that the crRNA (CRISPR RNA) produced by the CRISPR/Cas system of Pseudomonas aeruginosa carried the genetic information of the conserved domains of bacteriophages and Pseudomonas, including drug resistance. After the phage lysed the Pseudomonas, the crRNA was released and attached to the fungal spores, and then invaded the patient's cells along with the spores or hyphae. nsp3 synthesized and assembled 4Fe-4S, iron-containing molecules bound to the cas4 domain, in the mitochondria of phagocytes. The iron came from hemoglobin attacked by the SARS-COV-2 virus protein. The nsp3 protein bound the crRNA in the phagocytic cytoplasm. It targeted the negative-strand RNA of SARS-COV-2, inserting conserved domain gene fragments into the negative-strand RNA through editing and splicing. Since the Cas protein had no codon checking function, the cutting and splicing would destroy the protein-coding information in the original RNA coding region, causing mutation and recombination of the SARS-COV-2 virus genome. If crRNA carried the drug resistance gene fragments of bacteria or phage, SARS-COV-2 would have similar drug resistance. Because of the growing problem of drug resistance in COVID-19 patients, we should pay attention to preventing fungi and bacteria co-infection. Avoid the CRISPR/Cas-like system of the novel coronavirus to cause rapid mutation and recombination and increased the drug resistance problem of SARS-COV-2.

Keywords: Fungi and bacteria infection; White-nose syndrome (WNS); Pseudomonas aeruginosa; CRISPR/Cas system; crRNAs (CRISPR RNAs); nsp3.

1. Introduction

The patients with COVID-19 pneumonia often have bacterial and fungal infections, and empirical broad-spectrum antibacterial drugs have been widely adopted in treatment(1). Increasing

drug resistance issues arising from the pandemic pose significant challenges for clinicians and antibiotic management(2, 3), and the global healthcare system ushered in unprecedented pressure. Both fungi and bacteria have primitive immune defense systems, which promote the their genome's evolution to resist their parasitic viruses (such as bacteriophage) or drugs. Simultaneously, the SARS-COV-2 virus has a rapid mutation phenomenon, and some evolutionary recombination gene coding regions may be related to the drug resistance of the virus. Therefore, studying the relationship between fungi, bacteria, bacteriophage and the novel coronavirus could help clarify the evolutionary mechanism of the SARS-COV-2 virus.

The mutation rate of RNA viruses is too high, several orders of magnitude higher than that of DNA-based organisms, and most mutations are not suitable for health(4). The SARS-COV-2 virus and the bat SARS-like coronavirus (isolated in 2015) are homologous and similar, but the S glycoprotein and nucleocapsid protein have mutations(5). ORF1a protein of the novel coronavirus has the highest homology with the bat coronavirus isolate RaTG13(6). It shows that the coronavirus may mutate in bats to form a new type of coronavirus and spread from the bat, giving it the ability to infect humans. The mutation patterns of human SARS-COV-2 and Bat RaTG13 coronavirus genomes are strongly biased towards C>U transition, showing the rapid evolution of SARS-COV-2 in the host(7). Different human SARS-COV-2 genomes have many mutations and deletions in the coding and non-coding regions, and they also show genetic diversity and rapid evolution(8). The rapid development of SARS-COV-2 has caused significant difficulties in tracing the intermediate host of the virus. Pangolins, snakes, tortoises (Chrysemys picta bellii), Che (Chelonia mydas), and Chinese mitten crabs (Pelodiscus sinensis) have all been speculated as potential intermediate hosts for the spread of SARS-COV-2 to humans(9). The controversy about the intermediate host of SARS-COV-2 virus from bats to humans is still inconclusive. If we focus on the diseases and changes of bats themselves, we will find new clues.

Aspergillus fumigatus and Candida are common fungi in COVID-19 patients(10, 11). Fungal diseases have become a significant cause of morbidity and death in plants, animals, and humans(12). The white-nose syndrome (WNS) threatens contemporary bats(13). The white-nose syndrome is a severe skin infection in bats caused by the fungus Pseudogymnoascus destructans. Hibernating bats wake up frequently because of the itching caused by infection and consume the fat stored in the body to maintain a high body temperature for a time(14). They fly around, usually starving to death or freezing to death. The white-nose syndrome may be an emerging fungal infection disease that has only appeared in modern times(15). Since the introduction from Eurasia around 2006(16), some North American bat species hibernated have been almost devastated(17, 18). European and Asian bats have shown some resistance to the deadly white-nose syndrome(19). Pseudomonas on the surface of bats have antifungal properties(20). Pseudomonas is also the most common bacterium that causes opportunistic infections in bats(21). The fluorescein cluster secreted by Pseudomonas fluorescens produces fungal decomposing enzymes(22), which can colonize fungal mycelium and conidia, and inhibit fungal pathogens' growth. Therefore, Pseudomonas fluorescens has excellent potential for treating white-nose syndrome and other fungal infections(23). The study reports only single cupping erosions are observed in Miniopterus schreibersii, M. bechsteinii and Rhinolophus euryale(24). It shows that Rhinolophus bats have a special advantage in fighting white nose syndrome. It identifies Rhinolophus bats as the natural reservoir of SARS coronavirus(25, 26). SARS-COV-2 coronavirus is also originated from

them(27). The Pseudomonas of Rhinolophus bat may have a subtle relationship with the evolution of SARS-COV-2 coronavirus.

Pseudomonas aeruginosa was also found in patients with COVID-19(28). The bacteria is a conditional infection pathogen. Pseudomonas aeruginosa pneumonia is accompanied by cough, high fever, sepsis-like, respiratory dysfunction, and multiple spreading on both sides of the lungs. Some symptoms are like the novel coronavirus pneumonia. Pseudomonas aeruginosa is a microorganism that is difficult to control with antibiotics or disinfectants because of the resistance of cystic fibrosis (CF) strains to antibiotics(29). Pseudomonas may undergo modern compensatory mutations due to drug resistance, which indirectly promotes parasitic virus's adaptive evolution, such as phage ϕ6(30).. Emerging viruses may transition from the original host to new microbial species(31) or influence other microbes' genome evolution and adaptability through horizontal gene transfer(32). This trend may seriously interfere with the growth of microbial communities in animals and cause pandemics. For example, the Vibrio cholerae phage may be one factor that regulates the cholera epidemic(33). The compensatory mutation of Pseudomonas and its adaptive phage evolution may also promote the development of the SARS-COV-2 virus.

To fight against bacteriophages, bacteria has developed many defense systems. Pseudomonas employs the CRISPR/Cas system to target the short characteristic sequence in the phage genome(34) and integrate it into the CRISPR site (short palindrome) in the host genome. It derives CRISPR from a spacer nucleic acid sequence homologous to the phage nucleic acid other than bacterial or archaeal chromatin, the spacer sequence(35, 36). Sequence near 5'and transcribed into crRNAs (CRISPR RNAs)(37). crRNAs carry the characteristic sequence information of the invading phage and the bacterial repetitive interval sequence information. Therefore, crRNAs can target and interfere with the invading phage DNA sequence and protect the host's DNA sequence(38). CRISPR/Cas III (Cmr / Csm)(*39*), CRISPR/Cas II (Cas 9)(*40*), CRISPR/Cas VI(Cas 13)(*41, 42*) are three RNA-targeting CRISPR/Cas systems, could cut and edit RNA. Specific type II CRISPR-Cas systems have natural nucleic acid lytic activity against ssRNA (Single-Stranded Ribonucleic Acid) targets, and they do not use scaRNA (Small Cajal body-specific RNAs), but its exact mechanism of action is still unknown(*39*). Studies have shown that this activity is crRNA (CRISPR-derived RNA) dependent and tracrRNA (trans-activating RNA) dependent, and RNA cleavage at a specific site is most likely to be completed by the HNH domain of Cas9(*43*). Neither PAM nor PFS sequences show the necessary conditions to regulate this activity (49). Staphylococcus aureus(*44*), Campylobacter jejuni(*43*) and Neisseria meningitidis(*45*) have this special active to target ssRNA.

Bacteriophages have also strengthened some escape systems, which are immune resistant against host bacteria. The phage mutates the protospacer sequence or motif to circumvent the CRISPR/Cas system(*46*). The phage also encodes anti-CRISPR-Cas genes to synthesize some proteins to interfere with the formation of the host CRISPR/Cas protein complex or inhibit its activity(*47*). It could hijack the bacterial CRISPR/Cas system to amplify itself. For example, some Vibrio cholerae bacteriophages have hijacked the entire CRISPR/Cas system to achieve their defense and persistence(*48*). If the phage gene fragments on crRNAs result from mutations or modifications, crRNAs lose their ability to target the phage genome. Therefore, because of the gene integration and immune resistance of bacteriophages to host bacteria, crRNAs carry conservative domain genes in evolution. It is not surprising if ancient viruses and archaea carry

similar CRISPR/Cas structures through defending and confronting each other in terms of evolution. The CRISPR/Cas-like system was integrated with the virus itself, driving the evolution of the virus itself.

The battle between bacteriophages and Pseudomonas aeruginosa subtly promoted the evolution of coronaviruses in bats. The lysis of Pseudomonas aeruginosa by phage facilitated the release and diffusion of crRNA. Bat cells infected with coronavirus inhaled crRNA through endocytosis. Fragments in crRNA (complementary to the characteristic sequence of the phage) were combined with the coronavirus RNA's complementary region. The coronavirus RNA carried the distinct sequence fragments of phage or Pseudomonas through the cutting, modification, and splicing of the CRISPR/Cas-like system. It was a migratory evolution of conserved domains. It mainly positioned the two kinds of RNAs with each other through partial sequence complementation. So the coronavirus RNA (if positive strand) was partially similar to the sequence of phage or Pseudomonas. This migratory evolution most likely occurred on synthetic negative-strand of coronavirus RNA. The fungal infection speeded up the migration of crRNA into bat cells. Fungi employed spores and hyphae to infect bat cells. Fungal spores entered bat cells through endocytosis, and directly invaded bat cells after growing into hyphae. Fungal spores absorbed attached small RNAs(*49, 50*). Therefore, crRNA was attached to fungal spores or hyphae to achieve large-scale transport to bat cells. This migratory evolution was not a small probability event for large-scale fungal and Pseudomonas aeruginosa infections in bat communities (such as the white-nose syndrome mentioned above). The migration and evolution caused mutations and recombination of the SARS-COV-2 virus genome, which made the SARS-COV-2 virus have resistance similar to Pseudomonas or phage.

This research will explore the driver force of migration and evolution of the SARS-COV-2 virus. We found that the SARS-COV-2 virus has CRISPR/Cas 9 (II-B)-like system through the search method of conserved domains, which could target and edit RNA. The SARS-COV-2 virus proteins had Cas (HNH Cas9, HD Cas3, Cas4, Cas6) and Fe-S domains. So, each protein had a trace of evolution for the Cas domains. Only the Cas-like structure of nsp3 had complete Cas functions, CRISPR/Cas 9 (II-B)-like system. These Cas domains of nsp3 all have nuclease cleavage activity. Existing literature shows that biochemical and structural data show that nsp3 protein of SARS-CoV-2 is a larger orf1ab sub-protein, which retains the ability to bind ADP-ribose(51). The nsp3 has single ADP-ribose (MAR) hydrolysis Enzyme activity(52), which is an essential feature of beta-coronavirus and alpha-coronavirus. Simultaneously, the nsp3 macrodomain of SARS-CoV-2 holds a poly ADP-ribose (ADPR) binding module, and it has a single ADPR lyase Activity(53). It shows that nsp3 is the best candidate for the CRISPR/Cas-like system. Based on a wide range of fungal and Pseudomonas aeruginosa infections, the immune resistance of phage to Pseudomonas produced rapidly mutated domain gene information, which migrated crRNA into bat cells through fungal spore (or hypha) invasion. With the driver force of the CRISPR/Cas-like system, the conserved domain in crRNA was integrated into SARS-COV-2 virus RNA.

2. Method

2.1. Data set

1. The sequence of SARS-COV-2 protein

The SARS-COV-2 protein sequences come from the NCBI database. Including S, E, N, ORF3a, ORF8, ORF7a, ORF7b, ORF6, ORF10, orf1ab sequence. Besides, the orf1ab protein also includes corresponding sub-proteins.

2. Bacterial Cas protein sequence

We downloaded the bacterial Cas proteins from the UniProt database. The keyword was "bacterial+cas".

2.2. Localized MEME tool to scan for conserved domains

We downloaded MEME from the official website and installed it in the virtual machine ubuntu operating system. For each the sequences in all bacterial Cas proteins, paired with the protein sequence of SARS-COV-2 by MEME tool to find conserved domains. Only motifs with E-value less than or equal to 0.05 were accepted.

3. Results

3.1 The Nsp3 protein has the conserved domains of the CRISPR/Cas-like system

crRNA and target RNA have partial sequence complementarity. If the target RNA was to carry information about bacterial and phage genome fragments, coronaviruses only used negative-strand RNA to bind to crRNA. If the viral protein had the Cas function, it could edit the coronavirus's negative-strand RNA. We downloaded 9,360 bacterial Cas proteins from the UniProt database. Then, the local MEME tool was adopted to compare each SARS-COV-2 protein with bacterial Cas proteins to search for cas conserved domains (Table 1). Table 1 shows that all viral proteins had HNH Cas9-type domains. Except for ORF3a, nsp11, and helicase proteins, all viral proteins have HD Cas3-type conserved domains. Except for ORF3a, nsp7, and nsp9 proteins, all viral proteins have Cas_Cas4 conserved domains. Some proteins also have Cas9-BH, CRISPR_Cas6, Cas6b_C, Cas6b_N, Cas_CXXC_CXXC, Cas3_C, Cas1_AcylT, 4Fe-4S domains. Cas1_AcylT(*54*) handles polysaccharide acetylation.

HD Cas3-type domain. The HD Cas3 domain protein cleaves endonucleolytically and exonucleolytically (3--5) single-stranded DNAs and RNAs, as well as 3'-flaps, splayed arms, and R-loopss(*55*). In Cascade-mediated R loop formation Later, the Cse1 subunit recruits Cas3, which catalyzes target DNA's nicking through its HD nuclease domain. Then gradually untie the target and the combined ATP-dependent helicase activity and the HD cleavage nuclease activity of the dependent Mg2+. Cas3 completes degradation of the target DNA and neutralization of the invaders(*56*). Cas3_C is the C-terminal domain of the Cas3 protein; the C-terminal domain (CTD) regulates N-terminal HD nuclease activity(*57*). Cas3_C takes part in the movement of explicitly blocking the action of caspase-3(*58*). So we believed that the HD Cas3-type domain completes the unwinding of the negative-strand RNA of coronavirus.

Cas6 domain. Cas6 has endonuclease properties and is used for CRISPR RNA (crRNA)

recognition and cleavage surround mechanism. Cas6 processes the single-stranded RNA in the surrounding model(*59*), and has bivalent capture and melting(*60*). Cas6 contains a tandem ferredoxin fold(*61*). Cas6b is a member of the Cas6 RNA-processing endoribonuclease found in bacteria and archaea, and Cas6b_C and Cas6b_N have C-terminal and N-terminal, respectively (*62*). Cas6b forms an interference complex with CRISPR-associated (Cas) protein to target the invading nucleic acid for degradation. We speculate Cas6 binds to the crRNA of Pseudomonas to target the coronavirus's negative-strand RNA. It unties the coronavirus's negative-strand RNA for precise processing.

HNH Cas9-type domain. HNH Cas9-type belongs to the Cas9 protein domain of CRISPR/Cas 9 (II B). PAM presenting oligonucleotides (PAMmers) guide and stimulate the cleavage of ssRNA targets by site-specific endonucleases(*63*), and the HNH Cas9 domain binds or cleaves RNA targets(*64*). AcrIIC2 interferes with RNA binding by binding to the Cas9 BH motif and prevents DNA from being loaded into Cas9(*65*). HNH Cas9 is divided into -DH--N or -HH--N-type. -DH-based HNH endonuclease binds to catalytic Mg 2+ ions. -HH--N-based HNH endonuclease has one or two invariant Zn-binding CxxC/CxxxC motifs(*66*). The CXXC_CXXC zinc finger domain is a reader of unmodified CpG dinucleotides. It plays a vital role in epigenetic regulation by targeting various activities to CpG islands(*67*). The viral protein specifically binds to the unmethylated CpG motif through its CXXC_CXXC domain. HNH endonuclease based on -HH--N has a CXXC_CXXC zinc finger domain(*68*). Therefore, the HNH Cas9 domain of viral proteins may belong to these two endonucleases, respectively.

Studies have found that HNH Cas9 activity is crRNA-dependent(43), and PAM and PFS sequences are not required(45). The HNH Cas9 domain of SARS-COV-2 virus protein would cleave the negative-strand RNA of SARS-COV-2 virus protein through crRNA guidance. crRNA came from Pseudomonas crRNA, entering into animal cells by fungal spores. The SARS-COV-2 virus proteins all had HNH Cas9 domains, showing that the migration and evolution trace. We thought that HNH Cas9 completed the cleavage of the negative-strand RNA of coronavirus.

Cas4 domain. Cas4 belongs to the self-synthetic transposon superfamily Casposons. It was an integrase (recombinase)(*69*). Cas_Cas4 is believed to form recombinant 3'-ssDNA overhangs in the protospacer, facilitating their subsequent incorporation into the CRISPR array as dsDNA spacers(*70*). All members of the Cas4 family contain a single structure---four conserved cysteines, Fe-S-cluster binding module. It has been shown that different proteins, Cas4, bind to either [2Fe-2S](*71*) or [4Fe-4S] cluster(*72*). Fe-S-cluster is an iron-sulfur protein (iron-sulfur proteins, Fe/S). The protein is an iron-containing protein and also a cytochrome protein. It is not heme but iron and sulfur that bind in the center of the iron-sulfur protein molecule called iron-sulfur centers. The melting of DNA depends on several residues near the [4Fe-4S] cluster. The Cas4 protein may add new CRISPR spacers through the formation of 3'-DNA overhangs and the degradation of foreign DNA(*72*). Cas4 also cleavage RNA through the RNA-guided of CRISPR RNA-Cas protein complex(38). Cas4 may belong to the class II VA system of the CRISPR-CAS system, and it also helps process the precursor of CRISPR RNA, i.e., precursor crRNA(*73*). We speculated that the Cas4 domain of the SARS-COV-2 virus protein employed the 3'-terminal of coronavirus's negative-strand RNA as a CRISPR array. The Cas4 connected the RNA fragment of the conserved domain(from the crRNA) to the nick on the coronavirus's negative-strand RNA.

The Cas domain was a trace of migratory evolution. In the CRISPR/Cas II B genome

structure, the Cas9 protein was on the left (5'-terminal), and the CRISPR region was on the right (3'-terminal). The coronavirus genome structure should be like that of CRISPR/Cas II B. Only S, ORF7b, nsp3, nsp4 had complete HNH Cas9-type, HD Cas3-type, Cas4, 4Fe-4S, Cas6 domains. It meant that the Cas4 domains (without 4Fe-4S) of other proteins could not independently transfer the conserved domains on cRNA to the virus's negative-strand RNA. We also found that the heme domains of ORF3a, N, and E searched by heme theory(*74*) were all in the conserved domain of HNH Cas9-type. If the HNH Cas9 protein edited the RNA region that encoded itself, its activity would be lost, so ORF3a, N, and E could not have Cas function. But ORF7b and ORF6 (with 4Fe-4S) are shorter and in the 3'-terminal of viral RNA. So ORF7b and ORF6 were also unlikely to have the Cas-like system function. The HNH Cas9 of S and the Cas4 of nsp4 are also very short, and it is impossible to have a complete role. Therefore, the Cas domains except the nsp3 protein should be the trace of migratory evolution and had not a full Cas function.

nsp3 had a CRISPR/Cas-like system. Non-structural protein 3 (nsp3) is the most significant protein encoded by the coronavirus (CoV) genome. The extracellular domain of nsp3 (3Ecto), also is known as "zinc finger domain"(*75*). HNH Cas9-type, HD Cas3-type , Cas4 domains of nsp3 protein were longer (Table 2). nsp3 was also the only viral protein with Cas9-BH domain. Cas9-BH prevented DNA from binding to the CRISPR region of the negative strand of viral RNA, and it also avoided the interference binding of other RNAs. The Cas functional protein was in the orf1ab protein region at the 5'-terminal of the viral RNA, and the CRISPR region was at the 3'-terminal of the viral RNA. Nsp3 also was in the orf1ab area and was also the most significant sub protein.Then, nsp3 had a complete CRISPR/Cas-like system function.

3.2 Migration and evolution of conserved domains of coronavirus RNA

According to the previous analysis results, the migration and evolution model of coronavirus (positive-strand RNA) could be derived. The detailed steps of the model were:

1. Pseudomonas crRNA entered the cytoplasm of bat cells through endocytosis or fungal hypha invasion;

2. Bat cells synthesized the negative strand RNA of coronavirus in the cytoplasm.

3. The nsp3 protein with HD Cas3, Cas_Cas4, Cas6, and HNH Cas9 domains bound to the crRNA to form a complex. This complex located the negative-strand RNA of the coronavirus.

4. HD Cas3 domain unwinded the negative-strand RNA of coronavirus.

5.With the action of Cas6, the partial complementary region of crRNA and the negative-strand RNA of coronavirus were combined

6. HNH Cas9 cut one side of the complementary region of the coronavirus negative-strand RNA.

7. Cas_Cas4 cleaved the crRNA, transferred the conserved domain fragments near the complementary sequence of the crRNA to the nick of the coronavirus's negative strand. Then it connected the negative strand of the coronavirus and the conserved domain fragment of the crRNA.

Table 1. SARS-COV-2 proteins have HNH Cas9, HD Cas3, Cas4 and Cas6 domains

Protein	HNH Cas9-type	HD Cas3-type	Cas_Cas4	Cas_CXXC_CXXC	4Fe-4S	Cas9-BH	CRISPR_Cas6	Cas6b_C	Cas6b_N	Cas3_C	Cas1_Acy1T
S	V	V	V		V		V	V		V	
E	V	V	V	V			V				
M	V	V	V								
N	V	V	V	V							
ORF3a	V										
ORF7a	V	V	V								
ORF7b	V	V	V	V	V		V				
ORF8	V	V	V	V							
ORF6	V	V	V	V	V		V				
ORF10	V	V	V	V			V				
nsp2	V	V	V				V				
nsp3	V	V	V		V	V	V	V			
nsp4	V	V	V		V		V	V			
nsp6	V	V	V								
nsp7	V	V		V					V		V
nsp8	V	V	V								
nsp9	V	V		V							
nsp10	V	V	V	V							
nsp11	V		V				V			V	
3C	V	V	V				V	V			
RNA-depend	V	V	V								
helicase	V		V								
3'-to-5'exonuclease	V	V	V								
endoRNAse	V	V	V	V					V		
2'-O-ribose	V	V	V	V			V				
leaderprotein	V	V	V								

Table 2. nsp3 protein has Cas and 4Fe-4S domains

Domain	Motif of Domain	Count
Cas_Cas4	CMMCYKRNRATRVEC	1
	FYVYANGGKGFCKLHNWNCVNCDTFC	1
	GVQIPCTCGKQATKYLVQQESPFVMMSAPPAQYE	1
	HFISNSWLMWLIINLVQMA	1
	HFISNSWLMWLIINLVQMAP	1
	HFISNSWLMWLIINLVQMAPISAMVRMYIFFASFYYVW	1
	HFISNSWLMWLIINLVQMAPISAMVRMYIFFASFYYVWKSYVHVVDGC	1
	MMCYKRNRATRVEC	1
	NMTPRDLGACIDCSARHINAQ	1
	QMAPISAMVRMYIFFASFYYVWKSYVH	1
	QMAPISAMVRMYIFFASFYYVWKSYVHVVDGCNSSTCMMC	1
	REMLAHAEETR	1
	RRSFYVYANGGKGFCKLHNW	2
	WCIRCLW	35
	WHVNNATNKATYKPNTWCIRCLW	1
	WLIINLVQMAPISAMVRM	1
	WLMWLIINLVQMAP	1
	WLMWLIINLVQMAPISAMVRMYIFFASFYYVWKSYVHVVDGCNSSTCMMC	1
Cas6b_C	HNWNCVNCDTFC	1
	IMQLFFSYFAVHFISNSWLMWLIINLVQMA	1
Cas9-BH	RMYIFFASFYYVWKSYVHVVDGCNSSTCMM	1
CRISPR_Cas6	WNLREMLAHAEETRKLM	1
HD Cas3-type	FFSYFAVHFISNSWLMWLIINLVQMAPISAMVRM	1
	RCLNRVCTNYMPY	1
	TWCIRCLW	1
HNH Cas9-type	MQLFFSYFAVHFISNSWLMW	1
	MWLIINLVQMAPISAMVRMYIFFASFYYVWKSYVHVVDGCNSSTCMMCY	1
	PRDLGACIDCSARHINAQVAKSHNIALIW	1
	WCIRCLW	1
4Fe-4S ferredoxin-type	CCMTSCCSCLK	1

The Pseudomonas crRNA and the coronavirus negative-strand RNA were partially complementary. The coronavirus positive-strand RNA and the coronavirus negative-strand RNA were complementary. The sequence in the Pseudomonas crRNA spacer region was complementary to the characteristic sequence of the phage. Certain sequences in Pseudomonas crRNA are complementary to those of Pseudomonas DNA. Therefore, the positive-stranded RNA of coronavirus was partly similar to the characteristic sequence of phage and Pseudomonas. In this way, the conserved domain realized the migration and evolution from phage or Pseudomonas to coronavirus.

The Cas protein could not have a codon checking function. The protein-coding information in the original RNA coding region would be disrupted during the cutting and splicing process.

Therefore, SARS-COV-2 undergoes mutation or recombination through this migration and evolution of gene information. There would be various crRNAs that could target different sites or regions of the coronavirus negative-strand RNA. There were more coronavirus positive-strand RNA coding regions affected by mutation or recombination. Of course, the 5'-terminal of the positive-strand RNA of coronavirus was affected by mutation or recombination to a relatively small degree.

4. Discussion

4.1 The large-scale migratory evolution process of SARS-COV-2 occurred in critically patients' phagocytes

COVID-19 patients who are dually infected with fungi and bacteria are also at risk of SARS-COV-2 virus migration evolution, i.e., virus mutation and recombination. Iron-sulfur protein synthesizes and assembles [4Fe-4S]2+ cluster sites in mitochondria(*76*), and chaperone proteins take part in this synthesis reaction(*77*). 4Fe-4S was an active rate-limiting molecule of the Cas4 domain of nsp3. The heme theory found that the SARS-COV-2 virus protein attacked hemoglobin and dissociated the iron of heme(*74*). Activated phagocytes produced extreme phagocytic behavior, including red cells and iron. Phagocyte contained much iron. The time point should be when the condition of COVID-19 patients deteriorates. ROS immune damage theory(*78*) found that the SARS-COV-2 virus parasitized in phagocytes by decomposing or producing ROS. Clinical evidence shows that the SARS-COV-2 virus also infects phagocytes, completing the synthesis and replication of viral RNA. Therefore, nsp3 would synthesize and assemble 4Fe-4S in the mitochondria of phagocytes. Then, it bound to the bacterial crRNA in the cytoplasm and targeted the negative-strand RNA of the SARS-COV-2 virus to complete migration and evolution. So the SARS-COV-2 virus genome was mutated and recombined.

4.2 nsp3 could transfer host cell gene fragments to the positive-strand RNA of SARS-COV-2?

This study found that the nsp3 protein had a CRISPR/Cas-like system, which Pseudomonas crRNA guided. nsp3 targeted the negative-strand RNA of coronavirus to complete the domain's migration evolution. This migration led to mutations and recombination of the coronavirus genome. A study reported that coronavirus might steal host genes from Western European hedgehogs through EriCoV genetic characteristics(*79*). But the specific mechanism is not clear. If this migration and evolution behavior is also through the CRISPR/Cas-like system of nsp3, then it meant that nsp3 protein could not depend on bacterial crRNA. The gene fragment of the EriCoV domain in the host cell mRNA would be directly transferred to the coronavirus RNA's positive-strand by the nsp3 protein. Of course, EriCoV mRNA and coronavirus RNA positive strands had partial complementary regions, and it positioned and combined the two. But it needed more evidence to support that the nsp3 protein targeted the positive strand of coronavirus RNA through mRNA.

5.Conclusion

The patients of the novel coronavirus pneumonia often have bacterial and fungal infections. Empirical broad-spectrum antibacterial drugs have been widely used in treatment. The pandemic-derived drug resistance problem has become increasingly severe. For COVID-19 patients, Aspergillus fumigatus and Candida pneumonia are common fungal infections, while Pseudomonas pneumonia is a refractory bacterial infection. Contemporary bats are also facing fungal infections such as white-nose syndrome. The fluorescent matter secreted by the pseudomonas has an inhibitory effect on the growth of fungi. Species with the white-nose syndrome include Rhinolophus bats, the natural host of SARs, or SARS-COV-2. It found that in these similar bats, the SARS-COV-2 virus has a rapid evolution phenomenon. Therefore, studying the relationship between combined bacterial and fungal infections and the rapid evolution of SARS-COV-2 has important guiding significance for preventing pandemics.

We found that the SARS-COV-2 virus had CRISPR/Cas 9 (II-B)-like system through the search method of conserved domains, which could target and edit RNA. The SARS-COV-2 virus proteins had Cas (HNH Cas9, HD Cas3, Cas4, Cas6) and Fe-S domains. So, each protein had a trace of evolution for the Cas domains. Only the Cas-like structure of nsp3 had complete nuclease cleavage activity, CRISPR/Cas 9 (II-B)-like system. We speculated that the crRNA (CRISPR RNA) produced by the CRISPR/Cas system of Pseudomonas carried gene information on the conserved domains of the phage and the Pseudomonas genome. The crRNA targeted the phage genome segment and protected the pseudomonas genome. However, because of the immune resistance of phage to Pseudomonas, crRNA was affected by mutation and lost its ability to target the phage genome. The crRNA was released with the lysis of Pseudomonas by phages. In the mass reproduction of fungi and Pseudomonas, the crRNA was attached to fungal spores, and the spores or hyphae are invaded to bat cells. crRNA was combined with coronavirus negative-strand RNA fragment. The conserved domain on crRNA was inserted into the coronavirus negative-strand RNA through RNA editing by nsp3 protein. Therefore the CRISPR/Cas 9 (II-B)-like system of nsp3 promoted domains' migration evolution, leading the mutant recombination of SARS-COV-2.

There may be multiple crRNAs that can target different regions of a coronavirus negative-strand RNA. Since the Cas protein had no codon checking function, this cutting and splicing would disrupt the protein-coding information in the original RNA coding region. The way of migration and evolution caused mutations and recombination of the SARS-COV-2 virus genome. 4Fe-4S was an active rate-limiting molecule of the Cas4 domain of nsp3. Nsp3 would synthesize and assemble 4Fe-4S in the mitochondria of phagocytes. Iron comes from hemoglobin attacked by the SARS-COV-2 virus protein. Then the mutant and recombination of SARS-COV-2 occurred in the phagocytes of critically patients. Since the SARS-COV-2 virus could spread latent in phagocytes, the virus's genetic mutation and recombination were also latent.

If crRNAs carried the drug resistance genes of bacteria or phages, SARS-COV-2 could have similar drug resistance. In short, given the increasingly severe drug resistance problem, we should pay attention to the co-infection of fungi and bacteria in COVID-19 patients to cause SARS-COV-2 drug resistance through mutation and recombination of the novel coronavirus.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets and results supporting the conclusions of this article are available at https://pan.baidu.com/s/1odSOhrbc0KyBwkQm2PCSUg , code: 8vkr
Or: https://mega.nz/folder/NrJyjZ6L#30u6r_fzhAV6luMdXg8YUQ

Competing interests

The authors declare that they have no competing interests.

Funding

This work was funded by a grant from the National Natural Science Foundation for the Talent Introduction Project of Sichuan University of Science and Engineering (award number: 2018RCL20, grant recipient: WZL).

Author's contribution

Funding was obtained by WZL. Besides, design, analysis and writing are finished by WZL, while data curation and manuscript check are undertaken by HLL. Both authors have read and agreed to the published version of the manuscript.

Acknowledgements

Thanks readers for free review and suggestions.

Author details

[1] School of Computer Science and Engineering, Sichuan University of Science & Engineering, Zigong, 643002, China.
[2] School of Life Science and Food Engineering, Yibin University, Yibin, 644000, China;

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