Structural basis for neutralization of SARS-CoV-2 Omicron variant using engineered ACE2 decoy

The coronavirus disease (COVID-19) caused by sever acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in Wuhan, China, at the end of 2019 and then spread worldwide. Coronaviruses have large trimeric membrane proteins called spike proteins on their surface. The spike protein binds to angiotensin-converting enzyme 2 (ACE2) on the surface of human cells via its receptor binding domain (RBD), triggering viral infection. Therefore, inhibiting the binding of spike proteins to ACE2 is one of the most promising therapeutic strategies for COVID-19. Following this concept, multiple vaccines and therapeutic agents have been developed to date. However, the pandemic is still ongoing three years after the outbreak and many cases are reported every day, causing a significant impact on our lives. One reason for this is the high mutation rate of SARS-CoV-2 (~1×10^{-3} substitutions per site per year) [1]. Since the first SARS-CoV-2 appeared in Wuhan, various mutants of the virus have emerged over the past three years, and there is concern that spike protein-based vaccines and monoclonal antibody therapeutics targeting the RBD may reduce or nullify their efficacy because of the mutations.

We previously developed a therapeutic candidate that neutralizes SARS-CoV-2 using the extracellular region of ACE2, soluble ACE2 (sACE2) (Fig. 1) [2]. The main advantage of using an sACE2-based decoy receptor is its resistance to virus escape mutations. The virus mutant escaping from the sACE2 decoy should also have a limited binding affinity toward native ACE2 receptors on the cell surface, resulting in diminished or eliminated infectivity. Since the affinity of wild type (WT) sACE2 for the RBD is relatively low, we first attempted to increase the affinity by directed evolution. Random mutations were introduced to the amino acid residues 18–102 of ACE2, which form the RBD-binding interface by error-prone PCR. They were then expressed on human cells and ACE2 mutants with increased affinity were selected by flow cytometry. After repeating these procedures three times, we obtained a high-affinity ACE2 mutant, ACE2(3N39), with seven mutations, A25V, K26E, K31N, E35K, N64I, L79F, and N90H. Quantitative measurements of binding affinity to the Wuhan RBD showed that sACE2(3N39) has an approximately 100-fold increased affinity compared with WT (K_D values of WT and the 3N39 variant are 17.63 nM and 0.29 nM, respectively). We further characterized each mutation and found that only four of the seven mutations (A25V, K31N, E35K, and L79F) were sufficient to achieve high affinity comparable to those of conventional antibody drugs (K_D value of the A25V/K31N/E35K/L79F mutant is 0.64 nM). We next conducted the structural analysis of sACE2(3N39) complexed with the Wuhan RBD. Initial crystallization screening yielded thin rod-shaped crystals, and the optimization of crystallization conditions resulted in thick hexagonal plate-shaped crystals. X-ray diffraction data were collected at SPring-8 **BL44XU**, and the complex structure was solved at a resolution of 3.2 Å. The crystal structure revealed how the mutated residues in 3N39 contribute to the affinity enhancement for the Wuhan RBD [2].

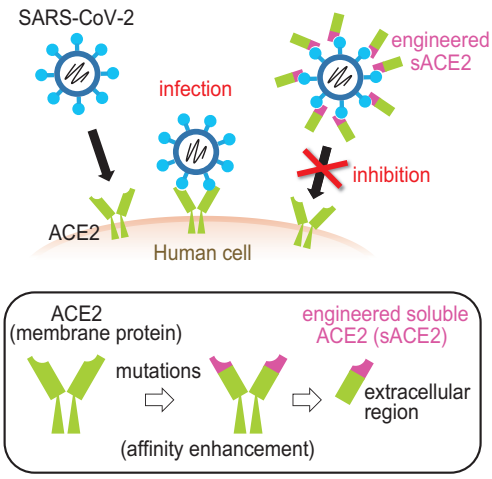

Fig. 1. Strategy for SARS-CoV-2 neutralization using engineered sACE2 decoy.

However, as mentioned above, SARS-CoV-2 rapidly undergoes mutations, and various variants of concern (VOCs) have emerged thus far. The Omicron variant of SARS-CoV-2, which was detected for the first time in South Africa in November 2021, has a high number of mutations in the spike gene. More than 30 amino acid residues are mutated in the Omicron spike protein, 15 of which are in the RBD (Fig. 2). These mutations significantly reduced the activity of several therapeutic antibodies developed to target the Wuhan strain [3,4]. The complex structure of the Omicron RBD and WT ACE2 was first deposited in the PDB in December 2021 and has since been reported by several research groups. The structures showed that multiple Omicron mutations are located at the binding interface of these molecules (Fig. 3(a)). To understand the difference in interaction mechanism among the RBD/ACE2 pairs, we compared the structures of Omicron RBD-WT ACE2, Wuhan RBD-WT ACE2, and Wuhan RBD-ACE2(3N39) complexes [5]. In WT ACE2, K31 and

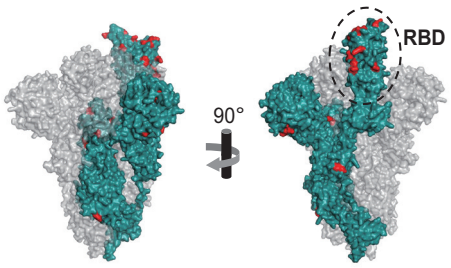

Fig. 2. Mapping of the mutation sites introduced in the Omicron spike protein. One subunit within the trimeric Omicron spike protein (PDB: 7wvn) is shown as a dark cyan surface, and the other two subunits are shown as gray transparent surfaces. Omicron mutations are highlighted in red.

E35 form an intramolecular salt bridge (Fig. 3(b)), whereas in our mutant, the simultaneous mutation of K31N and E35K disrupts this interaction, allowing E35K to exclusively form a hydrogen bond with Q493 of the Wuhan RBD (Fig. 3(c)). We speculated that this is one of the reasons for the improved affinity in 3N39. On the other hand, in the Omicron RBD, Q493 was substituted with arginine, which forms a salt bridge with E35 of WT ACE2 (Fig. 3(d)). This salt bridge may contribute to the affinity enhancement of the Omicron RBD for WT ACE2. However, when ACE2(3N39) binds to the Omicron RBD, there is concern that electrostatic repulsion between the E35K mutation site of ACE2(3N39) and R493 of the Omicron RBD could occur. To predict the effect of the Omicron mutations on ACE2(3N39) binding, we built a homology model of the Omicron RBD-ACE2(3N39) complex using structures of PDB IDs 7t9l and 7dmu. The complex model suggested that the electrostatic clash between K35 and R493 can be easily avoided by the rotation of their side chains. It was also found that the side chain of R493 could form a direct hydrogen bond with N31 of ACE2(3N39) instead of K35 (Fig. 3(e)). Thus, it was structurally predicted that 3N39 could retain a high affinity for the Omicron RBD.

In later studies, we evaluated various aspects of ACE2 decoys and attempted to optimize the design of ACE2 mutants. Finally, we succeeded in designing ACE2(3N39v4) with improved stability and reduced potential immunogenicity while maintaining the high affinity of the original ACE2(3N39). The degree to which sACE2(3N39v4) neutralized Omicron was comparable to the case of the Wuhan strain and showed a therapeutic effect against Omicron infection in hamster and human ACE2 transgenic mice [5]. These facts indicate that the decoy strategy is more effective against viruses with a high mutation rate than antibody-based therapeutics. The ACE2 decoy developed in this study is expected to show high neutralizing activity against any SARS-CoV-2 VOCs that may emerge in the future.

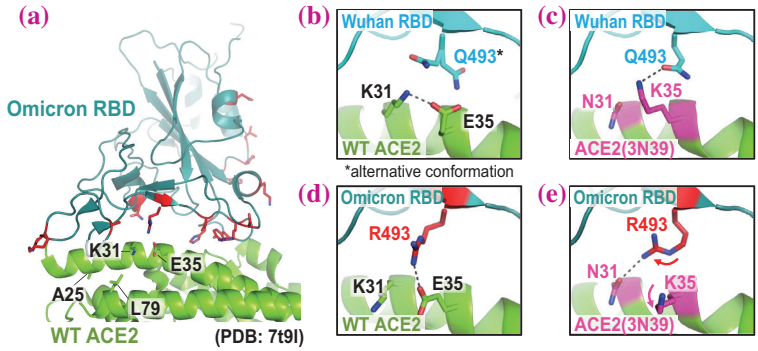

Fig. 3. Difference in interaction mode among the RBD/ACE2 pairs. **(a)** Cryo-electron microscopy structure of the Omicron RBD-WT ACE2 complex (PDB ID: 7t9l). Residues mutated in Omicron are shown as red sticks. Four residues (A25, K31, E35, and L79) of which mutations are essential for enhancement of affinity in the ACE2(3N39) variant are shown as sticks. **(b** to **e)** Close-up views of the interface between **(b)** Wuhan RBD and WT ACE2 (PDB ID: 6m0j), **(c)** Wuhan RBD and ACE2(3N39) (PDB ID: 7dmu), **(d)** Omicron RBD and WT ACE2 (PDB ID: 7t9l), and **(e)** Omicron RBD and ACE2(3N39) (simulated model) are shown. Potential hydrogen-bonding and salt-bridge interactions are indicated by dashed lines.

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