



Engineering a cell-penetrating hyperstable antibody scFv(Ras) – An extraordinary approach to cancer therapeutics

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ABSTRACT

In the modern pharmaceutical industry, monoclonal antibodies are often used as therapeutic agents. However, they are restricted to cell surface antigens due to their inability to penetrate the outer cell membrane and maintain normal function in the reducing environment. Additionally, it can lead to cytotoxicity since it attacks cancerous cells by mimicking the human immune system. As an alternative, this study modifies the hyperstable single-chain fragment variable(scFv) antibody to eliminate cancer using its linear shape. The scFv(F8) antibody model was modified to recognize human Ras protein by altering residues in the antigen-binding site. Furthermore, a cell-penetrating peptide (CPP) was attached to the scFv(Ras) antibody model to allow entrance to the cell, creating CPP-scFv(Ras). Sodium dodecyl sulfate polyacrylamide gel electrophoresis(SDS-PAGE), western blotting, and the binding assay were performed to prove its effectiveness. As a result, CPP-scFv(Ras) was successfully engineered and bound to the antigen, HRas(G12V).

1. Introduction

1.1. Conventional antibodies and their limitations

Engineered Antibodies have been used as a major therapeutic mechanism since the United States Food and Drug Administration approved its use in 1986. Their ability to attach to target receptors is used for disease diagnostics and eliminating mutated cells. Conventional antibodies are composed of variable heavy (VH) and variable light (VL) chains linked by disulfide bonds in a ‘Y’ shape. The disulfide bond dissolves in the reducing environment, which prevents the normal function of antibodies, and its ‘Y’ shape prevents entrance through the cell membrane even with the CPP tag. This prohibits effective extermination of the proliferating cancerous cells while triggering immune responses.

One of the most widely used antibodies is naked monoclonal antibodies(mAbs), engineered to replicate the mechanism of the human immune system to remove cancerous cells. However, monoclonal antibodies in cancer therapeutics are imperfect since they attach to outer cell receptors when target antigens are often located inside the cell. Furthermore, mimicking the immune system can cause serious side

effects such as cytotoxicity and illness to immunosuppressed patients [1].

1.2. Hyperstable antibody and its significance

As an alternative to mAbs, a CPP tagged single-chain fragment variable antibody(scFv) can be used to create an effective antibody. The CPP-scFv can penetrate the cell membrane and bind directly to the problematic antigen [2]. The flexible peptide chain that links the VH and VL in a single strand format allows penetration and normal functioning in the reducing environment [3,4]. Due to its unique structure, the stability of scFv antibodies is unusually high; therefore, it is called ‘hyperstable.’ The scFv(F8) model used in this study is also hyperstable due to the presence of a flexible peptide rather than a disulfide bond. When scFv(F8) antibody is tagged with a CPP, the antibody can penetrate the cell membrane and execute therapeutic roles. The specific CPP in this study is extracted from the Porcine circovirus type 2(PCV2), a small DNA virus that can infect pigs. The PCV2 capsid protein produces the nuclear localization signals, which form positively charged residues that ultimately act as a CPP [5]. The chain reaction of endocytic processes allows the CPP tagged scFv to internalize across the cell interior

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Abbreviation

CPP	Cell-Penetrating Peptide
GDP	Guanosine Diphosphate
GTP	Guanosine Triphosphate
LIC	Ligation-Independent Cloning
mAbs	Monoclonal Antibodies
PCR	Polymerase Chain Reaction
PCV2	Porcine Circovirus Type 2
P13K	Phosphoinositide 3-kinase
RALGDS	Ral Guanine Nucleotide Dissociation Stimulator
scFv	Single-Chain Variable Fragment
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TBS-T	Tris Buffered Saline with Tween 20
VH	Variable Heavy Domain
VL	Variable Light Domain

rapidly. Therefore, CPP-scFv(F8) eliminates cancerous cells with minimal damage to the normal cell population. The target-specific binding of the engineered antibody avoids binding to other intracellular components that can lead to significant side effects [6–8].

1.3. The Ras protein and CPP-scFv(Ras)

Ras is a signaling pathway found in most eukaryotic cells that is responsible for average cell growth, proliferation, and differentiation. The Ras protein is oncogenic, meaning that it is part of the cell's major signaling pathway controlled by guanosine triphosphate(GTP) active and guanosine triphosphate(GDP) inactive states. Mutated Ras or downstream effectors such as Phosphoinositide 3-kinase(P13K) and Ral Guanine Nucleotide Dissociation Stimulator(RALGDS) can indicate Ras is left in a constant active state, leading to abnormal cell proliferation that causes cancer [9]. The Ras protein is found in most human cells, and Ras mutation is identified in 30% of the human population. It is observed most often in colon, pancreas, lung, and adenocarcinoma cancers [10]. In this study, the CPP-scFv(F8) antibody model is engineered to bind to the Ras protein, creating CPP-scFv(Ras). scFv(Ras) is engineered from the scFv(F8) antibody model by grafting Ras. The grafting is conducted based on the Ras protein complex structure (PDB ID: 2vh5) and anti-Ras antibody. Once the scFv(Ras) binds to the active site of Ras, the Ras protein goes inactive. The CPP-scFv(Ras) minimizes the destruction of normal cells while it effectively controls the mutant cell population.

2. Material and methods

2.1. PCR amplification of insert HRas(G12V)

The Polymerase Chain Reaction(PCR) mix (TOYOBO, Japan) was prepared by mixing 42 μ L of sterile double distilled H₂O, 5 μ L of reaction buffer, 0.5 μ L of dNTP mix at 2.5 mM, 0.5 μ L of forward primer at 100 μ M, 0.5 μ L of reverse primer at 100 μ M, 1 μ L of plasmid DNA, and 0.5 μ L of DNA polymerase. Then, the PCR Program was operated. The initial denaturation occurred at 94 °C for 5 min. Then, the 2nd denaturation took place at 94 °C for 1 min. After the denaturation, annealing was performed for 30 s at 55 °C to allow the primer to bind to the template. During the progress, the extension time for DNA polymerase took place, which took 1 min with kb target sequence. The four steps listed above for the PCR Program were repeated 25 times. The final extension was performed at 72 °C for 5 min, and the mixture was stored at 4 °C until it was used [11].

2.2. DNA gel electrophoresis

DNA gel electrophoresis was used to check the PCR product. First, 0.36g of agarose was dissolved in 30 ml of Tris-acetate-Ethylenediaminetetraacetic acid (TAE) buffer. The mixture was microwaved until coming to a boil. It was cooled down to a point below 60 °C, and 0.6 μ L of Ethidium Bromide was added. The mixture was stirred well until it was evenly mixed and was poured into a gel cast. After the gel cast was filled, the comb was inserted, and it was left at room temperature for 30 min for solidification. Once it was fully solidified, the comb was removed, and the PCR samples from 2.1 were added. The gel electrophoresis was operated for 15 min at 120V [12,13]. The results confirmed that the HRas(G12V) insert was present. The length of the DNA fragment was 610 base pairs.

2.3. LIC vector cloning

The gel containing the DNA was cut with a knife, and the DNA was extracted using the extract kit. The blunt end DNA was created to create a sticky end DNA to insert into the Ligation-Independent Cloning(LIC) vector. The LIC vector was also treated to convert the blunt end to a sticky end vector to complete this action. There were 18, 22 overhand on the vector and DNA. 2 μ L of DNA and 1 μ L of the vector were put together and left for 5 min at 22 °C. 1 μ L of EDTA was added, and the mixture was left for 5 min at 22 °C. After annealing, the vector transformed to *E. coli* DH5 α (Real Biotech Corporation, Taiwan). The *E. coli* DH5 α containing the scFv(Ras) vector was cloned.

2.4. Transformation and recombination of plasmid to *E. coli* competent cell

The Plasmid, LIC vector-scFv(Ras), was transformed and combined to the *E. coli* competent cell BL21(DE)3 (Real Biotech Corporation, Taiwan). First, a mixture was created using 20 μ L of *E. coli* competent cells and 1.4 μ L of the plasmid. The mixture was cooled in a bucket filled with ice for 20 min. Then, a heat shock was applied for 1 min at 42 °C. The mixture was once again stored in a bucket filled with ice for 20 min [14]. Then, 200 μ L of LB (Duchefa Biochemie, Kingdom of the Netherlands) was added. The completed mixture was inserted into shaking incubation at 37 °C for 1 h at 200 rpm. After the incubation, it was centrifuged for 3 min at 3000 rpm. 150 μ L of the supernatant was discarded through pipette use. The remaining mixture was gently tapped to resuspend the cell pellets. Finally, it was plated by streaking, and the labeled plates were stored in an incubator at 37 °C overnight.

2.5. Purification of histidine tagged scFv(Ras)

The histidine-tagged scFv(Ras) was separated using Nickel-nitrilotriacetic acid resin (Cytiva, Belgium) affinity chromatography. The column was 5 column volume(CV) brought into equilibrium by 20 mM of Tris-HCl with a pH of 7.5 and 250 mM of NaCl buffer. The soluble fraction was passed through the column. The column was washed with 5CV buffer, created by adding 20 mM of imidazole (Sigma Aldrich, USA) to a buffer. The first elution was done by using a buffer with 5 ml of 100 mM imidazole. Then, the second elution was done by using a buffer with 5 ml of 400 mM imidazole. Elutions were used as two different histidine-tagged scFv(Ras) samples [15,16].

2.6. SDS-PAGE of purified histidine-tagged scFv(Ras)

6x Sodium Dodecyl Sulfate(SDS) sample buffer was added for WA, 100 mM, and 400 mM samples. The total volume was 48 μ L. Soluble fraction(Sol) and loading through(LT) were diluted by 50%. All samples were boiled before Sodium dodecyl sulfate polyacrylamide gel electrophoresis(SDS-PAGE) for 5 min [17]. 12% SDS-PAGE gel was performed. The gel was stained with Coomassie Brilliant Blue.

2.7. Western blotting

1L of 1x transfer buffer mix was prepared to perform western blotting. The buffer mix had 680 ml of distilled H₂O to 170 ml of 5x transfer buffer and 150 ml of MeOH. The Polyvinylidene Fluoride membrane (Merck Millipore, USA) was placed on methanol for about 1 min for activation. Then, the membrane was connected to the cathode, and the gel was placed on the anode. The gel was transferred at 100V for 60 min. The transferred membranes were placed on methanol and dried to remove residual alcohol. The membrane was incubated in the Tris Buffered Saline with Tween 20 (TBS-T) buffer at 4 °C for the next experiment.

The TBS-T buffer was removed, and the membrane was again incubated in the primary antibody solution, made of 4 µL anti-His6 from mouse IgG1 (Roche, Swiss) in 20 ml 5% milk (Becton Dickinson, USA) for 24 h at 4 °C. The membrane was rinsed with TBS-T three times for 10 min. The membrane was washed with the TBS buffer. Afterward, the membrane was incubated in the secondary antibody solution, Goat anti-Mouse IgG (LI-COR, USA), in 20 ml 5% milk for 30 min at room temperature. The membrane was rinsed three times for 10 min with TBS-T. Finally, the individual bands were analyzed by comparing the original amino acid sequence length and the incubated antibody solution's sequence length to see if they were identical [18–20].

2.8. Protein-protein docking modeling

The online docking server 'ClusPro' was used to conduct the protein-protein docking modeling [21]. The Protein Data Bank format of antibody CPP-scFv(Ras) and antigen HRas(G12V) was used for docking. The software's advanced docking mode allowed masking options for antibodies to maximize accuracy. Aligning with the characteristics of antibodies, the antibody mode supports the modeling process by removing the assumption of symmetry as an antibody-antigen has a flat and asymmetrical surface compared to other complexes such as the enzyme-inhibitors complementary surface [22]. Additionally, by masking the non-complementary determining regions, results improved accuracy [23]. Such options were necessary considering the CPP segment of the antibody can interfere with the antibody-antigen interaction that would produce inaccurate models. The CPP segment is a flexible peptide that conforms to the antibody-antigen interaction rather than interfering with the binding. Therefore, by masking the CPP segment, the model replicates a similar binding function.

2.9. Binding assay

A binding assay was conducted *in vivo* to validate the function of CPP-scFv(Ras) to bind to HRas(G12V) successfully. The sample of CPP-scFv(Ras) was prepared and loaded by gel filtration.

3. Theory/calculation

3.1. Proof of product

The binding assay through the size-exclusion column proved that the CPP-scFv(Ras) antibody was successfully attached to the mutated Human Ras protein, HRas(G12V), in an *in-vitro* environment. The binding assay verified that the engineered CPP-scFv(Ras) could penetrate and target the HRas(G12V) inside the cell. However, to use the CPP tagged scFv(Ras) antibody as cancer therapy, additional assays must be conducted to ensure its function and safety. Two additional assays are suggested here to prove its safety: the cell penetration and Ras signaling assays.

The cell penetration assay can be conducted *in vivo* using a human cell containing mutated Ras, HRas(G12V). By tagging a second fluorescent part onto the CPP-scFv(Ras), the experimenter can screen whether or not the antibody successfully entered the cell membrane.

Once the antibody binds together with HRas(G12V), the experimenter will identify the fluorescent tag tied to the inner cell membrane. If observed, CPP-scFv(Ras)'s ability to bind within the reducing environment is proven.

In addition, the Ras signaling assay can be performed to verify that the antibody restricts cell proliferation. As a control variable, the average cell proliferation rate of the mutated human cell containing HRas(G12V) can be measured and calculated. Then, it can be compared with the average cell proliferation rate of the cell after the antibody is attached. The comparison will successfully determine if the antibody achieved its engineered role of restricting Ras signaling, which should stop the proliferation of the mutated cell.

During the Ras signaling assay, the rate of cell proliferation can also investigate the duration of the CPP-scFv(Ras) antibody's effect on the mutated cell. An antibody is a protein, and therefore, the cell will eject the antibody similar to many other cell parts. If it is ejected, there is a high possibility that the mutated Ras protein will be activated without CPP-scFv(Ras) and restart the uncontrolled proliferation process. The change in the rate of cell proliferation will depend on whether or not the antibody is disposed of before or after a complete cell cycle. If the residing cell dies without proliferating, while if it is disposed of, there is a high chance that cell proliferation will occur at a similar rate to that of the control variable.

The assays will further prove the validity of the hyperstable CPP-scFv(Ras). However, specific data on binding is required to extend the antibody research for clinical use. The CPP-scFv(Ras) antibody mimics the X-ray structure of the 2vh5 antibody model. Therefore, the binding properties are anticipated to be identical to that of the verified 2vh5 model. Comparison of the two antibody-antigen binding models will be an effective method to verify the binding properties of scFv(Ras) on a molecular level. While an experimental approach can complete this process, protein-protein docking modeling can be used to visualize the antibody and antigen interaction.

3.2. Docking process

The monoclonal antibody scFv(Ras) was engineered from the hyperstable scFv(F8) antibody segment. The amino acid sequence of the protein data bank format was received through an external company, while the HRas(G12V) version of the human Ras protein was extracted from a known protein data bank of 2vh5 using AutoDockTools [24]. The CPP masked scFv(Ras) was modeled using antibody mode. The CPP mask allowed minimal interference of the flexible peptide with the actual antibody and antigen binding.

The final results were from 'scFv with CPP (antibody mode, CPP masked)' with the model evaluated with the model in cluster 0 with the lowest energy level of -359.7 and the largest members of 159 out of the 27 possible arrangements created through the 'ClusPro' software. Fig. 1 shows the receptor CPP-scFv(Ras) and ligand HRas(G12V) docking model overlapped with the 2vh5 antibody model.

3.3. Docking analysis

The CPP-scFv(Ras) and HRas(G12V) binding locations are positioned on the conventional antibody binding site, as shown with the similarities with the overlapped 2vh5 model. However, there is a difference in the exact positioning of the antibody and antigen-binding site. The ligand's unparalleled and slightly different posting shows a difference in the binding, calling for further investigation of what caused such differences and how this might affect the antibody's function.

4. Results and discussion

4.1. PCR of HRas and scFv(Ras)

The CPP-scFv(Ras) antibody and HRas(G12V) were amplified using

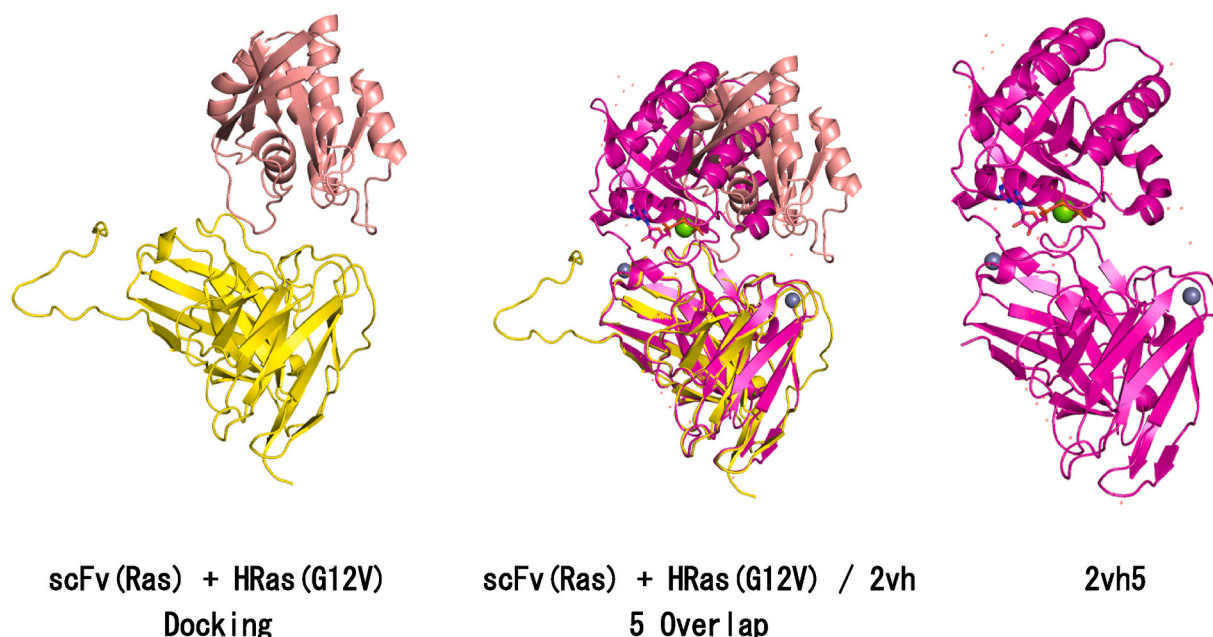


Fig. 1. Protein-Protein Docking between scFv(Ras) + HRas(G12V)/scFv(Ras) + HRas(G12V) + 2vh5 overlap/2vh5 (in order).

PCR and cloned into an expression vector. Fig. 2 shows the electronically obtained amino acid sequence of scFv(Ras) from DH5 α sequencing. The scFv(Ras) sequence was identical to the original scFv(Ras) protein sequence initially used in the experiment. Therefore, the sequence confirms PCR success. Fig. 3 is the western blotting result of HRas (G12V). The black strands on each column are located between 500 bp and 1000 bp. The original size of HRas was 610 bp. As a result, western blotting confirms that the cloned HRas has the same sequence as the initial HRas sequence, which indicates cloning success. Fig. 4 is the Western blot of a CPP-scFv(Ras) colony PCR. The black strands, which indicate protein size, are positioned between 500 bp and 1000 bp. The original sequence of CPP-scFv(Ras) was 805 bp. Thus the Western blot confirms the successful cloning of CPP-scFv(Ras). The experimental confirmation of scFv(Ras), HRas(G12V), and CPP-scFv(Ras) is an important indicator to start experimenting to confirm the function of scFv(Ras) through scFv(Ras) and HRas(G12V) binding.

4.2. Transformation and the recombination of plasmid LIC vector - scFv (Ras) to *E. coli* competent cell BL21(DE)3

The LIC vector, scFv(Ras) was inserted into the *E. coli* BL21(DE)3 by introducing the two parts in a liquid medium. The liquid medium was incubated at an optimal temperature of 37 °C overnight. The *E. coli* containing the CPP-scFv(Ras) was placed on the agar plate through streaking to grow overnight. Several different colonies were created on the petri dish overnight. One colony was scraped and placed in a liquid medium for proliferation in means of future protein extraction. Isolation of a single colony ensured that the *E. coli* expressed a scFv(Ras) antibody with an identical gene sequence. This minimized any inaccuracies in the following experimental steps that may result from differences in the antibody sequence that produces different antibodies once the gene is expressed.

4.3. SDS-PAGE of purified histidine-tagged scFv(Ras)

The proteins were separated based on their molecular weight and confirmed that the CPP tagged scFv(Ras) exists by the SDS-PAGE technique. HRas(G12V) was also included in the SDS-PAGE process to verify experimental success. The protein expression took place in four different conditions. There were two options for temperature and two

options for IPTG concentration (Duchefa Biochemie, Kingdom of the Netherlands) for both HRas(G12V) and CPP-scFv(Ras) test expression. The two provided temperatures were 18 °C and 24 °C. Then, the provided concentrations were 0.1 mM and 1 mM, like shown in Fig. 5.

Both HRas(G12V) and CPP-scFv(Ras) bands were marked clearly in their sizes. In Fig. 5, there were multiple marks between 20 kDa and 25 kDa. In the 18 °C and 0.1 mM conditions, it was most evident that there is a mark at 23.4 kDa. Such results confirmed that the purified HRas(G12V) harvested from *E. coli* BL21(DE)3 was accurate and can be used for later experimental steps which include antibody and antigen-binding experiments.

In Fig. 6, the result strands highlighted in the red box are between 25 kDa and 35 kDa. The initial size of CPP-scFv(Ras) was 28 kDa, which falls in this region of molecular size. Therefore, it was confirmed that there is a protein with a size of 28 kDa, which was the expected size of the CPP-scFv(Ras) antibody. It was proven that there were proteins with sizes of 23.4 kDa and 28 kDa. However, those proteins were not guaranteed to be the wanted proteins, HRas(G12V) and CPP-scFv(Ras). Therefore, further verification was done through Western Blotting (see Figs. 7 and 8.).

4.4. Western blotting

Western blotting was used to once again confirm the identity of the protein, scFv(Ras).

The results show that the desired protein, scFv(Ras) exists, and positive control is detected. The His-6HRas(G12V) was identified to be between and in the lower ranges between 20 kDa and 25Da, which equates to the 23.4 kDa protein size. The black arrow on the right-hand side of each graph indicates the resulting strand. This confirmed the His-6HRas(G12V) obtained through SDS-PAGE. Thus the confirmation that both scFv(Ras) and His-6HRas(G12V) obtained from previous steps were accurate allows progression to check scFv(Ras) function in binding with the HRas(G12V) antigen.

4.5. Size-exclusion column

We had three different trials using the superdex 75 100/300 GL column, using 20 mM Tris-HCl pH 7.0 and 250 mM NaCl as buffers. The first trial or the tube only contained scFv(Ras). It is labeled in blue. The

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Ras-scFv1-T7      CTCACCATCACCATCACCATGAAAACCTGTACTTCCAATCCAATGCAATGACATACACAC
Ras-scFv          -----ATGACATACACAC
                      *****

Ras-scFv1-T7      GGAGAAGATTCCGCCGCCGTGGCACCGGCCCGGAGCCAGGTACAATTACAAGAGTCCG
Ras-scFv          GGAGAAGATTCCGCCGCCGTGGCACCGGCCCGGAGCCAGGTACAATTACAAGAGTCCG
                      *****

Ras-scFv1-T7      GAGGTGACTTAGTTCAGCCGGGTGGTAGCCTTAAACTGAGCTGTGCAGCCTCCGGTTTCA
Ras-scFv          GAGGTGACTTAGTTCAGCCGGGTGGTAGCCTTAAACTGAGCTGTGCAGCCTCCGGTTTCA
                      *****

Ras-scFv1-T7      CGTTCTCTTCATATGGTATGTCATGGGTCCGGCAAACGCCCGACAAGCGTTTGAAGCTGG
Ras-scFv          CGTTCTCTTCATATGGTATGTCATGGGTCCGGCAAACGCCCGACAAGCGTTTGAAGCTGG
                      *****

Ras-scFv1-T7      TGGCTACAATAAATAGAAGCTGGGAAGACTACATACTACCCCGACTCGGTCAAAGGTAGAT
Ras-scFv          TGGCTACAATAAATAGAAGCTGGGAAGACTACATACTACCCCGACTCGGTCAAAGGTAGAT
                      *****

Ras-scFv1-T7      TTAATATCTCAGCGACAATGCAAAAAACACCTTGTATTTGCAGATGTCAAGTTAAAAA
Ras-scFv          TTAATATCTCAGCGACAATGCAAAAAACACCTTGTATTTGCAGATGTCAAGTTAAAAA
                      *****

Ras-scFv1-T7      GTGAAGACACCGCCATGTATTACTGTGCTAGAGGGCGCTTCTTTGACTATTGGGGCCAGG
Ras-scFv          GTGAAGACACCGCCATGTATTACTGTGCTAGAGGGCGCTTCTTTGACTATTGGGGCCAGG
                      *****

Ras-scFv1-T7      GAACTACAGTTACTGTCTCTTCGGGAGGAGGCGGTAGCGGTGGCGGGGTCTGGTGGCG
Ras-scFv          GAACTACAGTTACTGTCTCTTCGGGAGGAGGCGGTAGCGGTGGCGGGGTCTGGTGGCG
                      *****

Ras-scFv1-T7      GAGGCTCGGACATTGAATTAACGCAATCCCCAGCCTCTTTGGCGGTAGTTTAGGACAAA
Ras-scFv          GAGGCTCGGACATTGAATTAACGCAATCCCCAGCCTCTTTGGCGGTAGTTTAGGACAAA
                      *****

Ras-scFv1-T7      GAGCAAACAATCTCATGCCGGGCATCTGAAAGTGTGACAGCTACATGCACTGGTATCAGC
Ras-scFv          GAGCAAACAATCTCATGCCGGGCATCTGAAAGTGTGACAGCTACATGCACTGGTATCAGC
                      *****

Ras-scFv1-T7      AGAAACCGGGGCAGCCGCCGAAATTAAGTATTTACAGAGCGCTTAACCTGGAAGCGGCA
Ras-scFv          AGAAACCGGGGCAGCCGCCGAAATTAAGTATTTACAGAGCGCTTAACCTGGAAGCGGCA
                      *****

Ras-scFv1-T7      TCCTGCGAGATTTTCAGGATCTGGTAGTCGCACTGATTTACATTAACGATCAACCCTG
Ras-scFv          TCCTGCGAGATTTTCAGGATCTGGTAGTCGCACTGATTTACATTAACGATCAACCCTG
                      *****

Ras-scFv1-T7      TCGAAGCTGACGACGTAGCCACCTATTATTGTGACGCAATCCGTCGAGGACCCCTGGACTT
Ras-scFv          TCGAAGCTGACGACGTAGCCACCTATTATTGTGACGCAATCCGTCGAGGACCCCTGGACTT
                      *****

Ras-scFv1-T7      TCGGGGTGGGACGAAGTTAGAAATTAACGGTAATAACATTGGAAGTGGATAACGGATC
Ras-scFv          TCGGGGTGGGACGAAGTTAGAAATTAACGG-----
                      *****
    
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Fig. 2. Amino acid sequence of CPP-scFv(Ras).

second trial or the tube was labeled red and included HRas(G12V) only. Finally, the last trial with scFv(Ras) and HRas(G12V) attached was labeled in black.

From the three trials, the shift of the scFv(Ras) and HRas(G12V) peak, observed as the black line, to a higher molecular size was

confirmed (see Fig. 9). The size of the molecule increased because there was a successful binding between scFv(Ras) and HRas(G12V). Then, comparing the observed value of scFv(Ras) + HRas(G12V) and the original HRas(G12V) graph, the HRas(G12V) graph has slightly shifted to the left. The HRas(G12V) graph acts as a control variable to observe

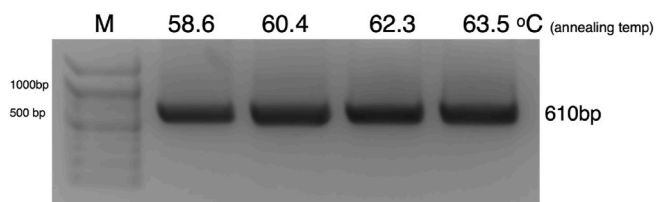


Fig. 3. Western blotting of HRas(G12V).

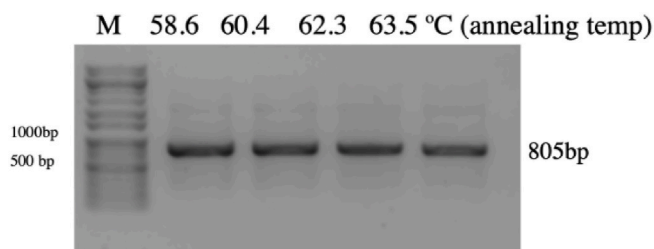


Fig. 4. CPP-scFv(Ras) colony PCR.

the shift of the scFv(Ras) + HRas(G12V) graph. The shift results from the increased molecular size after scFv(Ras) and HRas(G12V) bind together compared to the molecular size of the antigen or antibody alone. Thus it takes longer for the molecule to pass through the column that contains beads which prevent larger molecules to freely pass through. Therefore, we confirmed that the binding between scFv(Ras) and HRas(G12V) was successful, ensuring that our antibody model effectively binds to its

antigen, HRas(G12V).

5. Conclusion

The results of the experiment confirm that the engineered CPP-scFv (Ras) antibody successfully binds to cancerous cells. Such findings can go hand in hand with the results derived from other experiments, including those discussing the basic characteristics of the scFv(F8) antibody. Further studies and approvals must be taken to advance CPP-scFv(Ras) for clinical use. Those considerations include the method of delivery, amount of delivery, duration of therapy, and cost analysis which can become subjects of later study. Furthermore, The target specificity of CPP-scFv(Ras) must be evaluated for its effects on the normal cell population surrounding the cancerous cell as the antibody can limit the propagation of all cell populations in the range of antibody

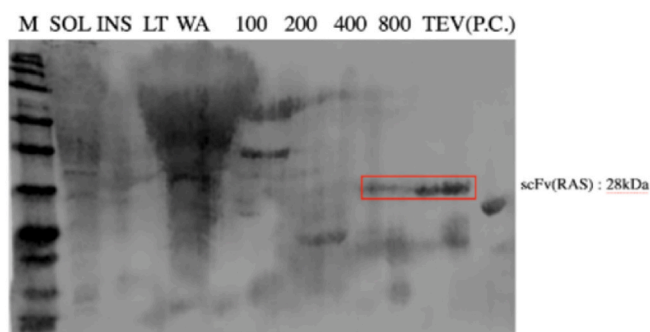
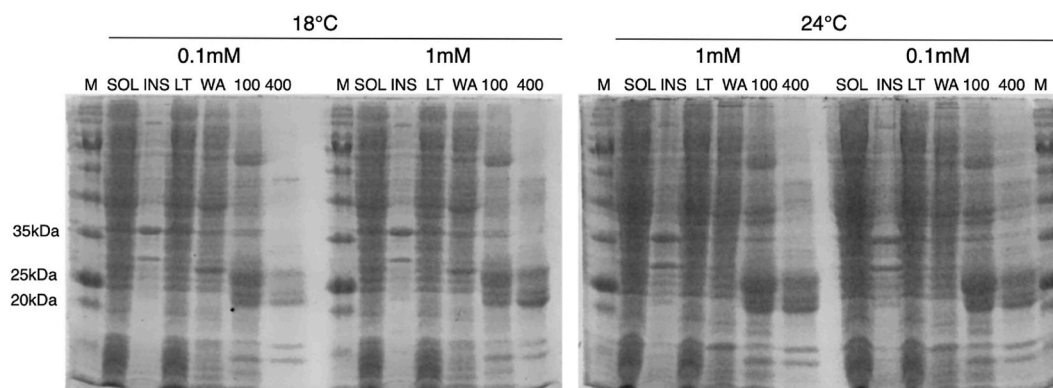
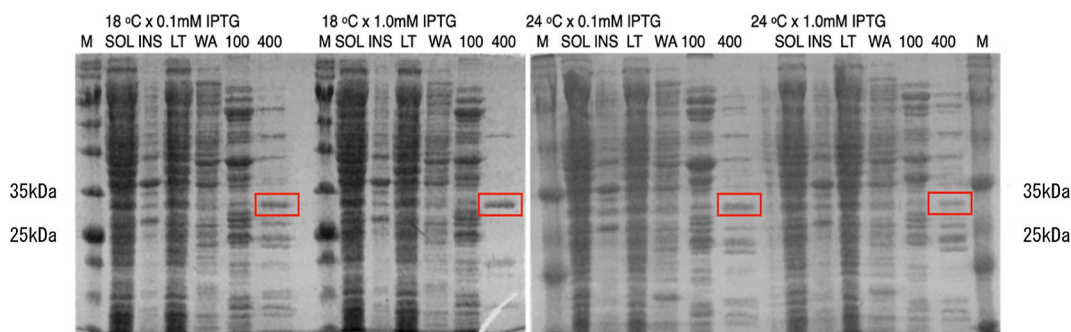


Fig. 7. scFv(Ras) Western Blotting Expression.



His6-HRas(G12V) = 23.4kDa

Fig. 5. HRas(G12V) SDS page expression.



CPP-scFv(Ras) : 28kDa

Fig. 6. CPP-scFv(Ras) SDS page test expression in four conditions.

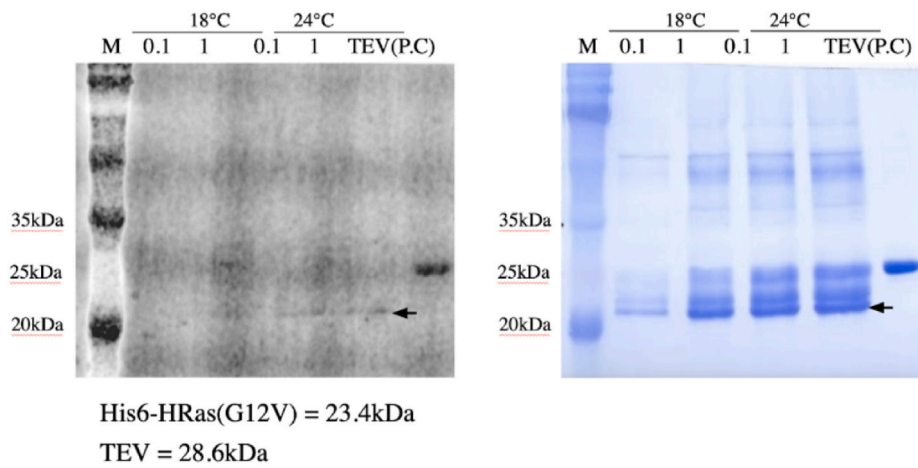


Fig. 8. His-6HRas(G12V) western blotting expression.

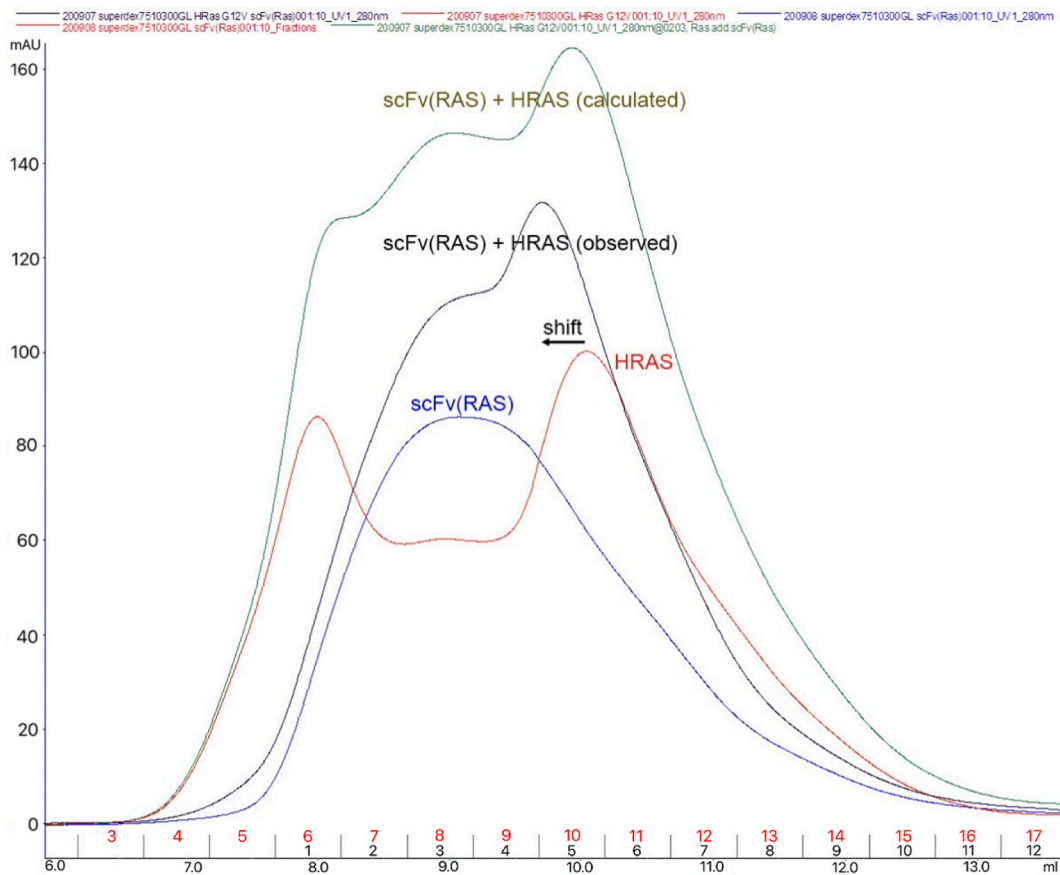


Fig. 9. scFv(Ras) /HRas(G12V)/scFv(Ras) + HRas(G12V) Size Comparison.

application.

CRedit authorship contribution statement

Jina Bae: Writing – original draft, Writing – review & editing, Visualization. **Yoonyee Song:** Writing – original draft, Writing – review & editing, Visualization.

Declaration of competing interest

We declare we have no conflict of interest.

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