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Binding specificity of HuScFv to cholangiocarcinoma cells; New avenues for diagnosis and treatment

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Abstract

Background: Cholangiocarcinoma (CCA) is a very aggressive cancer of the bile ducts. Recent advances in immunotherapy, particularly with human single-chain variable fragments (HuScFv), have shown promise in the treatment of solid tumors by targeting cancer cells or improving the immune response.

Objective: This study aimed to select and produce human single-chain antibody fragments (HuScFv) specific to CCA cells (HubCCA1, RMCCA) from phage display HuScFv libraries with minimum or no binding to cholangiocytes (MMNK1).

Methods: Phages that displayed HuScFv to CCA cells were selected by bio-panning and each phagemid was transduced to HB2151 *E. coli*. The presence of *huscfv* was determined by direct colony PCR. HuScFv was produced in the *E. coli* and detected by Western blot analysis and confirmed their specificity and binding capacity to CCA by flow cytometry.

Results: From biopanning, 196 of 350 colonies (56%) of HB2151 *E. coli* harboring the *huscfv* gene, and 106 of these (30%) produced the protein. Flow cytometry testing with 14 clones confirmed the presence of the HuScFv protein. The result showed that five HuScFv clones (25, 33, 61, 68, and 80) exhibited stronger binding to CCA cell lines (HubCCA1, RMCCA) compared to cholangiocytes. Furthermore, each clone possessed a distinct amino acid sequence, suggesting unique binding specificities.

Conclusion: HuScFv specific to CCA cells were successfully selected from phage display HuScFv libraries which offer new revenues to develop a pan-CCA immunotherapy and diagnosis of CCA

Key words: Single-chain variable fragment, flow cytometry, CCA, immunotherapy, targeted cancer therapy

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Abbreviations:

HuScFv	Human single-chain antibody variable fragments
CCA	Cholangiocarcinoma
Fab	Fragment antigen-binding
VH	Variable heavy chain
VL	Variable light chain
LB	Lysogeny broth

Introduction

Cholangiocarcinoma (CCA) is a cancer of the epithelial lining of the intrahepatic or extrahepatic biliary tract.[1] It has a high mortality rate associated with its rapid spread and diagnostic difficulties. CCA often has no symptoms in the early stages or presents with nonspecific symptoms and is usually diagnosed at a stage when the cancer has already spread. Chemotherapy has been a conventional treatment for unresectable CCA.[2] The chemotherapy drugs used most frequently for CCA were gemcitabine, cisplatin, 5-fluorouracil,

capecitabine, and oxaliplatin.[3] The challenge in chemotherapy was drug resistance to gemcitabine and cisplatin, the widely used chemotherapeutic drugs for metastatic CCA. Some patients developed resistance to these drugs due to mutations in cancer cells.[4] Mucin-type and mixed-type CCA responded differently to chemotherapy and molecular-targeted agents. This finding highlights the importance of tailoring treatment strategies based on the specific subtype of CCA to improve patient outcomes.[5] Immunotherapy has emerged as a treatment modality in cancer therapy, including CCA.[6] Antibody and antibody fragments, e.g., fragment antigen-binding (Fab), single-chain variable fragment (scFv), and variable heavy chain (VH), play crucial roles in cancer immunotherapy. These molecules directly target and destroy cancer cells by either recruiting immunological components or linking to cytotoxic drugs.[7] Some antibodies, such as ipilimumab, recover exhausted cells that express immune checkpoint inhibitors, specifically T-lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1), enhance the immune response against cancer.[8] Antibody fragments also serve as components of the chimeric antigen receptor in the T cells or NK cells.[9,10] A key challenge in developing effective immunotherapy is specificity, because tumor antigens usually originate from self-antigens that have developed abnormally or undergone mutation.[11,12] The discovery of antibodies specific to cancer cells, without binding or minimally binding to normal cells, would minimize the side effects of normal cells with a high capacity for development into an effective therapeutic tool.

A phage display antibody library is a useful tool for finding efficient antibodies against specific targets, including viruses, bacteria, toxins, cancer, and self-proteins.[13-15] In this study, a phage display HuScFv library was used to select antibodies specific for proteins on the surface of CCA by biopanning with a CCA cell line. The binding of HuScFv across different CCA cell lines was determined using flow cytometry. An HuScFv capable of recognizing multiple CCA cell types could enable swift and precise diagnosis and treatment.

Materials and methods

Cells and culture media

Intrahepatic CCA cell lines, HubCCA1 (CL-6) and RMCCA, were cultured and maintained in DMEM High Glucose (Gibco, Paisley, Scotland, UK) supplemented with 10% FBS, 50 units/mL of penicillin and 50 µg/mL of streptomycin. Cholangiocytes, MMNK1, were cultured and maintained in RPMI with 10% FBS, 50 units/mL of penicillin, and 50 µg/mL of streptomycin. Cultured cells were collected using Accutase (Merck KGaA, Darmstadt, Germany) after reaching 70-80% confluence and used for biopanning.

Selection of CCA-bound HuScFv using phage biopanning

The naïve human HuScFv phage display, constructed from 60 Thai blood donors by Kulkeaw et al[13] at ~10^{11} cfu was used for phage bio-panning. The library was blocked with 800 µL 2% BSA in PBS at 4°C for 15 min. Phage clones bound to cholangiocytes were subtracted after mixing the library with MMNK1 cells at 4°C for 1 h, twice. The unbound phages were collected by centrifugation at 100×g for 15 min at 4°C and mixed with HubCCA1 that had been blocked for nonspecific binding by preincubation with 1% BSA in PBS at 4°C for 1 h. HubCCA1 bound to phages were collected by centrifugation, washed 10 times with PBS (pH 7.4), and mixed with log-phase HB2151 *E. coli* at room temperature for 15 min to allow infection of CCA-bound phages. The mixture was diluted and spread on selective Lennox agar plates containing 100 µg/mL ampicillin.

Screening for huscfv-pCANTAB5e harboring HB2151 E. coli by PCR

The *E. coli* colonies on LB agar plates obtained from phage biopanning were screened for the presence of the *huscfv* gene by direct colony PCR using *Taq* polymerase (ThermoFisher Scientific, MA) at 55°C for annealing. The pCANTAB5e phagemid-sense primer (pCANTAB5-R1) was 5′-CCATGATTACGCCAAGCTTTGGAGCC-3′; the antisense primer (pCANTAB5-R2) was 5′-GCTAGATTTCAAAACA GCAGAAAGG-3′.[16] PCR products were analyzed by agarose gel electrophoresis. Positive clones that produced the product size of approximately 1000 bp were selected for the expression of HuScFv.

Production of HuScFv to CCA cell lines

The *E. coli* clones of strain HB2151 that harbor *huscfv gene* were cultured in 1 mL of 2×YT supplemented with 100 µg/mL ampicillin, and 2% glucose (2×YT-AG) overnight. The overnight culture was inoculated at 1% in 10 ml of 2×YT-AG until the optical density (OD) at 600 nm reached 0.3-0.5. The medium was then changed to 10 ml of 2×YT with ampicillin (2×YT-A). The HuScFv expression was induced by adding 0.1-0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown at 37°C for 3-5 h. The bacteria were harvested by centrifugation at 4,500×g, 4°C for 15-30 min, resuspended in 1 ml of PBS and homogenized using an ultrasonic processor (VCX130, Sonics & Materials Inc., Newtown, CT) with a 6-mm diameter tip at 30% amplitude for 2 min (2 sec on/2 sec off cycle) on ice. *E. coli* cell homogenates were centrifuged at 12,000×g, 4°C for 15-30 min. The supernatants were collected and subjected to SDS-PAGE and Western blot analysis to detect the HuScFv protein. The HuScFv was probed with rabbit anti-E-tag (Abcam, Cambridge, UK) and goat anti-rabbit conjugated alkaline phosphatase (Abcam, Cambridge, UK). The HuScFv bands were revealed by adding BCIP/NBT substrate (SeraCare, Milford, CT). The *E. coli* cell lysates that had soluble HuScFv can be stored at 4°C for up to 3 weeks or -20°C for long-term storage.

Measuring HuScFv concentration using image analysis

The intensity of the protein band from Western analysis was analyzed using AlphaEaseFC (Genetic Technologies, Miami, FL). The HuScFv band intensity was normalized by the intensity of the protein marker. The amount of HuScFv in each well (10 µL loading volume) was calculated by multiplying the normalized intensity by the concentration of marker protein (0.2 µg). The amount of HuScFv amount in each sample was adjusted to be equal using PBS, pH 7.4, for a binding experiment.

Selection of HuScFv specific to CCA cell lines by flow cytometry

HubCCA1, RMCCA, and MMNK1 cells were seeded onto 10-cm dishes until sub-confluent. Cells were harvested, washed with FACS buffer (2.5% FBS in PBS, pH 7.4), and seeded in a 96-well plate at a density of 2×10^5 cells/well. Cells were blocked with 10% human AB serum in FACS buffer at 4°C, 400 rpm for 1 h, washed twice with FACS buffer, mixed with 3 µg of HuScFv in 200 µL FACS buffer, centrifuged at 400 rpm for 1.5 h at room temperature, washed twice with FACS buffer. HuScFv was probed with 0.2 µg of anti-E-tag primary antibody and 0.3 µg of goat anti-rabbit-Phycoerythrin (GARPE) secondary antibody. The studied samples included: 1) CCA cells treated with HuScFv probed with GARPE alone; 2) CCA cells treated with HuScFv probed with anti-E-Tag and GARPE; 3) HB2151 *E. coli* lysate (without HuScFv) treated CCA cells probed with anti-E-Tag and GARPE. These samples consisted of fluorescence minus one (FMO) and negative binding controls. The samples were analyzed by flow cytometry (CytoFlex A001-1-1102, Beckman Coulter, Inc., USA).

Data Acquisition and Analysis

Flow cytometry data was analyzed using the FlowJo software version 10.7.1. The singlets were gated using an FSC height - FSC area dot plot. The FSC-SSC dot plot was gated to exclude debris. The percentage of cells positively bound to cholangiocyte or CCA cells was gated on histograms based on unstained CCA cells. Data from the FlowJo software were overlaid with the background of CCA cells exposed to HB2151 lysate. The bar graphs were constructed using GraphPad Prism version 8.3.0 (GraphPad Software, Boston, USA). The numerical data was analyzed with multiple *t*-tests to compare population means, with adjustments for multiple comparisons using the Holm-Sidak method to control the overall error rate. Results with *p*-values less than 0.01 and 0.05 were considered statistically significant.

Results

Phage bio-panning and selection of HB2151 E. coli that harboring huscfv gene

Each HB2151 *E. coli* was assigned a unique number during the selection of colonies on the agar plate. *E. coli* colonies were screened for the presence of the *huscfv* gene by direct colony PCR. Of 350 colonies that were tested for the presence of the *huscfv* gene, 196 clones (56%) were positive (**Figure 1**). Then all 196 clones were tested for HuScFv protein expression. Approximately 30% (106 clones) produced the protein.

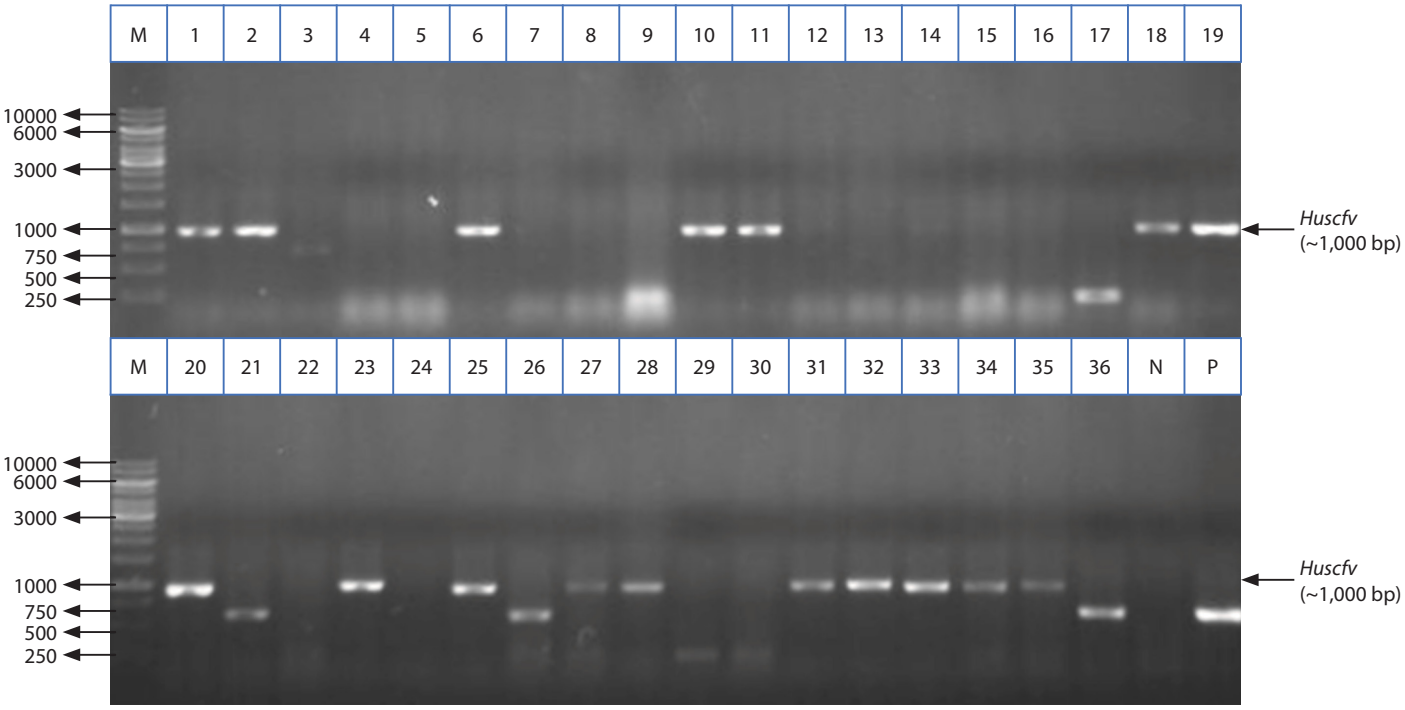

Figure 1. PCR amplicons of representative HB2151 *E. coli* clones were analyzed for the presence of *huscfv* gene by agarose gel electrophoresis. Lane M was a 1 kb DNA ladder. The positive clones were those yielded the product size of 1000 bp (arrow head).

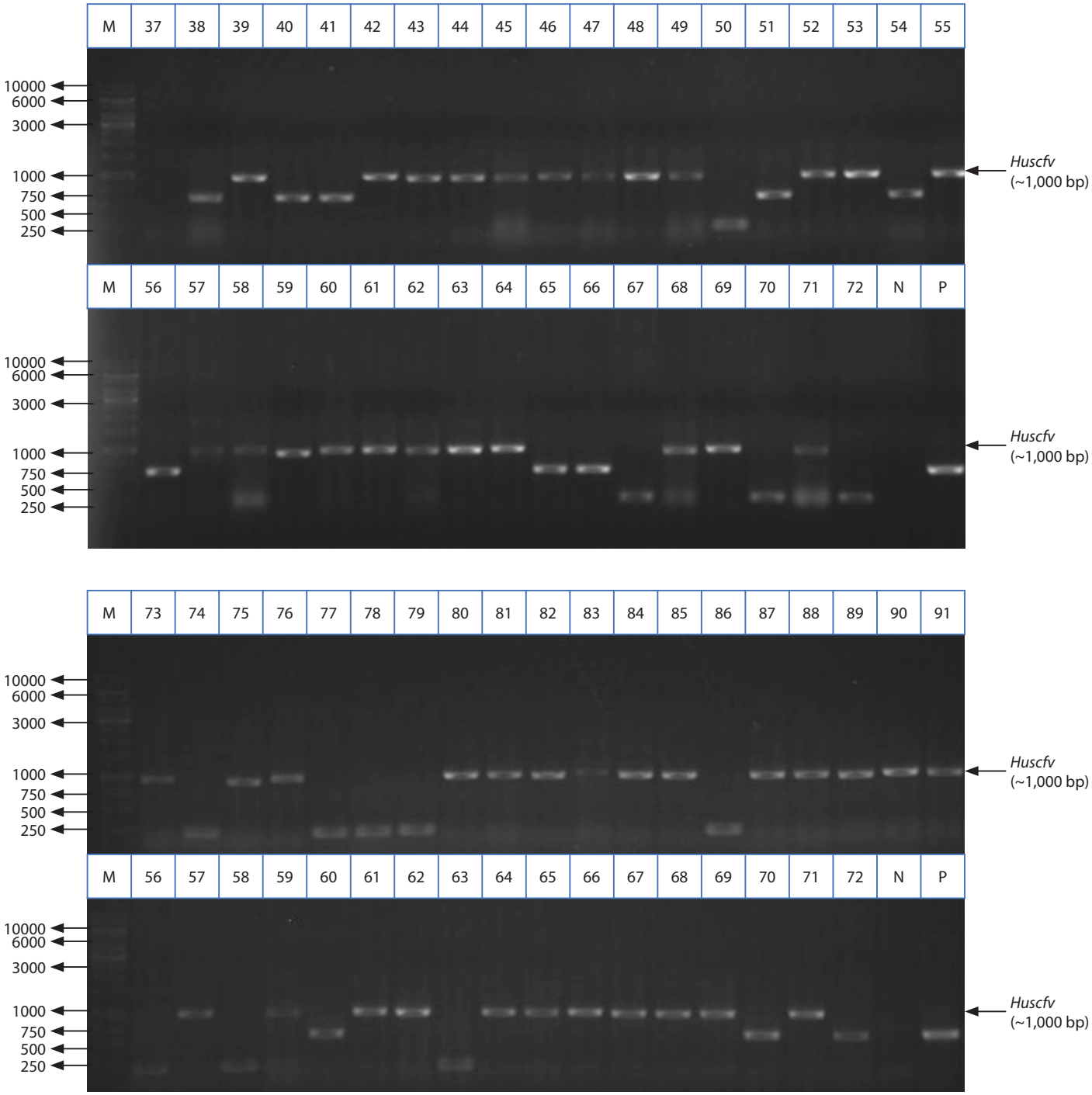

Figure 1. (Continued)

The presence of HuScFv and its level in HB2151 E. coli lysate

Western blot analysis showed that 14 representative *E. coli* clones (1, 2, 25, 27, 31, 32, 33, 47, 55, 60, 61, 68, 73, and 80) contained the predicted 26 kDa band of HuScFv (**Figure 2A**). The intensity of the HuScFv band of each clone was shown (**Figure 2B**). The normalized band intensity was used to adjust the amount of each HuScFv clone to achieve an equivalent amount (**Table 1**).

Binding specificity of HuScFv to CCA cell lines

The adjusted amount of HuScFv in the *E. coli* lysate was tested for its ability to bind CCA cell lines using flow cytometry. Unstained samples and fluorescence minus one (FMO) controls were used to exclude autofluorescence and nonspecific antibody binding. The results were demonstrated as histograms of flow cytometry data (**Figure 3**).

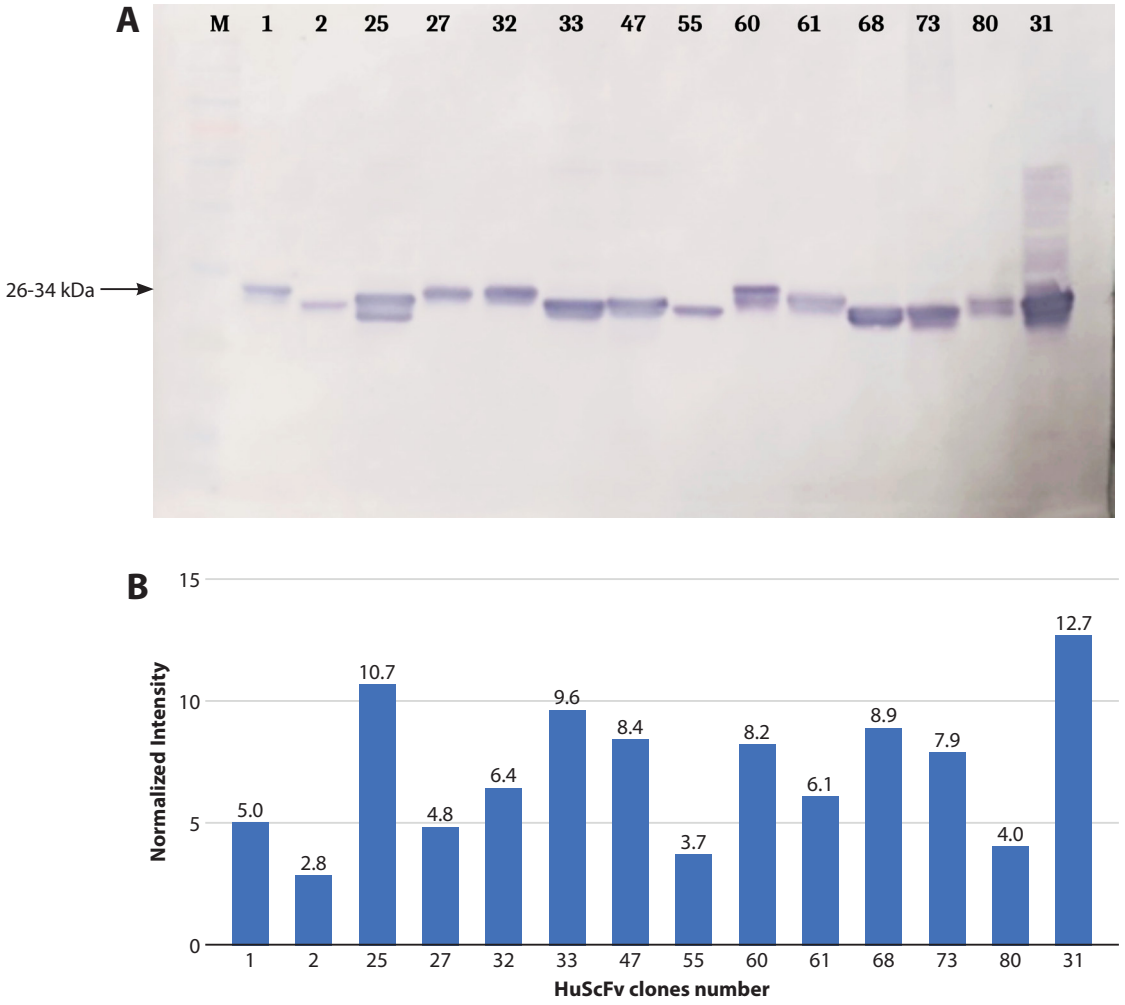

Figure 2. HuScFv protein in HB2151 *E. coli* and its intensity. (A) Western blot analysis of *E. coli* cell lysates. HuScFv was detected by rabbit anti-E-tag, goat anti-rabbit-conjugated-alkaline phosphatase, and BCIP/NBT substrate. The HuScFv-positive clones show a protein band of approximately 26-34 kDa. (B) The intensity of HuScFv bands was analyzed with AlphaEaseFC.

Table 1. Normalized intensity of HuScFv bands and concentration.

Samples	Band Intensity	Normalized intensity	(µg/10 µL)
markers	0.9	1.000	0.200
1	5.0	5.556	1.111
2	2.8	3.111	0.622
25	10.7	11.889	2.378
27	4.8	5.333	1.067
31	12.7	14.111	2.822
32	6.4	7.111	1.422
33	9.6	10.667	2.133
47	8.4	9.333	1.867
55	3.7	4.111	0.822
60	8.2	9.111	1.822
61	6.1	6.778	1.356
68	8.9	9.889	1.978
73	7.9	8.778	1.756
80	4.0	4.444	0.889

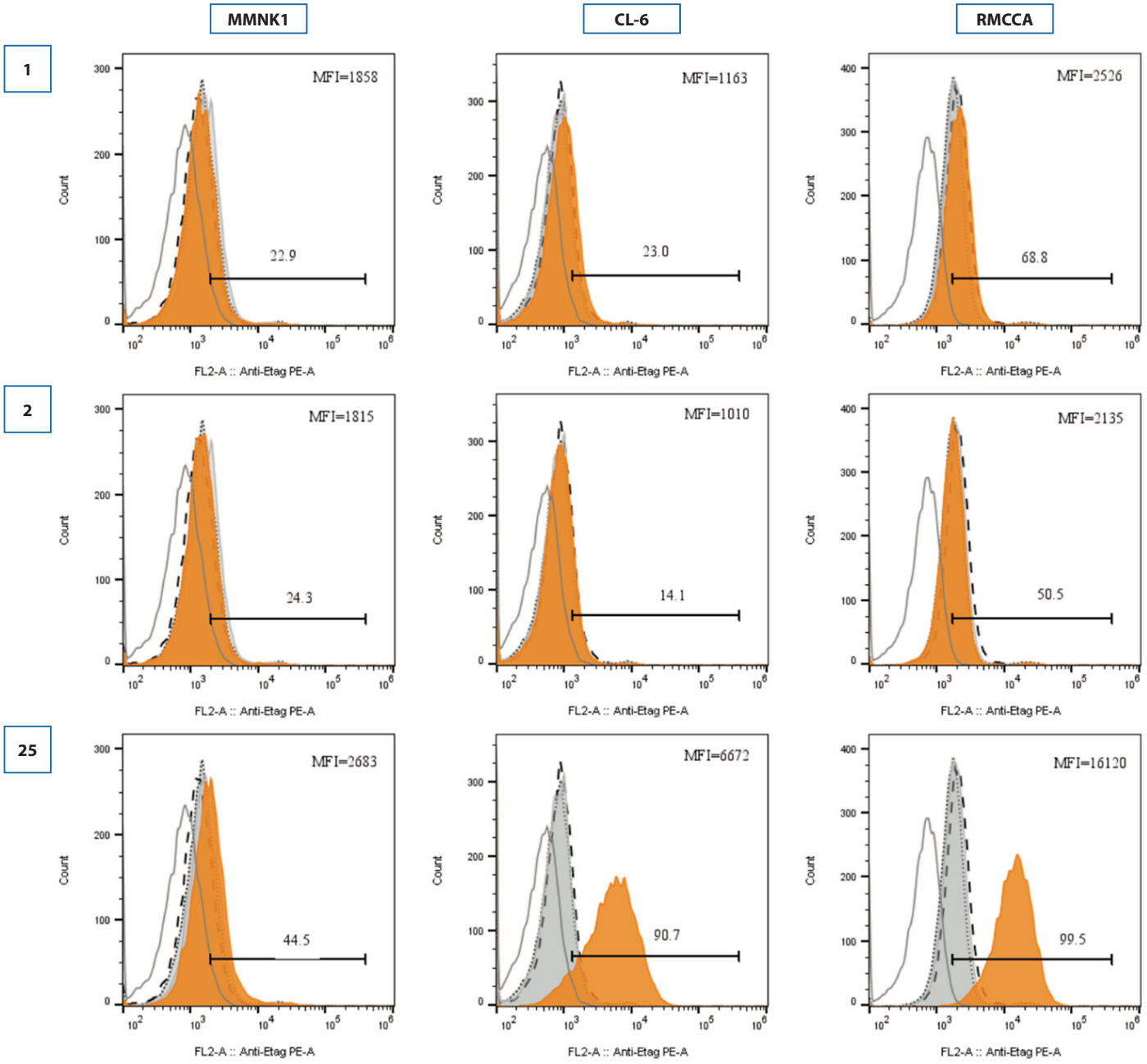

Figure 3. Clones of HuScFv with comparable binding to cholangiocarcinoma cells (HubCCA1 and RMCCA) and cholangiocytes (MMNK1). Control groups are shown in histograms with a gray border indicating unstained CCA/cholangiocytes, the positive binding population was gated based on this histogram. The histogram with a dashed line indicates cells incubated with GARPE, while the histogram with a dotted line shows cells incubated with anti-E tag and GARPE without HuScFv. The histogram with gray shading represents cells incubated with HB2151 cell lysate instead of HuScFv. The sample group, shown in an orange-shaded histogram, represents samples incubated with HuScFv, anti-E tag, and GARPE. The number above the gate represents the percentage of positive binding cells.

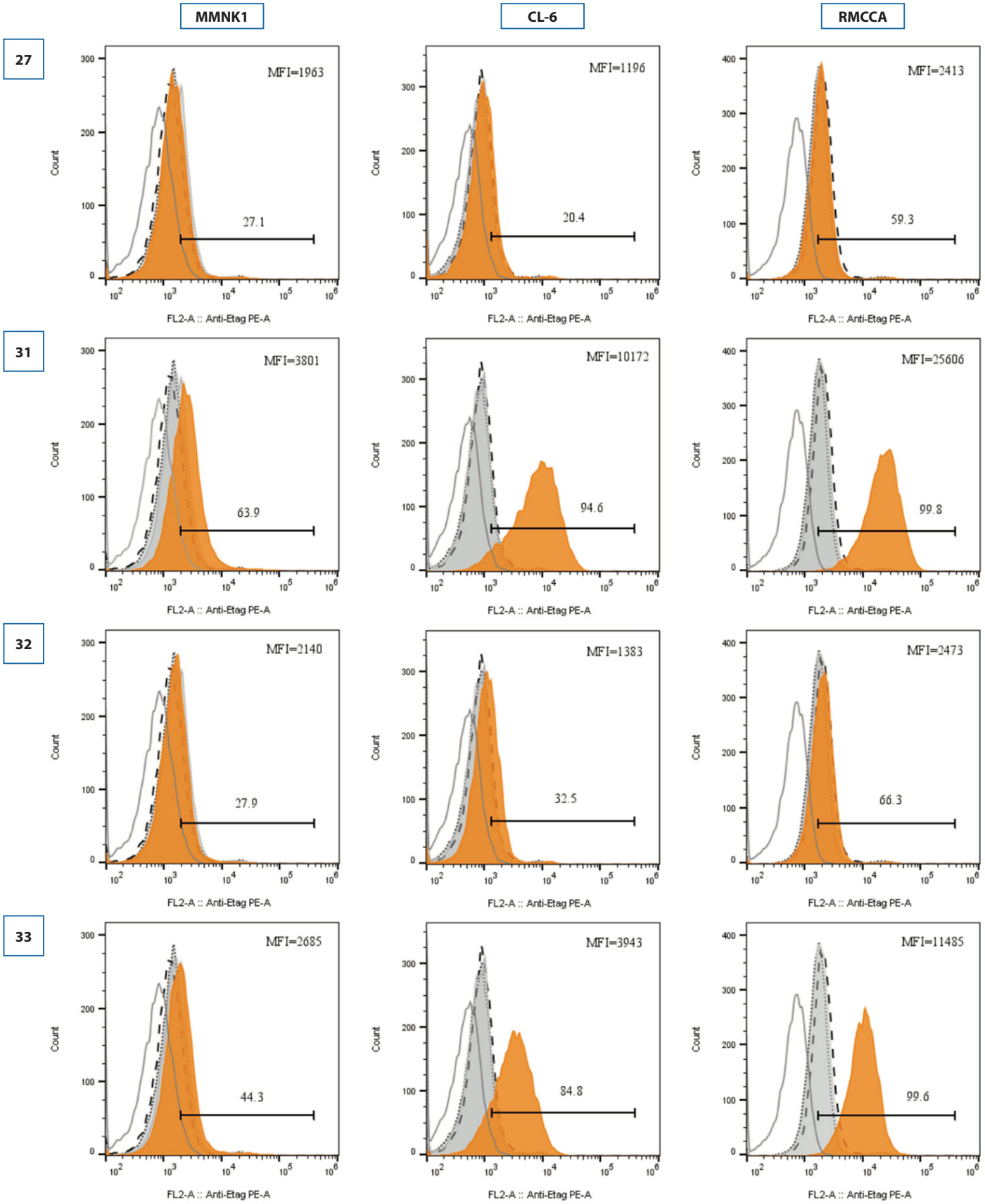

Figure 3. (Continued)

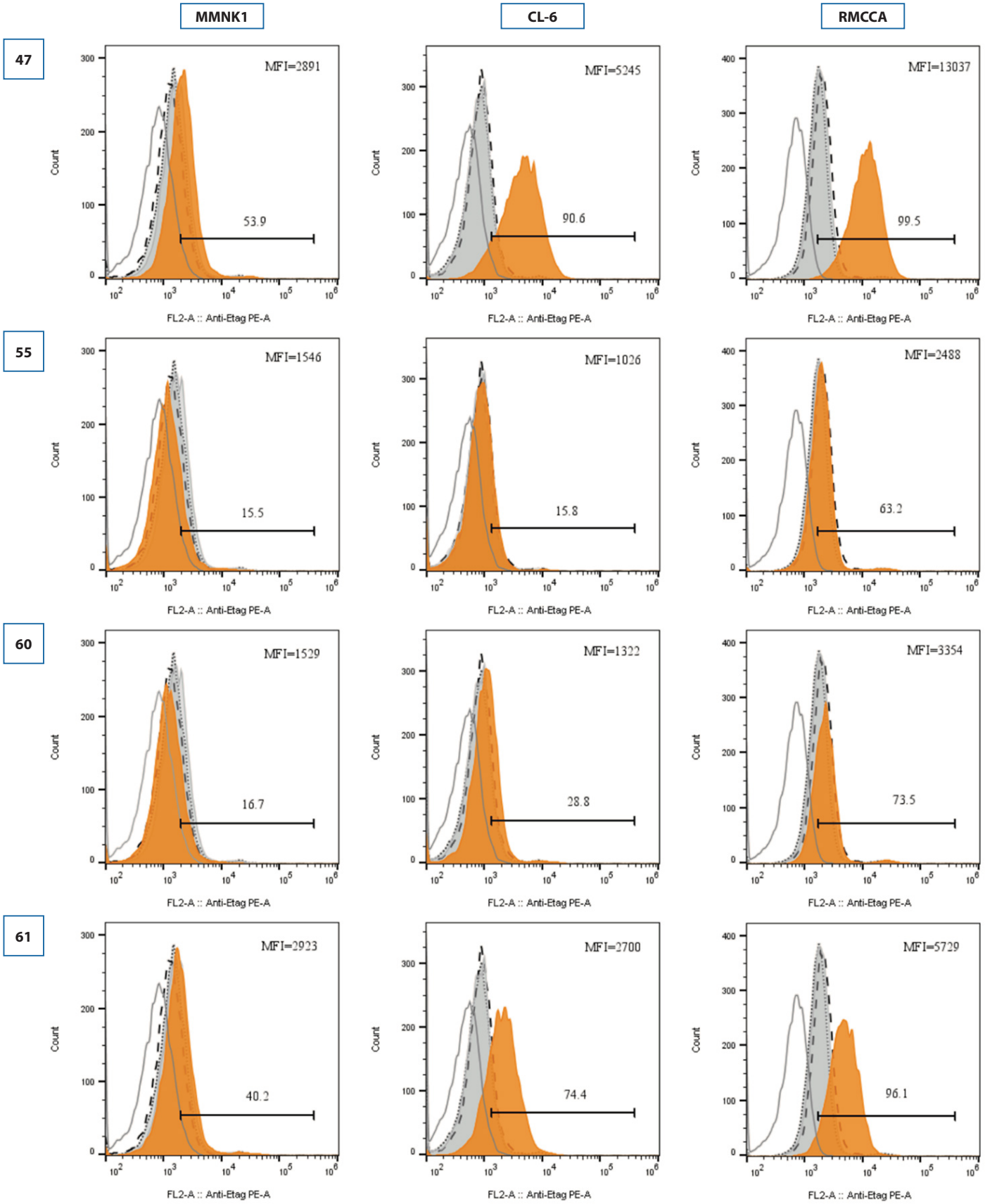

Figure 3. (Continued)

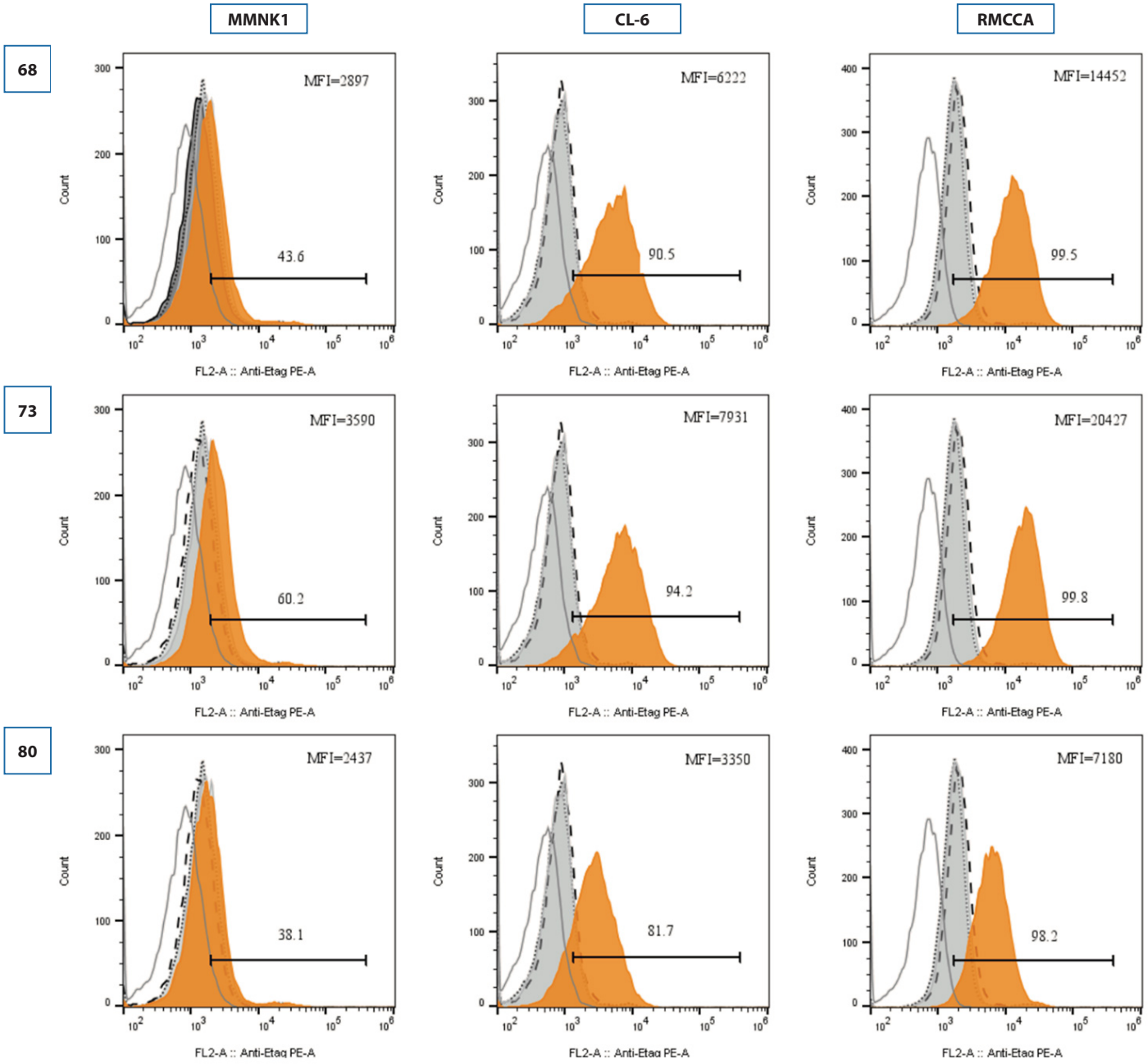

Figure 3. (Continued)

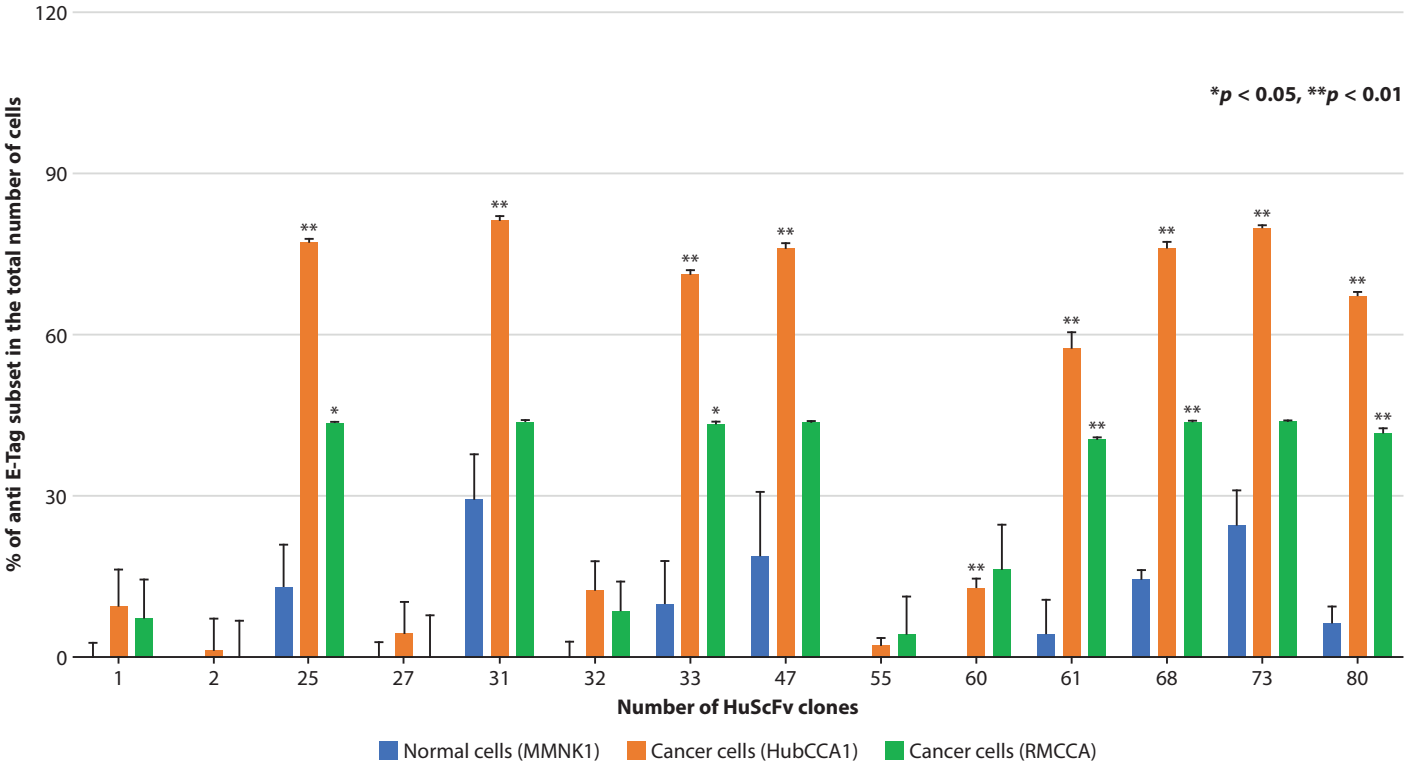

Figure 4. Percentage of positive binding to each CCA or cholangiocyte cell line. Data from each sample was subtracted with that of HB2151 cell lysate and analyzed by GraphPad version 8.3.0. The blue bar represents the positive binding to MMNK1 cells. The orange bar and the green bar represent the positive binding to HubCCA1 and RMCCA, respectively. HuScFv clones #25, 33, 61, 68, and 80 bound to both CCA cells significantly higher than cholangiocytes. HuScFv clones #31, 47, 60, and 73 bound significantly to HubCCA1 but not RMCCA. Statistical analysis was performed with a multiple *t*-test and statistical significance was determined using the Holm-Sidak method.

The percentages of HuScFv binding to the MMNK, HubCCA1, and RMCCA cell lines were presented with background subtraction (**Figure 4**). Among the 14 clones tested, 9 HuScFv clones (25, 31, 33, 47, 60, 61, 68, 73, and 80) showed stronger binding to HubCCA1 cells compared to cholangiocytes. Furthermore, HuscFv clones 25, 33, 61, 68, and 80 exhibited stronger binding to RMCCA cells than to cholangiocytes. Clones bound to both HubCCA1 and RMCCA were selected for further testing. All five of these clones contained different amino acid sequences (**Supplement figure 1**). Clones that did not show a difference in binding between CCA cells and cholangiocytes were not further tested.

Discussion

This study aimed to select specific to various CCA cell lines from the phage display human scFv library. After the bio-panning and screening for the *huscfv*-positive HB2151 *E. coli*, Western blot analysis was performed to detect HuScFv expression. The result showed the HuScFv presented as a doublet band approximately 26-34 kDa in size which suggests the presence of two forms of the HuScFv: the upper band represents immature HuScFv with a signal peptide, while the lower band represents mature HuScFv without a signal peptide.[17] In the binding assay, the amounts of HuScFv were adjusted equally by band intensity analysis. Flow cytometry was used to evaluate the specificity of HuScFv binding to CCA cells compared to cholangiocytes. The results demonstrated that four HuScFv clones (31, 47, 60, and 73) bound significantly to HubCCA1, the bio-panning target, but not RMCCA. Furthermore, five HuScFv clones (25, 33, 61, 68, and 80) bound to both HubCCA1 and RMCCA, with greater binding than to normal cholangiocytes (MMNK1). The amino acid sequence of five HuScFv clones were analyzed to predict complementarity-determining regions (CDRs) using TMGT/V-QUEST.[18] The variable chains, both heavy and light, exhibited distinct CDRs in each clone, reflecting differences in amino acid sequences. This diversity was crucial for antigen specificity, as these regions are responsible for the binding to specific antigens.

The advantage of flow cytometry was its ability to precisely determine cell counts and analyze the fluorescence expression level on an individual cell. The fluorescence expression level was directly associated with the protein expression level, which offers a deeper dimension of the analysis compared to the screening with ELISA. Normally, when flow cytometry is performed to analyze intracellular or extracellular targets, cells could be fixed before or after staining. Fixation sometimes affects surface staining due to loss or masking of epitopes, and crosslinking.[19,20]

To avoid the effects of fixative on the CCA-specific epitope, cells were stained and analyzed by flow cytometry immediately without fixation. In our experience, the use of ELISA to screen live CCA cells without fixation had faced the problem of unequal cell numbers due to irregular detachment.

CCA cell lines were heterogeneous[21,22] with unique characteristics[23] including significant genetic diversity due to mutations in genes such as KRAS, TP53, and FGFR2.[21] This could lead to antigenic variation among CCA cell lines and pose a challenge in immunotherapy development.[5] Another concern in developing cancer immunotherapy has been specificity. On-target, off-tumor toxicity has been reported as prominent toxicity generated by CAR-T cell therapy.[24] The successful selection of HuScFv which are specifically bound to HubCCA1 and RMCA with low binding to cholangiocytes indicates their potential to identify unique targets on the cancer cell surface. These HuScFv should be tested for their binding potency to other CCA cell lines to see the potential to develop further as a "pan"-CCA targeted therapy and drug delivery to specifically attack cancer cells and reduce side effects.

Acknowledgments

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Author contributions

- Nathawadee Sawatpiboon: Investigation (lead); methodology (equal); formal analysis (lead); visualization (lead); writing – original draft preparation (equal)
- Monrat Chulanetra: Methodology (equal); validation (supporting); supervision (supporting); original draft preparation (equal); writing – review & editing (lead)
- Wanpen Chaicumpa: Funding acquisition (equal); supervision (supporting)
- Sunisa Duangsa-ard: investigation (supporting)
- Kanda Kasetsinsombat: Resources (lead)
- Adisak Wongkajornsilp: Conceptualization; validation (lead); funding acquisition (equal); supervision (lead)

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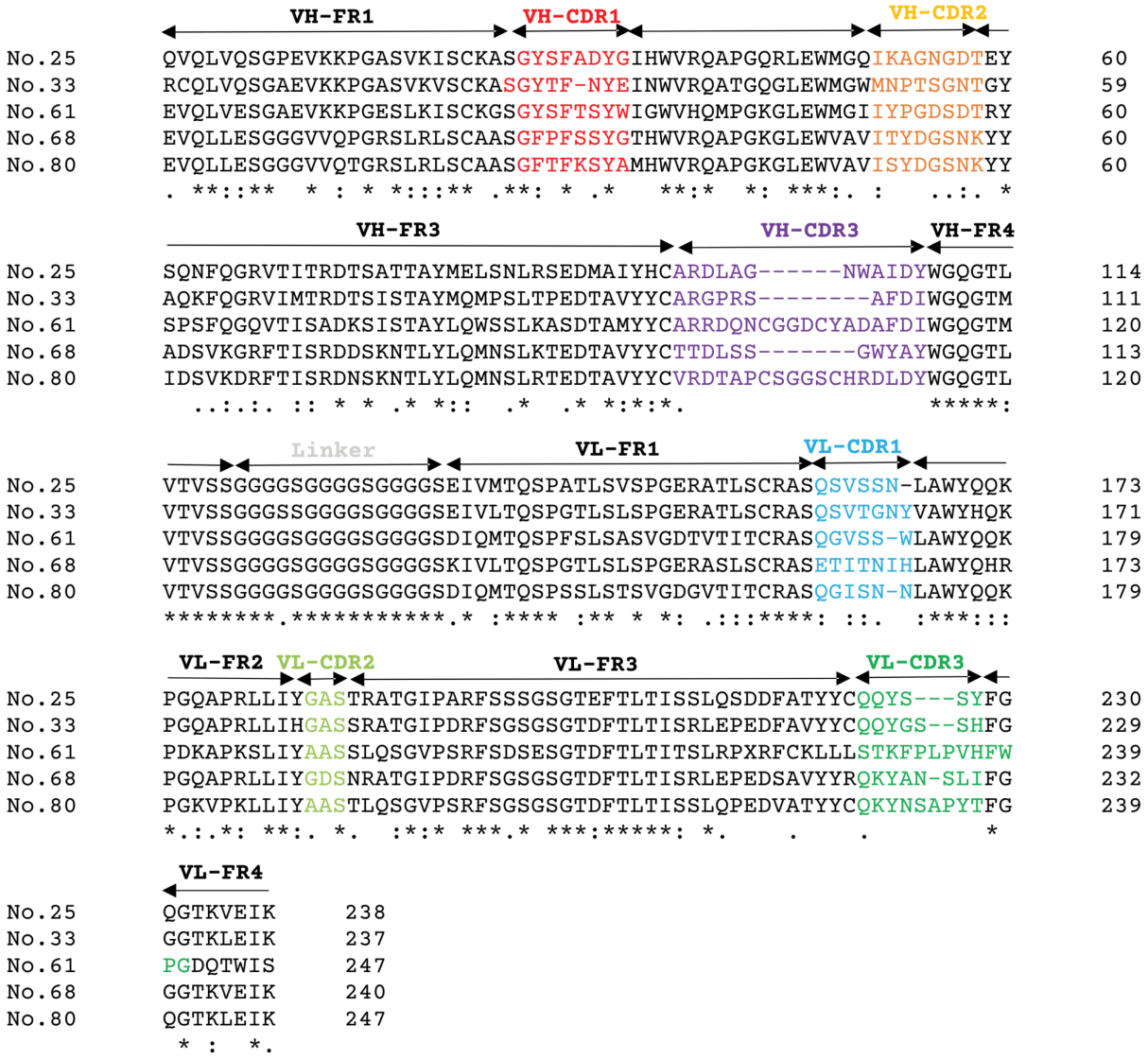
Supplementary Materials



Supplement figure 1. Multiple alignment of amino acid sequences for determining immunoglobulin frameworks (FRs) and complementarity determining regions (CDRs) of the 5 clones (25, 33, 61, 68 and 80) were verified by using CLUSTAL O (1.2.4) Region of CDRs and FRs of the HuScFv were identified by using The International ImMunoGeneTics Information System. (*) Asterisk indicates identical amino acids, (:) colon indicates conserved amino acid substitution, (.) and dot indicates a semiconserved amino acid substitution.