Screening of Single Chain Variable Fragment (ScFv) Specific to Expressed PD-L1 by Cholangiocarcinoma KKU-213 cell line for Cancer Immunotherapy

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Abstract

Cancer immunotherapy to harness the patient own immune system fighting against cancer is a breakthrough of cancer treatment. T-cell therapy, which has been successful for treatment of leukemia and melanoma, driving immunotherapy to possibly be front line of cancer treatment. However, the rate of its successfulness *in vivo* has been reported to be dramatically affected by immune escape mechanism, a major of which is through the interaction between PD-1 and PD-L1 that results in induction of T cell exhaustion and promotion of cancer evasion from the body immune surveillance. Interruption of PD-1/PD-L1 interaction is a crucial approach to enhance T-cell immunotherapy *in vivo*. This study, therefore, aimed to identify single chain variable fragment (ScFv) specific to PD-L1 for inhibition of PD-1/PD-L1 interaction. Initially, anti-PD-L1 ScFvs were screened from human ScFv-phage display 'YAMO' library. After testing protein expression, 11 unique ScFv clones were identified by DNA fingerprint using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Binding specificity of ScFv proteins to PD-L1 on KKU-213 cholangiocarcinoma cells was examined by flow cytometry. Top 5 ScFv clones were selected to analyze by DNA sequencing. The deduced amino acid sequences were obtained and predicted by using IMGT/V-QUEST software. The deduced amino acid sequences were aligned and predicted for putative ScFv structures by using Clustal Omega software and SWISS-MODEL webserver, respectively. The amino acid sequence alignment showed that all 5 clones had critical differences in CDR2-VH and CDR3-VH domains, which might be a key factor affecting their binding capacities to PD-L1. The results in this study are beneficial for further development of immunotherapy to disrupt PD-1/PD-L1 interaction.

Keywords: *Immunotherapy, T-cell therapy, PD-1/PD-L1 interaction, Human ScFv-phage display library, Bio-panning.*

1. Introduction

Cancer is a group of diseases involving abnormal cell growth that can invade and spread in the body. Cancer is currently the second leading cause of death globally that leads many researchers to find curative treatment in the patients. Conventional treatments for cancers, including surgery, chemotherapy, and radiation, are facing with obstruction attributable to that some cancers do not respond to these treatments. Recently, cancer immunotherapy has been developed and becomes a promising approach to use the patient own immune system to fight against cancer. One immunotherapy approach is an improving of T-cell function to enhance killing ability to cancer. However, the efficiency of killing by T-cell is limited according to the presence of immune checkpoint proteins that cancer cells use to inhibit T-cell function. The interaction between programmed death-1 (PD-1) on T-cells and programmed death-ligand 1 (PD-L1) on cancer cell is a mechanism that has been reported to possess a vital role in tumor survival by inducing T-cell exhaustion resulting in escaping of cancer cells from the body immune surveillance (Ma et al, 2017). PD-L1 is an antigen presented vastly on cancer cell surface. Its interaction with PD-1 on T-cells contributes to the weakening of anti-tumor activity by promoting T-cell exhaustion. Previously, there were several studies reported that the administration of inhibitors of PD-1/PD-L1 interaction could significantly increase the efficiency of immunotherapy (Mitchell et al, 2019). This suggests that combination of PD-1/PD-L1 inhibitors and another

immunotherapy treatment would be beneficial to enhance immune cell function. Thus, the present study aims to screen human single chain variable fragment (ScFv) specific to PD-L1 for enhancing immunotherapy. ScFv protein that neutralizes PD-1/PD-L1 interaction can be used in various platform of immunotherapy, such as incorporating into bi-specific T cell engager (BiTE) or fusing with T-cell receptor to generate chimeric antigen receptors (CARs). Herein, we report the results of screening of ScFv from a human ScFv-phage display library, namely 'YAMO' library (Pansri, Jaruseranee, Rangnoi, Kristensen & Yamabhai, 2009). The obtained anti-PD-L1 ScFv clones would be further characterized for their DNA sequences and protein structures. Their binding abilities have been confirmed to bind with PD-L1 protein expressing on KKU-213 cholangiocarcinoma cells by flow cytometry. The data from this study will be useful for development of cancer immunotherapy.

2. Objectives

This study aims:

1. To screen anti PD-L1 ScFv from human ScFv-phage display 'YAMO' library.
2. To determine binding specificity of the anti-PD-L1 ScFvs obtained from bio-panning by flow cytometry.
3. To examine the diversity of the anti-PD-L1 ScFvs obtained by DNA fingerprint using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method, DNA sequencing and deduced amino acid sequence analysis, and SWISS-MODEL protein structure modeling.

3. Materials and Methods

3.1 Bio-panning, colony PCR and restriction fragment length polymorphism (RFLP)

The screening of anti PD-L1 ScFv was carried out by bio-panning of human ScFv-phage display 'YAMO' library (Pansri et al, 2009). In YAMO library, the phages contained pMOD1 phagemid vector which the ScFv fragments were genetically fused in-framed with Myc-tag. Phage clones that could specifically bind to recombinant PD-L1 protein were harvested and used to infect *E. coli* HB2151. Colony PCR was carried out to determine inserted ScFv genes in *E. coli.* HB2151 clones. The PCR products were demonstrated by agarose gel electrophoresis and stained with GelRed dye® (Biotium, CA, USA). The diversity of anti-PD-L1 ScFv was analyzed by RFLP technique. The PCR products from previous experiment were purified and digested with 10 units of *Bst*NI restriction enzyme (NEB, MA, USA). The digested PCR products were separated on agarose gel electrophoresis. The DNA fingerprints were visualized on UV transilluminator and recorded by gel documentation.

3.2 Protein expression and immunoblot analysis

The positive *E. coli* HB2151 clones that contained PD-L1-specific ScFv genes were inoculated into 10 mL of M9ZB medium and incubated at 37˚C until the OD600 absorbance reached 0.6. The protein expression was induced by addition of 0.5 mM IPTG and incubation at 30˚C for 20 hours. Anti-PD-L1 ScFv produced and secreted into culture supernatant was detected by immunoblot analysis. Mouse-anti-Myc tag antibody (Thermo Fisher Scientific, MA, USA) and HRP-conjugated rabbit-anti-mouse antibody (DAKO, CA, USA) were used as primary antibody and secondary antibody, respectively. The signal was generated by incubating membrane with SuperSignal® West Pico Chemiluminescent substrate (Thermo Fisher Scientific, MA, USA) and exposing to X-ray film.

3.3 Flow Cytometry

The anti-PD-L1 binding specificity test was performed by flow cytometry using PD-L1 expressing cholangiocarcinoma (KKU-213) cell line as target cells. In each treatment, the target cells ($1.5x10^5$ cells) were incubated with culture supernatant from each clone for 60 minutes whereas FITC conjugated PD-L1 monoclonal antibodies (eBioscience, Thermo Fisher Scientific, MA, USA) were used as positive control. The flow cytometry was conducted by BD Accuri™ C6 Plus (Becton, Dickinson and Company, NJ, USA). The result was analyzed and prepared by using Flowjo™ (Becton, Dickinson and Company, NJ, USA).

3.4 Amino sequence alignment and protein structure prediction

The plasmid DNAs from the selected clones were extracted and subjected to DNA sequencing. The IMGT/V-QUEST online software was used to obtain deduced amino acid sequences and predict for the framework region (FR) and complementarity-determining regions (CDR). Clustal Omega software was used for alignment of deduced amino acid sequences. Anti-PD-L1 protein structures of the selected clones were predicted by homology remodeling by using SWISS-MODEL server (http://swissmodel.expasy.org).

4. Results and Discussion

Anti PD-L1 ScFv clones were screened from human ScFv-phage display 'YAMO' library by bio-panning. About 300 colonies were obtained and selected for PCR screening colony to confirm the presence of ScFv coding DNA. The results of PCR colony showed that 12 *E. coli.* HB2151 clones carried ScFv genes with approximately 900 bp in size, which is an approximate size of ScFv gene containing VH and VL domains (Mala et al, 2017) (**Figure 1A**). The PCR-RFLP was carried out by using *Bst*NI fingerprint analysis which was commonly used for ScFv DNA fingerprint analysis (Pansri et al, 2009). The result revealed 11 unique DNA fingerprint patterns from 12 tested clones (**Figure 1B**). In protein expression assay, all of the 12 positive HB2151 clones from the bio-panning result were cultured in M9ZB medium, and the protein expression was induced by IPTG at the concentration of 0.5 mM. The culture supernatant was used for ScFv detection by immunoblot analysis by using Myc antibody as primary antibody since ScFv was cloned in frame with MYC tag. The expected size of anti-PD-L1 ScFv is approximately 30 kDa (Marcotte & Hammarström, 2015). However, the actual protein bands observed from immunoblot were slightly varied due to their differences in amino acid composition and length (data not shown).

In order to determine anti-PD-L1 ScFv binding specificity, KKU-213 cholangiocarcinoma cells were used as target cells due to their highly expression of PD-L1 protein on cell surface which we confirmed the PD-L1 expression by flow cytometry (**Table 1**). KKU-213 cells were incubated with the culture supernatant of those 12 clones containing anti-PD-L1 ScFvs, followed by staining with FITC-tagged anti-Myc antibodies. The specificity of anti-PD-L1 binding was demonstrated by flow cytometry (**Table 1**). Positive control, KKU-213 cells stained with FITC-tagged anti-PD-L1 antibody, showed that KKU-213 has 99.5% PD-L1 expressed indicating that KKU-213 was a suitable target cell. There were 6 out of 12 clones, which were the clone numbers 5, 9, 29, 32, 103 and 117, that produced strong signals greater than those of other clones. Positive cells stained by anti-PD-L1 ScFv clone numbers 5, 9, 29, 32, 103 and 117 achieved 25.0%, 23.9%, 24.2%, 17.5%, 42.4% and, 11.3%, respectively (**Table 1**). Indeed, PCR-RFLP result showed that clone number 9 and 32 had the same fingerprint. Their differences in activity accessed by flow cytometry might be the impact of concentrations of proteins which affected the binding ability of ScFv to the target protein. However, the measurement of ScFv protein concentration in culture supernatant is difficult due to the impurity of proteins in culture supernatant. Since 9 and 32 were the same clone. We, therefore, selected only the clone numbers 5, 29, 32, 103, and 117 for the further studies.

To characterize the differences of anti-PD-L1 ScFv proteins from 5 selected clones. DNA sequencing was performed. The derived DNA coding sequences of variable domains of heavy chain (VH) and light chain (VL) from each selected clone was used for prediction of framework region (FR) and complementarity determining region (CDR). The amino acid sequence alignment from each selected clone is shown in **Figure 2**. According to the amino acid sequence alignment, all anti-PD-L1 ScFvs from these 5 clones consisted of four FRs and three CDRs between each VH and VL (Morea, Lesk & Tramontano, 2000). The critical differences of amino acid sequences were observed on CDR2-VH and CDR3-VH domains. Since CDR regions provide a recognition site on the antibody, the variations between the sequences especially in CDR2-VH and CDR3-VH domains might result the anti-PD-L1 ScFvs from the selected clones to bind to different epitopes and/or have different binding affinities to the same epitope (Li et al, 2017).

To demonstrate protein structures, the amino acid sequences of the 5 clones were submitted to SWISS-MODEL server for homology remodeling. The results showed that the anti-PD-L1 ScFv-5 has 71.72% sequence identity and 51% sequence similarity to reported anti-Mcl1 ScFv. The anti-PD-L1 ScFv-29 has 78.69% sequence identity and 54% sequence similarity to reported human Gankyrin in complex to the

single chain antibody F5. The anti-PD-L1 ScFv-32 has 72.06% sequence identity and 52% sequence similarity to reported anti-CXCL13 ScFv. The anti-PD-L1 ScFv-103 and anti-PD-L1 ScFv-117 shared the same template, scFv AM2.2, with achieved 77.82% sequence identity, 54% sequence similarity and 85.46% sequence identity, 56% sequence similarity, respectively. Five putative anti-PD-L1 ScFv structures consist of VH and VL domains linked by linker peptide were shown in **Figure 3**. However, the correlation of protein sequences/structures and binding affinities in addition to the neutralizing activities to inhibit PD-1/PD-L1 interaction still need to be further evaluated, and the potential anti-PD-L1 ScFvs will be further used for development of immunotherapy in future.

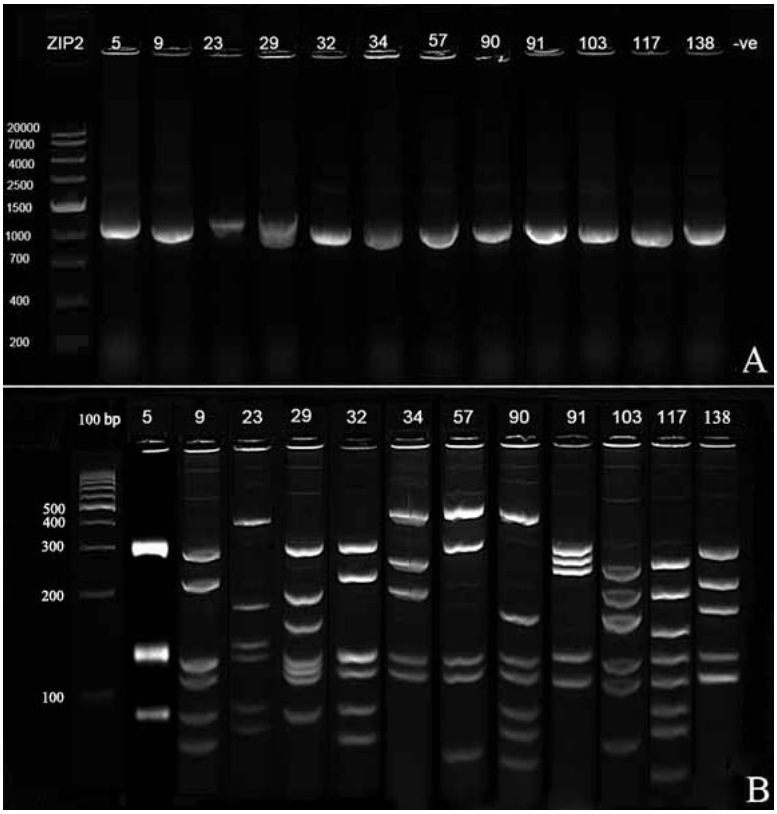

Figure 1 Gel electrophoresis stained with GelRed dye® detected under UV light
A) Screening of E. *coli* HB2151containing ScFv gene by colony PCR. All positive clones showed approximately 900-bp band indicating that they contained ScFV genes; ZIP2 (ZipRuler Express DNA Ladder) is standard board-range DNA marker, B) DNA fingerprint analysis of the PD-L1-specific ScFv diversity by PCR-RFLP using *Bst*NI. The result showed that most of positive clones were different except the clone numbers 9 and 32 that had the same fingerprint pattern, indicating that they might be the same ScFv.

Table 1 Binding specificity of anti-PD-L1 ScFvs determined by flow cytometry.

Clone Number	Anti PD-L1 binding percentage (%)
Positive Control	99.50
5	25.00*
9	23.90*
23	8.06
29	24.20*
32	17.50*
34	7.12
57	6.93

Clone Number	Anti PD-L1 binding percentage (%)
90	5.30
91	9.54
103	42.40*
117	11.30*
138	7.25

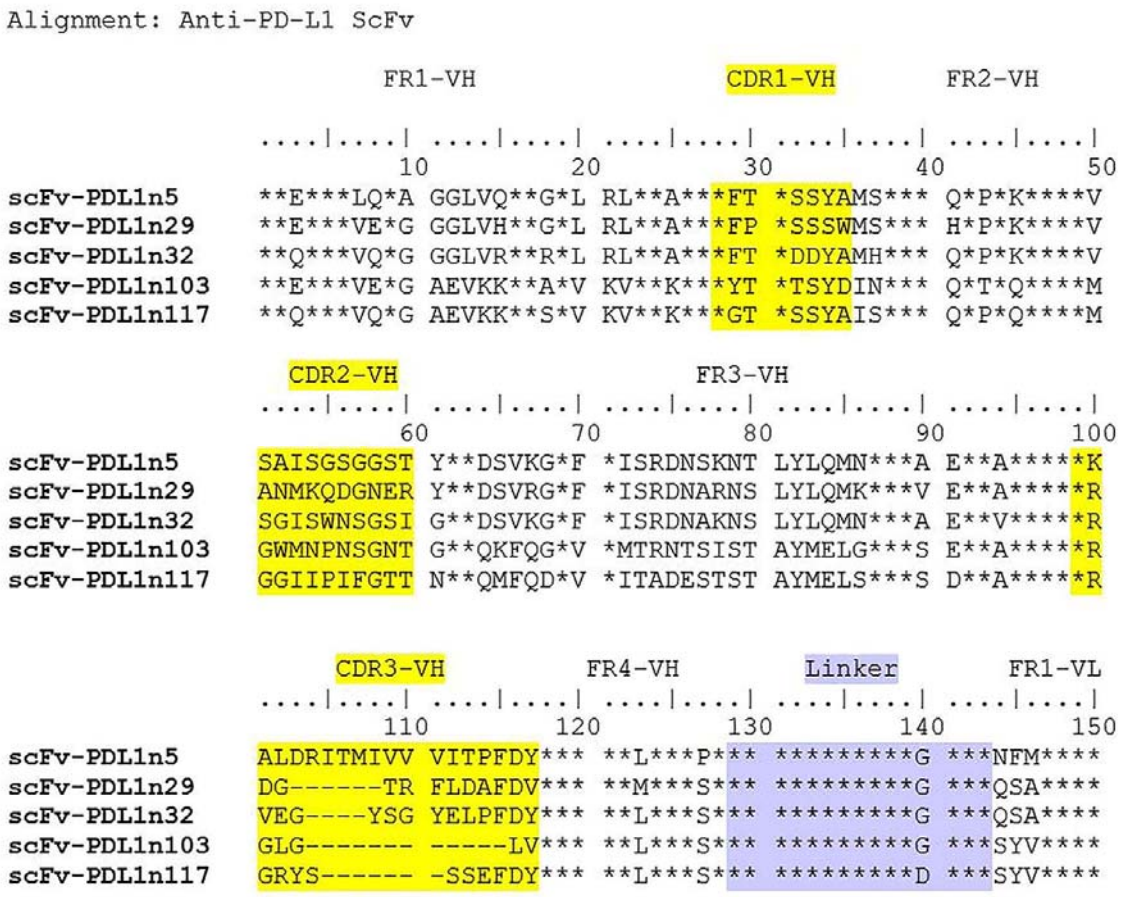

Figure 2 Amino acid sequence alignment of anti-PD-L1 ScFv genes.
These amino acid sequences, obtained from IMGT/V-QUEST online software, were aligned. Yellow highlight showed the complementarity-determining regions for variable domains of heavy chain (CDR-VH). Linker peptide to variable domains of light chain is highlighted with purple. (* is for consensus amino acid sequence.)

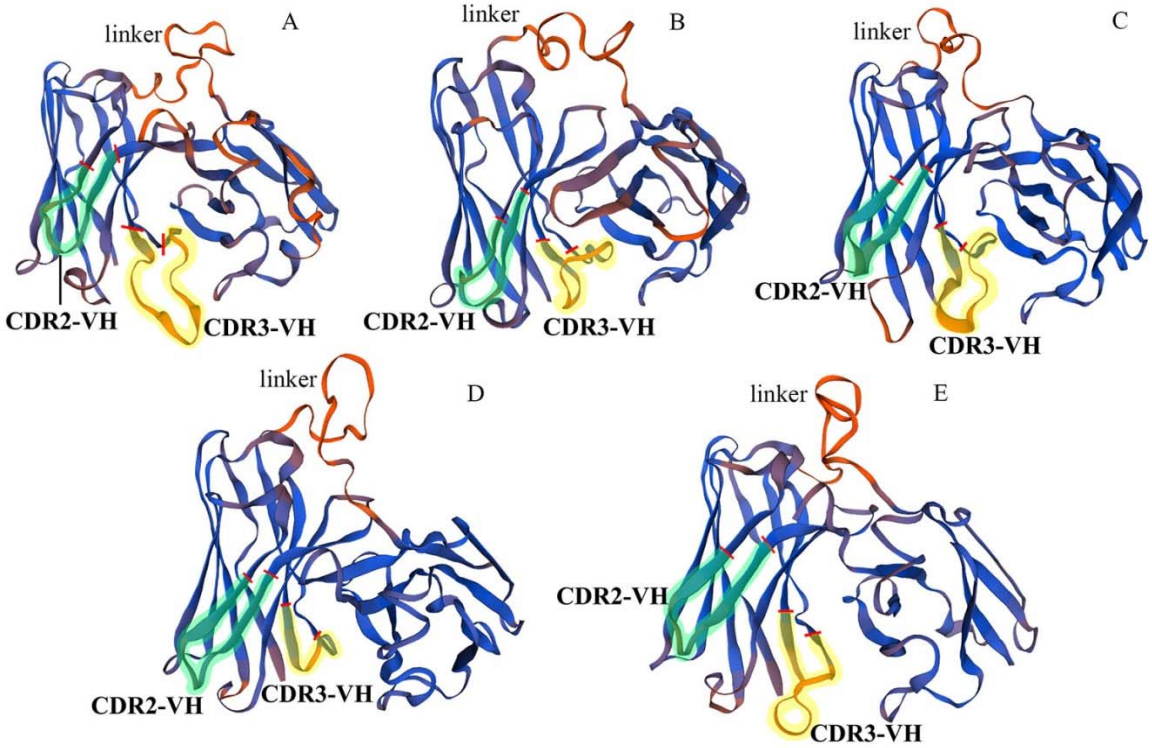

Figure 3 The predicted protein structure of Anti-PD-L1 ScFv using SWISS-MODEL server.
A) Anti-PD-L1 ScFv-5, B) Anti-PD-L1 ScFv-29, C) Anti-PD-L1 ScFv-32, D) Anti-PD-L1 ScFv-103, E) Anti-PD-L1 ScFv-117. CDR2-VH and CDR3-VH, critical difference region, were highlighted with green and yellow color, respectively.

5. Conclusion

Anti-PD-L1 ScFv clones were screened from human ScFv phage display 'YAMO' library. Totally, 11 unique clones were obtained. Their proteins were produced and confirmed for binding abilities to PD-L1 proteins by flow cytometry. Five selected ScFv clones were analyzed by DNA sequencing to obtain DNA sequences and corresponding deduced amino acid sequences. Deduced amino acid alignment and protein structure prediction demonstrated the differences of top five clones in CDR2-VH and CDR3-VH domains. All of 5 different clones will be used for further studied to improve immunotherapy for cancer treatment.

6. Acknowledgements

This study was financial supported by National Science and Technology Development Agency (NSTDA) (grant number P1650727), Chiang Mai University, and a Ph.D. scholarship from the Development and Promotion of Science and Technology Talents Project awarded to Methi Wathikthinnakon.

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