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A novel lentiviral scFv display library for rapid optimization and selection of high affinity antibodies

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ABSTRACT

Antibody display libraries have become a popular technique to screen monoclonal antibodies for therapeutic purposes. An important aspect of display technology is to generate an optimization library by changing antibody affinity to antigen through mutagenesis and screening the high affinity antibody. In this study, we report a novel lentivirus display based optimization library antibody in which Agtuzumab scFv is displayed on cell membrane of HEK-293T cells. To generate an optimization library, hotspot mutagenesis was performed to achieve diverse antibody library. Based on sequence analysis of randomly selected clones, library size was estimated approximately to be 1.6×10^6. Lentivirus display vector was used to display scFv antibody on cell surface and flow cytometery was performed to check the antibody affinity to antigen. Membrane bound scFv antibodies were then converted to secreted antibody through cre/loxP recombination. One of the mutant clones, M8 showed higher affinity to antigen in flow cytometery analysis. Further characterization of cellular and secreted scFv through western blot showed that antibody affinity was increased by three fold after mutagenesis. This study shows successful construction of a novel antibody library and suggests that hotspot mutagenesis could prove a useful and rapid optimization tool to generate similar libraries with various degree of antigen affinity.

1. Introduction

Monoclonal antibodies have high specificity and efficiency. But for therapeutic purposes, mAbs require optimization and maturation to eliminate unwanted side effects and increase efficiency. Although antibody generation process is mainly governed by its exposure to antigen, but, even in the absence of antigen, human body can maintain possibly 10^{12} diverse repertoire of antibodies [1]. Many laboratory experiments have been conducted to study and induce *in vitro* affinity maturation by using random mutations in CDRs of antibodies through error-prone PCR, radiations or some chemical mutagens. The effect of these mutations can be further studied by using display systems like phage display and lentivirus display systems. Viral vectors are a common molecular tool used for many medical practices. It has many advantages which makes it desirable to adapt over other techniques such as safety, less toxicity, high stability, target specificity and easy to screen after inoculation [2]. Different types of identification markers are used for screening process such as GFP which is one of the most common tagging proteins for identification purposes. Lentivirus based display system is a very proficient system for clinical purposes because of its simplicity and high efficiency [3].

Antibody display library can be used to screen antibody specific antigen. For higher efficiency, size of library matters a lot. By hotspot mutagenesis in variable chains of antibody, library can be diversified with large repertoire of mutants that could increase the size of any display system [4,5]. In our current study, we followed the similar approach to construct our novel library. Hotspots in CDRs of antibody variable light and heavy chains have been

Abbreviations: **AGR2**, anterior gradient 2; **CDR**, Complementary Determining Regions; **CopGFP**, copepod *Pontellina plumata* Green Fluorescence Protein; **scFv**, single chain fragment variable; **DsRed**, Discosoma coral Red; **NaAz**, Sodium Azide; **TM-EGFP**, Transmembrane- Enhanced Green Fluorescence Protein; **CDS**, coding sequence.

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identified in past and research is carried on involving the mutations in these sequences [6]. Other non-targeted mutagenesis studies have also shown highly positive effect on *in vitro* affinity maturation of antibodies and 450–6000 fold increase in antibody affinity has been reported [7]. Hotspot mutagenesis offers to enhance affinity and diversity of antibodies from site directed mutagenesis technique [8–10]. This is one of the main focuses in our research study under consideration.

In one of our previous study, we reported development and characterization of a humanized monoclonal antibody, Agtuzumab against Agr2 [11]. This investigation helped to further understand the role of Agr2 in tumor microenvironment and demonstrated that anti-Agr2 therapy in cancer treatment can be an effective way to treat cancer. In this study, we report Agtuzumab scFv based antibody optimization library by creating mutations in CDR3 hotspots of variable light and heavy chain. Successful characterization of different mutant clones showed great potential and efficacy of the library to screen high affinity clones.

2. Materials and methods

2.1. Sequence analysis of variable heavy and light chains and hotspots identification

Specific sequence in variable heavy chain of antibody was identified using NCBI online igBlast tool. Research focus was to look for two specific motifs which are most prone to somatic hyper mutation. These include RGYW (A/G-G-C/T-A/T) and AGY (AG-C/T) according to a previously published study [12]. NCBI Online igBlast tool was used to mark the exact sequence in variable light and heavy chains of antibody.

2.2. Primer designing for hotspot mutagenesis and sequencing

Mutagenesis primers were designed according to KOD Mutagenesis kit protocol (Table 1). Site directed mutations were introduced at 5′ ends of sense and antisense primers. Total six nucleotides containing hotspots were targeted for site directed mutagenesis. Nucleotide codon optimization was carried out according to a previously published work [13]. Primer 5 software was used to virtually set the primer parameters. Required primers were constructed by Shanghai HuaGene Biotech Co.,Ltd.

2.3. Mutagenesis of variable light and heavy chains and 18A4hu scFv library construction

KOD mutagenesis kit was purchased from TOYOBO Bio-Technology, CO., LTD, China. A site-directed mutagenesis based on inverse PCR (iPCR) was performed to create random mutations at specific sites in CDR3 of variable chains. Standard PCR protocol was followed provided by the manufacturing company. Mutant fragments were inserted in PCDH display vector by overlap PCR.

Table 1
Primers for hotspot mutagenesis.

Type (mutagenesis primers)	Primers (5′ to 3′)
Sense	GWVATATCCCATCATCGATCTTGCACAG
Antisense	VWGCCTATGGACTACTGGGGTCAAGGC
Sense	CWNATATCCCATCATCGATCTTGCACAG
Antisense	NWCCCTATGGACTACTGGGGTCAAGGC
Sense	GSNATATCCCATCATCGATCTTGCACAG
Antisense	NSGCCTATGGACTACTGGGGTCAAGGC

Three pairs (forward and revers) of primers were designed for hotspot mutagenesis. Random mutations were introduced at 5′ end to create site directed mutations in CDR3 of variable heavy and light chains of 18A4Hu scFv antibody.

2.4. Cell line and cell culture

HEK-293T cells were purchased from ATCC (American Type Cell Culture) and maintained in DMEM (gibco by Life Technologies Corporation) containing 10% NBS serum (Bio-sciences) and 1% antibiotic (Bio-sciences) at 37 °C.

2.5. Transfection, transduction and cre/loxP recombination in HEK-293T cells

Transfection of HEK-293T cells was performed using PEI (polyethylene amine). Growth media was replaced by DMEM w/o serum and antibiotics 2 h prior to transfection. Supernatant was collected at 48 and 72 h after transfection and used to infect HEK-293T cells. After 24 h, the culture media was replaced by full growth media and let the cells grow for next 24 h. Lentivirus titer was measured using FACS after 48 h of infection period and cells were harvested and cultured in full growth puromycin selection media for antibiotic selection. Cells were maintained in puromycin selection media for 9 days and then changed to full growth media (10% NBS, 1%Antibiotic). PBST-CRE-CMV plasmid encoding CRE recombinase was transfected to cleave transmembrane by CRE mediated cre/loxP recombination resulting in secretion of scFv antibody in supernatant.

2.6. Microscopic observation

HEK-293T cells were seeded overnight on coverslips in a 6 wells plate. Cells were washed and fixed with 4% PFA. Fixed HEK-293T cells were incubated with Rhodamine (TRITC)-conjugated Goat anti-human IgG (H + L) secondary antibody (1:100 dilution) for 1 h. Nuclei staining was performed by DAPI and coverslips were mounted on glass slide for microscopic observation.

2.7. Construction of DsRed-Agr2 protein plasmid and protein purification

Fluorescently tagged Agr2 protein plasmid was constructed by inserting DsRed monomer CDS into His-Agr2 bacterial expression system by overlap PCR. Plasmid was transformed into *E.Coli* and bacterial colony was screened and grown in liquid LB for protein extraction according to standard purification method.

2.8. Flow cytometery

Lentivirus titer in transduced cells was calculated by counting green fluorescent cells through FACS. Binding of antigen to cell surface displayed scFv was measured by incubating transduced Cells ($5*10^5$) with DsRed-Agr2 protein (10μg/ml) in ice cold 1% NaAz and 1× PBS for 2 h in 4 °C. Cells were washed with ice cold 1× PBS and performed FACS immediately.

2.9. Immunoblot and immunoprecipitation

Transduced cells were lysed and cell extract was used to perform immunoblot to check the antibody expression and concentration in cells. Expression of scFv antibody in supernatant was inquired by using immunoprecipitation. Agarose Protein G beads slurry was added to cell supernatant and put on shaker for 2 h. Protein G beads were collected by centrifugating supernatent at 900 rpm for 1 min and washed with 1× PBS. Agarose beads were re-suspended in 2× loading dye in 1× PBS and boiled at 95 °C for 5 min. After performing SDS-PAGE, immunoblotting was performed and membrane was incubated against goat anti-human IgG for 1 h. Membrane was washed and scanned at 800CW channel.