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**碩士論文**

**Department of Microbiology, Immunology and Biopharmaceuticals**

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**Master Thesis**

**以噬菌體呈現篩選並構築抗人類B型肝炎病毒外膜蛋白之單鏈變異區抗體**

**Construction and Screening of Anti-HBV Envelope Protein Single-Chain Variable Fragments by Phage Display**

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**中文摘要**

根據世界衛生組織2017最新的統計報告指出，在2015年時全球估計約有257,000,000人慢性感染B型肝炎病毒 (hepatitis B virus；HBV)，目前用於治療HBV的藥物包括類核苷(酸)反轉錄酶抑制劑 (nucleos(t)ide analogue reverse transcription inhibitors；NRTIs)以及重組干擾素(recombinant interferon) 而上述藥物只能抑制病毒的複製而無法完全清除病毒，主要是因為共價閉合環狀DNA (covalently closed circular DNA; cccDNA) 持續存在於被感染的肝臟細胞中而作為病毒複製的模板，因此目前急需其他有效且迅速清除病毒的治療方法。閒錢其他研究已證實利用抗原嵌合受體T細胞 (chimeric antigen receptor-T cell; CAR-T) 的方式能將被HBV 感染的人類肝臟細胞殺死，並且能將cccDNA清除。而在CAR的構築上最重要且具抗原辨識能力的即為單鏈變異區抗體 (single-chain variable fragment; scFv)，再加上外膜蛋白是最先與肝臟細胞進行交互作用之病毒蛋白，因此能有效的篩選並製備出針對B 型肝炎病毒外膜蛋白的單鏈變異區抗體將會為CAR-T細胞療法應用在慢性B型肝炎病毒感染奠定重要的基石。將從全血中分離出的PBMC利用第四型人類皰疹病毒 (Epstein-Barr virus; EBV) 感染後可使B細胞在體外存活並持續分泌抗體至上清液中，利用酵素免疫分析(Enzyme Immunoassay; EIA) 篩選能分泌anti-pre-S1抗體的細胞，再抽取其mRNA 並利用反轉錄聚合酶鏈鎖反應(Reverse transcription-polymerase chain reaction; RT-PCR)轉換成一個cDNA library。最後利用噬菌體呈現 (phage-display) 的方式篩選出anti-pre-S1or anti-HBsAg scFv。結果顯示由生物淘選過程中篩選出來的scFv 具有pre-S1或HBsAg專一性的親和力。

**關鍵字：**B型肝炎病毒、pre-S1蛋白、噬菌體呈現、單鏈變異區抗體、抗原嵌合受體T細胞

**Abstract**

According to the latest report from World Health Organization, it is estimated that 257 million people are chronically infected with hepatitis B virus (HBV) in 2015. Current treatments includes nucleos(t)ide analogue reverse transcription inhibitors (NRTIs) and interferons, however they are unable to eliminate the cccDNA inside the infected hepatocytes, which consistently provides as a replication template and results virus persistence. Developing new strategies to solve this problem is now an urgent need, and given the promising results from chimeric antigen receptor T cell (CAR-T) in treating leukemia patients; it is reasonable to apply CAR-T in curing chronic HBV infection. Single-chain variable fragment (scFv) is the most crucial part of a CAR molecule since it provides antigen-recognizing ability; so effectively designing a scFv, which can bind to HBV envelope proteins, is a key factor in paving the way for employing CAR-T cell therapy to treat chronic HBV infection.Using Ficoll® Paque density gradient centrifugation and followed by Epstein-Barr virus (EBV) infection to isolate B cells from peripheral blood mononuclear cell (PBMC) and culture *in vitro*. Further screening possible pools by analyzing culture supernatant using enzyme immunoassay (EIA). Then extract the mRNA from those possible B cells and reverse transcribe them into a specific cDNA library. Finally using phage-display and biopanning to select the possible scFvs that are able to bind pre-S1 or HBsAg. Results showed that the scFv we selected from biopanning procedures has the ability and specificity to bind pre-S1 pre-S1 or HBsAg protein.

**Keywords:** HBV, pre-S1, phage-display, scFv, CAR-T cell

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**Content**

**Literature Review**

* Hepatitis B Virus (HBV)

HBV is a virus belongs to genus *Orthohepadnavirus* and family *Hepadnaviridae*. These two Latin-words specify two characteristics: it is a DNA virus, and its tropism is hepatocytes. Its genome is constituted with 3182 nucleotides (genotype D) to 3248 (genotype G) nucleotides depending on the genotype (1). Inside the genome, there are four major genes: C, X, S, and P, and the last P gene, which is translated into polymerase(832 residues to 845 residues), overlaps the former three genes.

* + Vaccines and NRTIs
* pre-S1
  + possible receptor
* phage-display
  + History
  + Other system display
* CAR-T cell

The concept of CAR has been proposed since 20 years ago, it finally came to reality in the 2010 as the first clinical trial started. It attracted huge attention due to its extraordinary high rate and long duration of complete remission in r/r B-cell ALL. Last year, the USFDA approved the marketing of Novartis’ α-CD19 CAR-T cell therapy, KYMRIAH® (tisagenlecleucel). There are more and more ongoing CAR-T cell clinical trials not just for hematologic malignancies; they are pushing the frontier into solid tumors. The difficulties in eliminating solid tumors is that CAR-T cells are hard to penetrate into tumors owing to the adhesion molecules expression pattern of tumor endothelial cells is completely different from standard physiological one and the tumor microenvironment is favoring regulatory immune cells. One of the possible mechanism demonstrated by Ronald *et al.* in the human ovarian cancers is that the overexpression of endothelin B receptor (ETBR) is associated with the absence of tumor-infiltrated lymphocytes (TILs) by downregulating intercellular adhesion molecule-1 (ICAM-1) expression and increasing nitrogen oxide (NO) and NO synthase release. Despite the nature of sinusoidal endothelial cells (SECs) is acting as a barrier between sinusoid and parenchyma, they do not line as tightly as the endothelial cells in the vasculature because of lacking basement membrane. These pores are just perfect for leukocyte migrating from the sinusoid into the space of Disse, which lifts the possible restriction of hard infiltration for effector T cells into the foci of HCC. In addition, choosing a viral protein as the target for CAR-T will substantially decrease the possibility of “on target- off tumor” effect which cause severe side effects.

**Introduction**

Human hepatitis B virus (HBV) is one of the member in the family *Hepadnaviridea*. In Baltimore virus classification, it belongs to group VII, which means its genetic materials are constituted with partially double-stranded DNA (dsDNA) and it possesses the same ability as retroviruses: reverse transcribing RNA into DNA (2). The most significant difference between HBV and other viruses is that after entering the cell nucleus, its genome can be integrate into the host’s chromosome or can be converted into cccDNA by filling in the gapped dsDNA using its own polymerase (3). The cccDNA will further act as a replication template if the infected hepatocyte is not scavenged by host’s immune system, and it will become a reservoir of HBV proliferation, which is regarded as chronic infection (4). It gained notoriety for causing not merely inflammation of the liver tissues, but also inducing cirrhosis or even hepatocellular carcinoma (HCC) out of consistent inflammation (5).

There are 257,000,000 people chronically infected by HBV in 2015, according to the latest report in 2017 from World Health organization’s statistics (6). Despite of so many medical measures we have taken and invented, it is still a crucial public health burden because around 500,000 to 700,000 people died from chronic infection or other related complications every year (7). Vaccination is deemed as the most powerful prophylaxis in preventing HBV infection for the neonates (8), and it is recommended for all infants to take shot of long-established recombinant HBV surface antigen (HBsAg) vaccine shortly after birth (within 24 hours). Even though it shows great efficacy in the newborns, there are still few people did not respond after three doses of administration (9). The herd immunity might protect these non-responders, but in the high-prevalence countries, they are much more vulnerable and are high probable turning into HBV carriers(10).

Nowadays, for treating HBV infected patients, nucleos(t)ide analogue reverse transcription inhibitors (NRTIs)(11), recombinant interferons, and pegylated interferons (12) are applied clinically. However, they all failed in cccDNA clearance since the mechanism of these therapeutics are indirectly inhibiting the replication of the viruses, not straightly destroying the cccDNA itself. Interferon-α has been reported to be able to influence the stability of cccDNA through epigenetic modification in humanized mice model (13), moreover it has higher resistance threshold HBsAg clearance rate than NRTIs (14, 15). Nevertheless, the most prominent side effect of interferon therapies is that more than 30% of the patients would experience the flu-like symptoms, such as fever, chills, generalized aches and pains, and poor appetites (16-18). That is why some of the patients would rather choose NRTIs than interferons as treatment. On the other side, NRTIs have the advantage of oral consumption compared with intravenous, intramuscular, or subcutaneous infusion of interferons, and they should be used as first-line therapy due to the low cure rate of current licensed antiviral drugs, which means patients will need indefinite medication (19). The disadvantage of taking NRTIs in a long-term is that it acts as a strong and consistent selection pressure to induce viral mutation, which finally forming drug-resistant mutants (20-22). The G145R mutation in the “a” determinant is the mostly well-reported vaccine-induced immune escape (23-26), which does not only make the vaccine-induced anti-HBs lose its neutralizing ability (27) but also cause false-negative outcomes of the present test reagent (28).

To sum up, current strategies in treating HBV chronic infection are not effectively enough to cure this annoying and highly cancer-inducing disease because of the incapability to eradicate cccDNA, or even in preventing HBV infection and transmission since the its hypermutation nature (29). Therefore, we urgently require an accurate, quick, and effective therapy to cure this disease by eliminating the cccDNA at the onset of infection, or by blocking the entry of HBV particles into hepatocytes. There are many proposals to overcome this problem such as using siRNA (30) or CRISPR/Cas 9 (31) system to clear the cccDNA or barricade the route of entry by designing pre-S1 binding peptide (32) or small molecules interacting with NTCP (sodium-taurocholate cotransporting polypeptide), cyclosporine A and its derivatives (33, 34), or a NTCP-binding peptide (35, 36). However here, we tried to addressed this complicated issue by star ting with designing a neutralizing scFv targeting HBV envelope protein, and the advantages are 1) scFv itself alone can be a therapeutic drug by directly binding and interfering the first step of infection. 2) Transforming the scFv into a monoclonal antibody that could be used in diagnosis and research. 3) It could be adapted into CAR-T cell therapy by constructing the sequences into a CAR molecule, and the anti-HBs CAR-T cells are able to destroy the infected hepatocyte containing cccDNA. Additionally, in order to construct a completely novel scFv rather than borrowing published CDR sequences, phage display was applied to screen out possible candidates in specific cDNA library that are able to bind HBV envelope protein.

Phage display is a well-established technology for developing a brand-new antibody; it is also time and cost saving in comparison with using hybridoma system(37). Since its first visitation to the biotechnology field in 1985 (38), and further adaptation in expressing recombinant antibodies (39); it has already dramatically changed the way of producing monoclonal antibodies. Commercially available products are miscellaneous, and we chose Hyperphage from PROGEN as an efficient helper phage system (40). This system is featured with filamentous phage protein III (pIII) deletion in the helper phage genome; the major benefit of pIII-deletion is that the packaged phage will only contain scFv-pIII fusion protein but not the wild-type pIII from traditional helper phage, which discriminate those low-affinity binders in the eluted pool, which might interfere or even dominate the following panning processes. According to the original article, antigen-binding activity was increased about 400-fold by displaying oligovalent antibody on every phage particle. Apart from that, we also pre-screen the supernatants secreted by *in vivo* EBV-infectedPBMC, which attempts to increase the uniformity of cDNA library. This crucial step can raise the chances of positive clones to be successfully isolated during the panning procedures, rather than being outnumbered by those negative clones.

In conclusion, a neutralizing scFv alone can be a useful medicine in fighting acute virus infection, or it can further be integrated into a CAR molecule and be the “bullet” of CAR-T cells. Hopefully in the near future, the anti-HBsAg or anti-pre-S1 scFv we constructed as a potent weapon can be harnessed in CAR-T cell therapy.

**Materials and Methods**

*PBMC isolation and culture*

Whole human blood was kindly provided by Tainan Blood Center, Taiwan Blood Services Foundation. All of the experiments containing biological materials that is related to human tissue had been reviewed by Institutional Review Board, Tri-Service General Hospital (TSGHIRB No.: 2-105-05-071) and the approval was obtained. First, whole blood was aliquoted into 50 ml centrifuge tubes (P & C Biotech) and centrifuged at 25℃ at 2000 x g , and braking level was set as 2 for 30 minutes (5810R, Eppendorf). Plasma proportion was removed, and the buffy coat was diluted with 2 times volume of 0.02% EDTA (Avantor)/PBS (Life Technology) solution. 28 ml of the mixture were added onto 14 ml Ficoll-Paque™ PLUS (GE Healthcare) and centrifuged at 25℃ at 400 x g, and braking level was set as 0 for 40 minutes. Supernatant was removed and the PBMC were washed twice with two times volume of 0.02% EDTA/PBS by centrifuging at 25℃ at 300 x g for 10 minutes. Pellet was resuspended with 10 ml RPMI 1640 + 2.05 mM L-glutamine (GE Healthcare) supplemented with 1 mM sodium pyruvate (Life Technology), MEM non-essential amino acids solution (Life Technology), 50μg/ml gentamycin sulfate (TAI YU Chemical & Pharmaceutical), and 50μg/ml kanamycin sulfate (VWR Chemicals). Total cell number was calculated by hemocytometer (Reichert Technologies). Cell density was resuspended into 107 cells/ml after centrifuging at 25℃ at 200 x g for 10 minutes. L-leucyl-L-leucine methyl ester hydrobromide (LeuLeuOMe; Sigma-Aldrich) was added to a final concentration of 85 μg/ml. The solution was standed in room temperature (RT) for 15 minutes and the reaction was stopped by adding 2% of total volume fetal bovine serum (FBS; Life Technology). LeuLeuOMe was eliminated by centrifuge at 25℃ at 200 x g for 10 minutes and the pellet was resuspended with RPMI 1640 plus the same supplements as former mentioned and 2% of total volume FBS. Total cell number was counted and the pellet was resuspend into 107 cells/ml with EBV Buffer after centrifuging at 25℃ at 200 x g for 10 minutes. Solution was incubated in 37℃ water bath for 1 hour and inverted every 15 minutes. After adding 50 μl of 10% FBS in RPMI 1640 with the same supplements into 96-well cell culture plate (Guangzhou Jet Bio-Filtration), 200 μl of EBV infected cell solution was aliquoted into each well. Cultured in 37℃ incubator with 5% CO2 for around 2 weeks.

*Enzyme Linked Assay*

Each well was coated with 100 ng of bovine serum albumin (BSA, Sigma-Aldrich) or pre-S1 (MyBioSource; Large envelope protein; Hepatitis B virus genotype C subtype ad (isolate Japan/S-179/1988)) or HBsAg (GeneScript) dissolved in of 0.5 M bicarbonate buffer (15.9 g Na2CO3 (Sigma-Aldrich) and 29.3 g Na2HCO3 (Avantor) in 1 L Milli-Q water; pH 9.6). Plates were shaken at 4℃ at 150 rpm overnight. Plates were then washed with 200 μl washing buffer (29.22 g NaCl (Sigma-Aldrich) and 1 ml polysorbate 20 (tween 20; Sigma-Aldrich) in 1 L Milli-Q water) 3 times. Fifty μl of SD buffer (1.42g Na2HPO4 (Avantor), 29.22 g NaCl (Sigma-Aldrich), and 1 ml polysorbate 20 (Sigma-Aldrich) in 1 L Milli-Q water) was mixed with 50 μl of cell culture supernatant into each well and incubated at 37℃ for 1.5 hours. Each well was washed with 200 μl washing buffer 5 times. One hundred μl of 2500X diluted biotinylated mouse anti-human IgA + IgG + IgM (H+L) antibody (SeraCare KPL) in SD buffer was added into each well and incubated at 37℃ for 1 hour. Plates were washed 5 times at the same term. One hundred μl of 1000X diluted horseradish peroxidase conjugated streptavidin in 0.1% PBS-tween 20 was added into each well and incubated at 37℃ for 1 hour. Wells were all washed 5 times at the same condition. One hundred μl of 0.4 mg/ml *o*-phenylenediamine dihydrochloride (Sigma-Aldrich) dissolved in 0.05 M phosphate-citrate buffer with sodium perborate (Sigma-Aldrich; pH 5.0) was added into each well. Plates were incubated in the dark at 25℃ for 30 minutes. Reaction was stopped by adding 50 μl of 2 N H2SO4 (Avantor) into each well and optical density values were measured at 492 nm.

*CDNA Library construction*

Cells in wells that its OD492nm ratio to BSA is higher than 2 were collected and separated into 2 pools (i.e., pre-S1 and HBsAg) depending on its binding target. Total RNA were extracted by using Total RNA Miniprep Purification Kit (GMbiolab), and all of the procedures were strictly followed by manufacture’s protocol. MRNA was reverse-transcribed into cDNA by MMLV Reverse Transcription Kit (Protech) by using oligo-dT as primer. Also, all steps were accorded to the producer’s manual. After the reaction, all of the solution were combined into one tube and DNA concentration was quantified with spectrophotometer (NanoDrop™8000, Thermo Fisher Scientific). CDNA were aliquoted to the amount needed for the following experiments and diluted into 100 ng/μl, which was stored in the -20℃ refrigerator. The remaining were kept in -80℃ refrigerator for long-term storage.

*OE-PCR*

Variable heavy chains (VH) and light chains (VL) were amplified with independent primer pairs which contain restriction enzyme sites and partial linker sequence (Table 1), which was designed based on Human IgG/IgM Library Primer Set (PROGEN Biotechnik). This amplification step is termed as “extension PCR”. Every PCR were optimized by adjusting cycle number, annealing temperature, polymerase type, template concentration, and percentage of additives. Detailed conditions for each reaction are listed in Table 2. All of the VH or the VL reaction solution were pooled together and agarose (UniRegion Bio-Tech) gel-purified by AxyPrep™ DNA Gel Extraction Kit (Corning). Overlap PCR for pre-S1 scFv was performed with 50 ng of both VH and VL, and 1.25 units of *OneTaq* (New England Biolabs) at 94℃ for 2 minutes as preliminary denature, 5 cycles at 94℃ for 30 seconds, 56℃ for 30 seconds, and 72℃ for 1 minutes, and a final extension at 72℃ for 1 minutes. The purification PCR was carried out in a 50 μl reaction with 2 μl of previous overlap PCR product, 2.5 units of *OneTaq* at 95℃ for 3 minutes, 30 cycles at 95℃ for 1 minute, 56℃ for 45 seconds, and 72℃ for 1 minutes, and a final extension at 72℃ for 5 minutes. The forward flanking primer used is 5’- GAATAGGCCATGGCG -3’, and the reverse flanking primer used is 5’- TGACAACCTTGCGGCCGC -3’. The approximate 800bp product, scFv, was gel-purified by the same kit mentioned above.

*Competent Cell Preparation*

Chemically competent cells were prepared as following steps: one ml of wild-type XL-1 Blue: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacIqZ∆M15* Tn*10 (*Tetr*)*] (Agilent Technologies) overnight culture was added into 99 ml fresh 2YT (16 g tryptone (Laboratorios CONDA), 10 g yeast extract (Laboratorios CONDA), and 5 g NaCl in 1 L Milli-Q water) in a 500 ml Erlenmeyer flask. The culture was shaken at 200 rpm at 37℃ until OD600nm reached 0.4. The culture was divided into two 50 ml centrifuge tube and chilled on ice for 30 minutes. It was harvested by centrifuging at 6000 rpm at 4℃ for 5 minutes. Supernatant was disposed and the pellet was resuspend gently with 10 ml of ice-cold 0.1 M CaCl2 (Avantor)/tube. The solutions were incubated on ice for 30 minutes and combined into one tube before centrifuging at 5000 rpm at 4℃ for 10 minutes. The pellet was gently resuspended with 1 ml of 10% glycerol (Avantor) in 0.1 M CaCl2. At last, it was aliquoted into 100 μl/tube and stored in -80℃ refrigerator immediately. For electrocompetent cell preparation, air conditioner was set at 25℃ six hours before the experiment, and all of the equipment and materials were stood in the same room.(41): 1 ml of wild-type XL-1 Blue overnight culture was aliquoted into 99 ml fresh 2YT in a 500 ml Erlenmeyer flask. Cells were cultured at 200 rpm at 37℃ until OD600nm reached 0.6. The culture was separated into two 50 ml centrifuge tube and centrifuge at 6000 rpm, 25℃ for 5 minutes. The supernatant was decanted and pellet was resuspended gently with 50 ml of ambient temperature (25℃) 10% glycerol /tube. They were then centrifuged at 5000 rpm at 25℃ for 10 minutes. Pellets were washed with 25 ml of 10% glycerol /tube and solutions were combined into one tube before centrifuge at the same term again. Finally, pellet was resuspended with 1 ml of 10% glycerol and stored at 4℃.

*Ligation and Transformation*

One μg of gel-purified scFv was digested by 20 units of *NotI* (New England Biolabs) and *NcoI* (New England Biolabs) at 37℃ for 2 hours and terminated the reaction by heating at 80℃ for 20 minutes. Inserts were purified with MinElute PCR Purification Kit (QIAGEN). Plasmid pSEX81 (PROGEN Biotechnik) was transformed into XL-1 Blue by heat shock. PSEX81 was purified by using Gene-Spin™ Miniprep Purification Kit (Protech) and digest 10 μg of purified product with 40 units of *NotI* and *NcoI*, and 3 units of Shrimp Alkaline Phosphatase (rSAP; New England Biolabs) at 37℃ for 2 hours. Digestion/Dephosphorylation was stopped by heating for 20 minutes at 80℃ after adding EDTA (pH 8.0) to a final concentration of 5 mM. Digested/dephosphorylated pSEX81 was purified with crystal violet (CV; Sigma-Aldrich) and Orange G (OG; Sigma-Aldrich) in agarose gel (1.6 μg/ml of CV and 0.8 μg/ml of OG) and 10X DNA loading dye (300 μg/ml of CV and 150 μg/ml of OG)(42, 43). Purified product was served as vector. Fifty ng vector and insert were mixed at a molar ratio of 1 to 3 and heated at 65℃ for 5 minutes. Remaining materials needed were then added: T4 DNA ligase (Lucigen), T4 ligation buffer, and nuclease-free water to 20 μl and ligated at 16℃ overnight. For heat shock, 1 μl of ligation reaction was directly added into 100 μl of chemically competent cell, and the following steps were all operated as same as the manual’s description. For electroporation, one ligation reaction was precipitated by adding 2 μl of 3 M NaOAc (Sigma-Aldrich; pH 5.2), 55 μl of absolute ethanol (Avantor) and 20 μg of glycogen (Roche Diagnostics). After mixing thoroughly it was stored at -20℃ overnight. Precipitated ligation products were centrifuged at 4℃ at 13,000 x g for 30 minutes and pellet was washed with 500 μl of ice-cold 80% ethanol twice. Residual ethanol was evaporated by opening and inverting the tube in laminar flow for 10 minutes. Adjusted conditions are listed as follows: the dried DNA pellet was resuspended with 40 μl of electrocompetent cells and stood in a 4℃ refrigerator for 30 minutes rather than with 1-5 μl of nuclease-free water. Field strength was set as 1900 V/cm in place of 1700 V/cm. Competent cell/DNA were acclimated into ambient temperature 10 minutes before the electroporation. Finally, instead of proceeding in an ice-cold cuvette, electroporation was performed at RT. All of the transformants (heat shock & electroporation) were plated on Super Optimal Broth - Glucose/Ampicillin Agar plate (SOB-GA plate; 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 0.186 g KCl (Sigma-Aldrich), 0.952 g anhydrous MgCl2 (Alfa Aesar), 2.465 g MgSO4 ∙7H2O (Sigma-Aldrich); 18.5 g bacterial agar (Laboratorios CONDA), 100 mM of D-glucose (Sigma-Aldrich), and 100 μg/ml of ampicillin (Sigma-Aldrich) in 1 L Milli-Q water). Plates were all incubated at 37℃ for no more than 16 hours after recovery culture was spread.

*Phage packaging and collection*

Colonies on the SOB-GA plates were scratched down with 4 ml of 2X SOB-GA (40 g tryptone, 10 g yeast extract, 1 g NaCl, 0.372 g KCl, 0.952 g anhydrous MgCl2, and 2.465 g MgSO4•7H2O with the same glucose and ampicillin concentration described above in 1 L Milli-Q water) by glass cell spreader. Each plate was rinsed with extra 1 ml of 2X SOB-GA. Collected culture was shaken at 200 rpm at 37℃ for 1.5 hours as recovery. One ml of recovered culture was added into 33ml of 2YT-GAT (2YT medium with extra 100 mM D-glucose, 100 μg/ml of ampicillin, and 10 µg/mL of tetracycline (Sigma-Aldrich)) and shook at 200 rpm at 37℃ until OD600nm reached 0.5. Hyperphage M13 K07*ΔpIII* (PROGEN Biotechnik) was inoculated at a multiplicity of infection (MOI) = 20. Helper phage infection began with sitting in 37℃ without shaking for 30 minute and followed by 200 rpm shaking for 45 minutes. Infected Culture was centrifuged at 25℃ at 6000 x g for 10 minutes. Culture medium was replaced with 32 ml of 2YT-AKI (2YT medium with additional 100 μg/ml of ampicillin, 50 μg/ml of kanamycin sulfate, and 100 μM of IPTG (Sigma-Aldrich)), and shook in a 250 ml Erlenmeyer flask at 200 rpm at 30℃ for 24 hours. Phage particles were collected by using polyethylene glycol (PEG) -precipitation. The one-day culture was chilled on ice for 10 minutes, and it was centrifuged at 4℃ at 13,000 x g for 10 minutes. The supernatant (about 32 ml) was poured into 8 ml of ice-cold 5X PEG/NaCl (PEG-8000 20% and NaCl 2.5 M) and incubated on ice in 4℃ refrigerator for 1 hour after mixing properly by inverting 5 times. The mixture was centrifuged at 4℃ at 16,000 x g for 20 minutes, and the supernatant was discarded. In order to remove residual culture medium and gather the phage pellets, another centrifuge was performed at 4℃ at 13,000 x g for 5 minutes. The remaining supernatant was carefully taken out by pipette, and the pellet was resuspended with 1200 μl of PBS. It was vigorously pipetted and transferred into a 1.5 ml of chilled microcentrifuge tube. The phage solution was vortexed until the pellet was totally dissolved, and centrifuged at 4℃ at 13,000 x g for 5 minutes. The supernatant was transferred into a new microcentrifuge tube with the pellet undisturbed, which was stored in 4℃ refrigerator.

*Antibody-Phage ELISA*

Pre-S1, HBsAg, BSA, and human serum albumin (HAS; Sigma-Aldrich) were all coated at the same condition as EIA. Plates were all washed with 200 µl PBS 3 times and blocked with 100 µl of 2% skim milk (Fonterra) in PBS at RT for 2 hours. Plates were washed with 200 µl of 0.2% PBS-Tween 20 four times. Phage solutions were aliquoted and diluted with PBS into 150 µl containing 2% skim milk, 0.2% Tween 20, and 109 particles per well. After adding phages into wells for 1 hour, the plates were washed with 0.2% PBS-Tween 20 four times. One hundred µl of 1000X diluted anti-M13/fd/F1 Filamentous Phages Mouse Monoclonal Antibody (PROGEN Biotechnik) was added into each well and stood in RT for 1 hour. Wells were washed with 0.2% PBS-Tween 20 four times and 100 µl of 3500X diluted HRP Goat anti-mouse IgG (minimal x-reactivity) Antibody (BioLegend) was pipetted into each well for an hour of room-temperature incubation. Plates were washed with the same term for 4 times again. One hundred µl of 0.4 mg/ml o-phenylenediamine dihydrochloride dissolved in 0.05 M phosphate-citrate buffer per well was used as substrate. Plates were incubated at RT in the dark for 15 minutes. Fifty μl of 2 N H2SO4 was added into each well and optical density values were measured at 492 nm.

*Biopanning*

Each pool was panned for 4 times, and detailed description was listed in Table 3, which was based on the publication of Rahbarnia L*, et al*.(44). Protein coating, washing, and blocking procedures were as same as phage-ELISA, however at least 1011 phage particles in a pool were added into each well. After washing with the according times, bound phages were eluted with 100 μl of 0 .025% trypsin and 0.01% EDTA in PBS (Life Technology) at RT for 15 minutes. The solution was vigorously pipetted at least 50 times and transferred into a sterile microcentrifuge tube which was sit on ice. One hundred μl of freshly-prepared wild-type XL-1 Blue in 2YT- Tetracycline medium whose OD600nm was 0.4-0.5 was mixed with 10 μl of eluted phage solution and incubated in 37℃ for 20 minute without shaking. One μl of eluted phage solution was also serial-diluted with SM buffer (5.8 g NaCl, 2 g MgSO4, 50 ml Tris-HCl (1 M, pH 7.5), and 0.1 g gelatin (Sun-Island Coffee & Teas & Foods) in 1 L Milli-Q water) for titration. The phage-infected cultures were then all plated on SOB-GA plates. One round of biopanning is defined as finishing all the procedures above in this section. Moreover, colonies from each round were randomly picked for colony PCR using 200 nM of forward primer (5’- TTAGCTCACTCATTAGGCACCCCA -3’) and reverse primer (5’- GCTAAACAACTTTCAACAGTTTCAGC) to check the integrity of the scFv, whose length is expected to be around 978 bp.

*HepaRG Culture and HBV Infection*

Undifferentiated HepaRG cells were seeded at around 50% confluence into desired vessel (e.g., 2 × 106 cell/75 T flask). Undifferentiated culture medium (William’s E medium (Life Technology) supplemented with 1 mM sodium pyruvate, MEM non-essential amino acids solution, GlutaMAX™-I solution (Life Technology), 50μg/ml gentamycin sulfate, 50μg/ml kanamycin sulfate, 5 μg/ml insulin (Insulatard®, Novo Nordisk), 5 × 10−7 M hydrocortisone sodium succinate(Solu-Cortef, Pfizer), and 10% FBS) was applied until the cells reached to 90%-95% confluence. After that, culture medium was substituted with differentiating medium (The concentration of hydrocortisone sodium succinate was elevated to 5 × 10−5 M, and extra 1.8% of dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added, and the others remained the same) for 2 week. For HBV infection, the FBS in differentiating medium was substituted with human HBV positive serum. Four days later, cells were washed with PBS twice and culture medium was replaced back to differentiating medium. Medium of 8 days after infection was collected for secreted HBV DNA detection by PCR. Nucleic acids in the medium were firstly purified by LabPrep DNA Extraction Kit (Taigen Bioscience). One μl of eluted solution was used as template. 2X *Taq* DNA Polymerase Master Mix RED (Ampliqon), 200 nM of each forward primer (5′-TCA CCA TAT TCT TGG GAA CAA GA-3′) and reverse primer (5′-CGA ACC ACT GAA CAA ATG GC-3′) were used in the PCR, which was performed at 95℃ for 10minutes, and 40 cycles at 94℃ for 20 seconds, 55℃ for 20 seconds, and 72℃ for 1 minute(45).

**Results**

*Pre-screening potential candidate in PBMCs*

The total experiment flow chart is briefly diagrammed in the **Fig. 1**. In pre-screening step, the supernatant from successfully EBV-transformed lymphoblastoid cells were directly applied in enzyme immunoassay. For pre-S1 screening, 37 wells out of 100 wells had OD492nm at least 2 times higher than OD492nm at BSA. On the other hand, for HBsAg screening, 37 wells out of 100 wells had OD492nm at least 2 times higher than OD492nm at BSA. Additionally, these results match the Poisson distribution, which is around 2-4 wells in one 96-well plate will be positive ones **(Fig. 2)**.

*Construction of scFv insert using overlapped-extension PCR*

Reverse-transcribed cDNA was used as template of antibody variable genes for extension PCR. At first time of biopanning, multiplex PCR was performed to amplify VH and VL by adding 5 primers and 11 primers mixture respectively, and the annealing temperature for VH and VL extension PCR was set as 55℃ and 65℃ independently to obtain the greatest amount of product (**Fig. 5**). 15 cycles of purification PCR was performed at a gradient Ta after 18 cycles of overlap PCR, and the results showed that when Ta set as 50℃, the sensitivity is the highest amount them all (**Fig. 6**). At second time of biopanning, forward primer 2A, 2B, and 2C paired with reverse primer 2D+Yol were used to amplify variable heavy chain from IgG. Forward primer 2A, 2B, and 2C paired with reverse primer 2E+Yol were used to amplify variable heavy chain from IgM (**Fig. 3A**). Forward primer 2F+Yol, 2G+Yol, and 2H+Yol paired with reverse primer 2P were used to amplify variable kappa light chain. Forward primer 2I+Yol, 2K+Yol, 2L+Yol, 2M+Yol, 2N+Yol, and 2O+Yol paired with reverse primer 2Q were used to amplify variable lambda light chain (**Fig. 3B**). Finally 15 independent PCR was performed to amplify each variable regions. Each pre-S1 VH and VL amplifiedresult was cropped into **Fig.13**; and for HBsAg, they were all in **Fig. 14**. Purification PCR for pre-S1 scFv amplification, Ta was set as 52℃ without GC enhancer, and for HBsAg scFv amplification, Ta was set as 55.6℃ without GC enhancer.

*Library Construction Optimization*

Crystal violet and orange G were used as counter-ion dyes to stain the DNA molecules in agarose gel, rather than using traditional ETBR staining. Even though the sensitivity is not as high as ETBR or SYBRSafe (**Fig. 16**) under the ultraviolet visualization, it is convenient at restriction endonuclease digested vector gel-purification, which gives higher transformation efficiency. Ligation was also tested at variant vector to insert molar ratio, and the results indicated that under the ratio of 1 to 3 has the best transformation efficiency

*Biopanning makes interactive candidate become dominant in the pool*

*Identifying HBsAg-1 and pre-S1-binding single clones*

Isolated anti-HBsAg and anti-pre-S1 scFv have HBV neutralizing efficacy

**Discussion**

* multiplex pcr/ idependent pcr
* cloning vh and vl independently
* solid phase efficiency/ partial sequence
* ~~panning on fixed cells~~
* antibody family and cdr analysis
* ~~ISA-pcr is not that good as the authors proposed.~~

To date, there are abundant published methods to optimize phage display outcomes in order to construct a *de novo* antibody to against desired targets(46-50), however the efficiency and the so-called “theoretical possibility” needs to be tested and confirmed. In our lab, we simultaneously tried one out of many methods, independent strand amplification, proposed by Pablo H. Sotelo *et al*. (50), and the quantity of full-length scFv did not increase after an extra 30 cycles of independent strand primer amplification were introduced. Our data (not showed) were not consistent with the hypothesis the authors established, which might due to the differences from cDNA constitution, primer sequences and the DNA polymerase used. Additionally, we tried to develop a scFv that is able to directly neutralize HBV particles and possess the specificity to HBV envelope proteins by panning on 8 % formaldehyde-fixed differentiated HepaRG at the first round and panning on HBV-infected HepaRG for the following rounds. However, it did not work out, which might resulted from insufficient HBV envelope protein expression on the infected HepaRG cell surface or epitopes were not presented by this kind of fixation. Furthermore, most of the scFv in the phagemids using this approach were not complete, but the similar concept used in peptide library selection are much more efficient (51, 52), which indicates this process might not suitable for scFv selection. It is important to check the intactness of the scFv after each round of panning, because the bacteria harbor incomplete scFv have growth advantage, and might eventually dominate the pool, which was regularly occurred in our experiment.

In order to reach the maximal library size, tips for manipulating nucleic acids such as reducing the ultraviolet damage and elevating the recovery rate from agarose gel, PCR optimization with gradient annealing temperatures, touch-down methods increasing specificity, and increasing ligation efficiency had all been tested and applied.

Using gene therapies to achieve the so-called virological cure is attainable. For example, using the hot ticket, CRISPR/Cas9 system, to edit HBV genome inducing cccDNA degradation is better than using siRNA, which only provides inhibiting efficacy. It seems hopeful to solve this tough problem by applying this avant-garde technology; nonetheless, the first clinical trial is just about to start in this year. The safety and efficacy will be evaluated in the following years, but it is a great way to kick off. Therefore, adapting from currently United States Food and Drug Administration (USFDA) licensed therapies, it is reasonable using chimeric antigen receptor T cell (CAR-T cell) therapy to address this carcinogenic public health issue. Given the specificity it provides and encouraging clinical outcomes in the circumstances of refractory/relapse B-cell precursors acute lymphoblastic leukemia (r/r B-cell ALL), additionally, pre-S1 protein is the surface antigen which binds to the entering receptor, NTCP. Constructing a scFv that specifically binds to pre-S1 into a CAR molecule would be a fantastic strategy to manage the trouble we are facing now.

**Tables and Figures**

**References**

1. Kay A & Zoulim F (2007) Hepatitis B virus genetic variability and evolution. *Virus Research* 127(2):164-176.

2. Jones SA & Hu J (2013) Hepatitis B virus reverse transcriptase: diverse functions as classical and emerging targets for antiviral intervention. *Emerging Microbes &Amp; Infections* 2:e56.

3. Gao W & Hu J (2007) Formation of Hepatitis B Virus Covalently Closed Circular DNA: Removal of Genome-Linked Protein. *Journal of Virology* 81(12):6164-6174.

4. Villeneuve J-P (2005) The natural history of chronic hepatitis B virus infection. *Journal of Clinical Virology* 34:S139-S142.

5. Di Bisceglie AM (2009) Hepatitis B And Hepatocellular Carcinoma. *Hepatology (Baltimore, Md.)* 49(5 Suppl):S56-S60.

6. Anonymous (2017) Global Hepatitis Report 2017. (World Health Organization, Geneva).

7. Meireles LC, Marinho RT, & Van Damme P (2015) Three decades of hepatitis B control with vaccination. *World Journal of Hepatology* 7(18):2127-2132.

8. Andre FE & Zuckerman AJ (Review: protective efficacy of hepatitis B vaccines in neonates. (0146-6615 (Print)).

9. Saco TV, Strauss AT, & Ledford DK (Hepatitis B vaccine nonresponders: Possible mechanisms and solutions. (1534-4436 (Electronic)).

10. Zampino R*, et al.* (2015) Hepatitis B virus burden in developing countries. *World journal of gastroenterology* 21(42):11941-11953.

11. Clark DN & Hu J (2015) Hepatitis B virus reverse transcriptase - Target of current antiviral therapy and future drug development. *Antiviral research* 123:132-137.

12. Rijckborst V & Janssen HLA (2010) The Role of Interferon in Hepatitis B Therapy. *Current hepatitis reports* 9(4):231-238.

13. Belloni L*, et al.* (2012) IFN-α inhibits HBV transcription and replication in cell culture and in humanized mice by targeting the epigenetic regulation of the nuclear cccDNA minichromosome. *The Journal of clinical investigation* 122(2):529-537.

14. Perrillo R (2009) Benefits and risks of interferon therapy for hepatitis B. *Hepatology* 49(S5):S103-S111.

15. Dusheiko G (Treatment of HBeAg positive chronic hepatitis B: interferon or nucleoside analogues. (1478-3231 (Electronic)).

16. Kartal ED, Alpat Sn Fau - Ozgunes I, Ozgunes I Fau - Usluer G, & Usluer G (Adverse effects of high-dose interferon-alpha-2a treatment for chronic hepatitis B. (0741-238X (Print)).

17. Lau GKK*, et al.* (2005) Peginterferon Alfa-2a, Lamivudine, and the Combination for HBeAg-Positive Chronic Hepatitis B. *New England Journal of Medicine* 352(26):2682-2695.

18. Marcellin P*, et al.* (2004) Peginterferon Alfa-2a Alone, Lamivudine Alone, and the Two in Combination in Patients with HBeAg-Negative Chronic Hepatitis B. *New England Journal of Medicine* 351(12):1206-1217.

19. Tang LSY, Covert E, Wilson E, & Kottilil S (Chronic Hepatitis B Infection: A Review. (1538-3598 (Electronic)).

20. Fischer KP, Gutfreund KS, & Tyrrell DL (2001) Lamivudine resistance in hepatitis B: mechanisms and clinical implications. *Drug Resistance Updates* 4(2):118-128.

21. Tenney DJ*, et al.* (2004) Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to Lamivudine. *Antimicrobial agents and chemotherapy* 48(9):3498-3507.

22. Motahar M*, et al.* (2016) Evaluation of HBV resistance to tenofovir in patients with chronic hepatitis B using ZNA probe assay in Kerman, southeast of Iran. *Asian Pacific Journal of Tropical Disease* 6(7):513-516.

23. Rezaee R*, et al.* (2016) Impacts of the G145R Mutation on the Structure and Immunogenic Activity of the Hepatitis B Surface Antigen: A Computational Analysis. *Hepatitis monthly* 16(7):e39097-e39097.

24. Thakur V*, et al.* (Transmission of G145R mutant of HBV to an unrelated contact. (0146-6615 (Print)).

25. Komatsu H*, et al.* (Evaluation of the G145R Mutant of the Hepatitis B Virus as a Minor Strain in Mother-to-Child Transmission. (1932-6203 (Electronic)).

26. Datta S*, et al.* (2009) Genetic Characterization of Hepatitis B Virus in Peripheral Blood Leukocytes: Evidence for Selection and Compartmentalization of Viral Variants with the Immune Escape G145R Mutation. *Journal of Virology* 83(19):9983.

27. Carman WF*, et al.* (1990) Vaccine-induced escape mutant of hepatitis B virus. *The Lancet* 336(8711):325-329.

28. Sticchi L, Caligiuri P, Cacciani R, Alicino C, & Bruzzone B (2013) Epidemiology of HBV S-gene mutants in the Liguria Region, Italy: Implications for surveillance and detection of new escape variants. *Human vaccines & immunotherapeutics* 9(3):568-571.

29. Noguchi C*, et al.* (G to A hypermutation of hepatitis B virus. (0270-9139 (Print)).

30. Li G*, et al.* (Inhibition of hepatitis B virus cccDNA by siRNA in transgenic mice. (1559-0283 (Electronic)).

31. Seeger C & Sohn JA (2014) Targeting Hepatitis B Virus With CRISPR/Cas9. *Molecular Therapy - Nucleic Acids* 3.

32. He Y*, et al.* (2014) Selection of HBV preS1-binding penta-peptides by phage display. *Acta Biochimica et Biophysica Sinica* 46(8):691-698.

33. Shimura S*, et al.* (Cyclosporin derivatives inhibit hepatitis B virus entry without interfering with NTCP transporter activity. (1600-0641 (Electronic)).

34. Watashi K*, et al.* (2014) Cyclosporin A and its analogs inhibit hepatitis B virus entry into cultured hepatocytes through targeting a membrane transporter, sodium taurocholate cotransporting polypeptide (NTCP). *Hepatology (Baltimore, Md.)* 59(5):1726-1737.

35. Passioura T*, et al.* (2018) <em>De Novo</em> Macrocyclic Peptide Inhibitors of Hepatitis B Virus Cellular Entry. *Cell Chemical Biology* 25(7):906-915.e905.

36. Ye X*, et al.* (2016) Efficient Inhibition of Hepatitis B Virus Infection by a preS1-binding Peptide. *Scientific Reports* 6:29391.

37. Rami A, Behdani M, Yardehnavi N, Habibi-Anbouhi M, & Kazemi-Lomedasht F (2017) An overview on application of phage display technique in immunological studies. *Asian Pacific Journal of Tropical Biomedicine* 7(7):599-602.

38. Smith GP (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* 228(4705):1315.

39. McCafferty J, Griffiths AD, Winter G, & Chiswell DJ (1990) Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 348:552.

40. Rondot S, Koch J, Breitling F, & Dübel S (2001) A helper phage to improve single-chain antibody presentation in phage display. *Nature Biotechnology* 19:75.

41. Tu Q*, et al.* (2016) Room temperature electrocompetent bacterial cells improve DNA transformation and recombineering efficiency. *Scientific Reports* 6:24648.

42. Rand KN (1996) Crystal violet can be used to visualize DNA bands during gel electrophoresis and to improve cloning efficiency. *Technical Tips Online* 1(1):23-24.

43. Yang Y, Jung Dw Fau - Bai DG, Bai Dg Fau - Yoo GS, Yoo Gs Fau - Choi JK, & Choi JK (Counterion-dye staining method for DNA in agarose gels using crystal violet and methyl orange. (0173-0835 (Print)).

44. Rahbarnia L*, et al.* (2016) Development of a Novel Human Single Chain Antibody Against EGFRVIII Antigen by Phage Display Technology. *Advanced pharmaceutical bulletin* 6(4):563-571.

45. Naito H, Hayashi S, & Abe K (2001) Rapid and specific genotyping system for hepatitis B virus corresponding to six major genotypes by PCR using type-specific primers. *Journal of clinical microbiology* 39(1):362-364.

46. Løset GÅ, Bogen B, & Sandlie I (2011) Expanding the Versatility of Phage Display I: Efficient Display of Peptide-Tags on Protein VII of the Filamentous Phage. *PLOS ONE* 6(2):e14702.

47. Løset GÅ, Roos N, Bogen B, & Sandlie I (2011) Expanding the Versatility of Phage Display II: Improved Affinity Selection of Folded Domains on Protein VII and IX of the Filamentous Phage. *PLOS ONE* 6(2):e17433.

48. ’t Hoen PAC*, et al.* (2012) Phage display screening without repetitious selection rounds. *Analytical Biochemistry* 421(2):622-631.

49. Duan Z & Siegumfeldt H (An efficient method for isolating antibody fragments against small peptides by antibody phage display. (1875-5402 (Electronic)).

50. Sotelo P*, et al.* (2012) An efficient method for variable region assembly in the construction of scFv phage display libraries using independent strand amplification. *mAbs* 4(4):542-550.

51. Shukla GS & Krag DN (2005) Phage display selection for cell-specific ligands: Development of a screening procedure suitable for small tumor specimens. *Journal of Drug Targeting* 13(1):7-18.

52. Wang J*, et al.* (2011) Selection of phage-displayed peptides on live adherent cells in microfluidic channels. *Proceedings of the National Academy of Sciences* 108(17):6909.