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Screening of phage displayed human liver cDNA library against dexamethasone

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Abstract

This paper described an attempt to establish a new method to screen the target biomolecule from phage displayed cDNA library against small molecule drug insoluble in water. Dexamethasone was selected as the model drug, and the screening was carried out in an Eppendorf tube packed with the drug. The whole procedure was monitored by PCR with the enriched specific phage clone as the template. After four rounds screening, the PCR products of selected phages with the lengths of 400 and 600 bp were sequenced, and revealed identical sequences with cytochrome *c* oxidase subunit III and albumin respectively by GenBank searching. Furthermore, frontal analysis-capillary electrophoresis (FA-CE) was performed to study the interaction between dexamethasone and albumin, and the binding constant was calculated to be 1.153×10^3, validating the weak specific interaction between the drug and the target protein. All these results demonstrated that with insoluble drug as the solid phase directly, the screening of target large molecule expressed in phage display cDNA library was feasible, which might pave an easy way to screen the candidate drug targets.

Keywords: Phage display; cDNA library; Dexamethasone; Interaction

1. Introduction

Phage display technology is a technique that can display random peptide or protein sequence on the surface of the phage by the fusion of the foreign DNA sequence with the gene for the phage coat protein, thus linking a phenotype with its genotype [1–3]. Peptide or protein sequence on the surface of the phage can be replicated when the phage is infected and multiplied in the host cell. The displayed peptide or protein often behaves essentially as it would if it were not attached to the virion surface, so the affinity purification technique can be used to capture phages displaying ligand binding properties from the phage library. Random peptides or proteins from human tissues can be displayed on the surface of phage particles to generate a library with a capacity of 10^6–10^9 protein or peptide sequences, from which the target sequence can be screened with drugs or proteins as ligands by "biopanning" the phage pool [4,5], which generally includes three steps: the binding of phages displaying specific peptide or protein that interacts with the ligand on the solid phase, the elution of bound phages, and the amplification of the eluted phages in host cells. The process is repeated 3–5 rounds to enrich the peptides or proteins with the desired binding properties, and then the enriched sequence is determined from the coding region of the viral DNA.

Complementary DNA (cDNA) is the reverse transcriptase product of mRNA, and represents the coding sequence of all transcribed genes at the time that mRNA is isolated. cDNA has been reported displayed on the surface of phage particle [6-8]. Various methods have been developed for the screening of phage displayed cDNA libraries, and the most are based on the protein–protein interaction, in which the protein ligands are immobilized on the solid phase by noncovalent adsorption. Library screening with nonpeptidic small molecule drug as ligands remains few. Sche and his colleagues described the screening of binding proteins from a T7 phage

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displayed cDNA library of human brain tissue against FK506, in which biotinylated FK506 was bound to the monomeric avidin agarose for the affinity selection [9]. Jin et al. identified hNopp140 from human liver cDNA library as the target of doxorubicin by binding biotinylated doxorubicin on the surface of streptavidin-coated plate [10]. In both cases, the small molecules were first biotinylated, and then immobilized on the surface of various supports through the interaction between biotin and avidin or streptavidin. However, for some small molecule drugs, the functional groups to interact with biomolecules might be occupied during the modification or immobilization procedure, which might affect the screening efficiency.

Dexamethasone is a synthesized corticosteroid compound that has been widely used in clinical treatment for Addison's disease, systemic lupus erythematosus, periarteritis nodosa and other autoimmune disease. In addition, it is a model drug commonly used in studying drug–protein interaction. Therefore, in this paper, taken advantage of the insolubility of dexamethasone, a new method for the screening of candidate drug targets in phage displayed cDNA library was proposed, and after four rounds of screening, the phage clones that expressed albumin and cytochrome *c* oxidase subunit III were enriched. Furthermore, the specific interaction between dexamethasone and albumin was validated by the frontal analysis-capillary electrophoresis (FA-CE).

2. Experimental

2.1. Reagents

Dexamethasone was purchased from Tianjin Tianyao Pharmaceuticals (Tianjin, China). T7Select human liver cDNA library and bacteria strain BLT5403 were ordered from Merck (Darmstadt, Germany). Bacto Tryptone, Yeast Extract and human serum albumin Fraction V were bought from Sigma (St. Louis, MO). Carbenicillin disodium salt was obtained from Amresco (Ohio, USA). TaKaRa Taq polymerase, DNA Marker DL2000, agarose and the synthesized primers were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. (Dalian, China). All other reagents were of analytical grade.

2.2. Instruments

FTGENE5D PCR thermal cycler from Techne (Cambridge, UK) and ABI PRISMTM 377xl DNA Sequencer from Applied Biosystem (Foster City, USA) were used for the amplification of the enriched phage clones and the subsequent DNA sequencing. P/ACE 5010 CE system with UV detector from Beckman (Fullerton, USA) was used for the frontal analysis. HPLC experiments were performed on a system with Jasco PU-1580 intelligent pump (Jasco Co., Tokyo, Japan) equipped with a UV 200 II detector (Elite Analytical Instrument Ltd., Dalian, China) to study the solubilities of dexamethasone in water and various buffers.

2.3. Methods

2.3.1. Measurement of the solubility of dexamethasone

The solubilities of dexamethasone in various solutions were determined by HPLC with the following steps. First, a series of dexamethasone solutions dissolved in methanol with concentrations of 0.015625, 0.03125, 0.0625, 0.25 and 1 mg/mL were prepared, and analyzed by HPLC respectively to make the calibration curve. Then different dexamethasone solutions were prepared by adding excessive dexamethasone into Eppendorf tubes containing water, phosphate buffered saline (PBS), PBS containing 0.2% Tween-20 (referred hereafter as PBST) and 1%SDS respectively, and shaking vigorously to make dexamethasone dissolved. All these solutions were kept at room temperature overnight, and centrifuged to collect the supernatant. Finally, the solubilities of dexamethasone in various buffers were determined by HPLC.

2.3.2. Amplification of T7Select human liver cDNA library

Fifty millilitre LB medium with 50 μg/mL carbenicillin in a 250 mL conical tube was inoculated with a single colony of BLT5403, and cultured at 37 °C overnight. Then 1 mL culture was added to 50 mL LB/carbenicillin medium, and grown to an OD_{600} of 0.8. Subsequently, 5 μL T7Select human liver cDNA library was add to the medium and incubated with vigor shaking at 37 °C for 3 h until lysis was observed, which was clarified by spinning at 8000 × *g* for 10 min at 4 °C. The supernatant was collected, and the resulting amplified library was titered.

2.3.3. Screening of phage displayed cDNA library

The amplified phage library was diluted with PBS, and then poured into an Eppendorf tube packed with a certain amount of dexamethasone. The "binding-elution-amplification" selection process was performed with the procedure shown in Fig. 1. After incubated at room temperature for 30 min with gentle shaking, phages with specific interaction with dexamethasone bound to the drug particles, and the unbound ones were washed off with PBS. Subsequently, T7 phage host bacteria BLT5403 were added

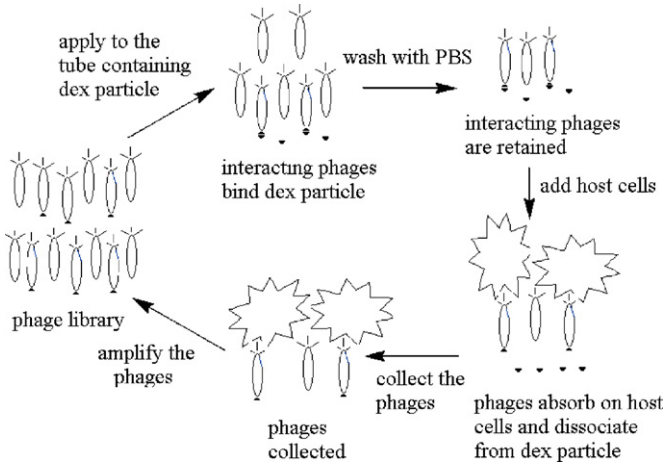

Fig. 1. Diagram of the screening procedures of phage displayed cDNA library using dexamethasone particle as solid phase. (⬮) Specific phage; (⬭) non-specific phage; (▬) dexamethasone particle.

Fig. 2. T7Select10-3b cloning region.

into the tube to competitively bind the phages retained on the dexamethasone particles, and the eluants were collected by centrifugation. The collected phages with specific interaction with dexamethasone were then amplified, and used for the next round of selection. The affinity selection process was carried out for four rounds.

2.3.4. Monitoring the enrichment of specific phages by PCR

The enrichment of specific phages by four rounds of "biopanning" process was monitored by PCR with the lysate of selected phages as the templates after each round of screening. Primers designed to anneal to the vector sequence flanking the cloning site was used for PCR to analyze the insert size (Fig. 2). Amplified phage lysate of each round screening was diluted 100 times with 10 mM EDTA, and then heated at 65 °C for 10 min before it was used as PCR template. The reaction solution of PCR was composed of 2 μL phage lysate, 5 μL 10× Taq buffer with $MgCl_2$, 1 μL T7Select up primer (20 μM), 1 μL T7Select down primer (20 μM), 4 μL dNTP mixture (2.5 mM for each), 0.25 μL TaKaRa Taq DNA polymerase (5 U/μL) and distilled water with the total volume of 50 μL. The PCR conditions were 94 °C for 4 min, and then repeated for 35 cycles as 94 °C for 50 s, 50 °C for 1 min and 72 °C for 1 min. The last cycle was extended to 10 min at 72 °C. The PCR products were analyzed by 1% agarose electrophoresis. The amplified phages after the fourth round of screening were titered and plated at low density. Individual, well isolated plaques were scraped up from the top agarose, dispersed in 10 mM EDTA solution, and heated at 65 °C for 10 min before they were used as the templates of PCR. Five PCR products of enriched phages with the inserts about 400 bp and six products with the inserts about 600 bp were sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit. BLAST program was used to compare the obtained sequences with those listed in GenBank [11].

2.3.5. Frontal analysis-capillary electrophoresis

Frontal analysis-capillary electrophoresis was employed to study the interaction between dexamethasone and screened potential protein target according to the method described in Ref. [12]. As the solubility of dexamethasone in PBS was poor, dexamethasone sodium phosphate was used in the analysis. The effective and total lengths of the separation capillary were 20 and 27 cm. Before the operation, the capillary was conditioned with 1 M NaOH, distilled water and 67 mM phosphate buffer (pH 7.4). A series of mixtures with albumin concentration of 100 μM and increasing dexamethasone concentration respectively of 150, 200, 250, 500 and 750 μM were prepared, and kept at room temperature for 2 h before analysis. Samples were injected at 0.5 psi for 10 s, and 8 kV was added during the separation. The UV detection wavelength was set at 214 nm. The data were processed according to Ref. [13]. In order to obtain good reproducibility, between each run the capillary was conditioned with the solutions mentioned above.

Table 1
Solubilities of dexamethasone in water and PBS analyzed by HPLC

	Water	PBS	PBS containing 0.2% Tween-20	PBS containing 1% SDS
Dissolved dex (mg/mL)	0.056	0.057	0.110	0.896

3. Result and discussion

3.1. Solubility of dexamethasone

To screen the candidate targets from phage displayed cDNA library with insoluble dexamethasone particles as the solid phase directly, it is important to ensure dexamethasone could not be soluble in water and buffers. In our experiments, the quantitative analysis of dexamethasone dissolved in water, PBS, PBS containing 0.2% Tween-20, and PBS containing 1% SDS was performed by HPLC to examine its solubility at room temperature. From Table 1, it could be seen that the dissolved dexamethasone in water and PBS was less than 0.057 mg/mL and the bulk of added dexamethasone existed as particles. Therefore, it could be used directly as the solid phase for screening.

3.2. Screening of phage displayed cDNA library

The amplified library with a titer of 1.86×10^{10} pfu/mL was used for the subsequent screening. As mentioned above, since the solubility of dexamethasone in PBS was low, in our experiments dexamethasone was put in an Eppendorf tube, and used as the solid phase directly to select phages with binding proteins. Compared to other solid phase commonly used in "biopanning" the library, such as ELISA plates, magnetic particles, globular agarose and glass beads, by our proposed method, all functional groups of the insoluble drug were kept free, enabling the enough interaction between the drugs and targets.

In the "biopanning" procedure, the amount of loaded phage displayed cDNA library and dexamethasone, as well as the washing buffer, were the key parameters that might affect the screening. The experimental conditions for the screening were listed in Table 2. For each round of screening, the amount of added phages and dexamethasone was increased to improve the recovery of specific phages that interacted with dexamethasone. For the washing buffer, usually Tween-20 was added to pre-

Table 2
Experimental parameters in each round of screening

	Titer of loading phages (pfu)	Dex (mg)	Non-specific washing times with 400 μL PBS
1st	9.3×10^7	10	6
2nd	6.2×10^8	20	12
3rd	8.2×10^8	30	18
4th	2.3×10^9	40	24

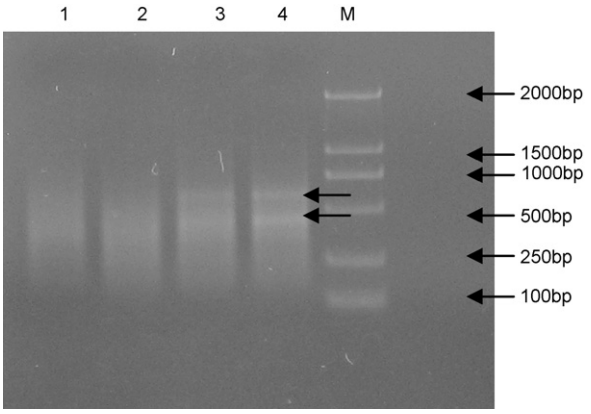

Fig. 3. Analysis of PCR products enriched in each round of screening. Lane1–4: PCR products with the lysate of the enriched phage after the 1st, 2nd, 3rd and 4th round of selection as the template, respectively. Lane M: DL2000 DNA marker (2000, 1500, 1000, 500, 250 and 100 bp).

vent the non-specific interaction between the solid phase and the phage coat proteins. However, from Table 1 it could be seen that the addition of Tween-20 could almost double the solubility of dexamethasone compared to that in PBS, therefore in our experiments only 400 μL PBS was used as the washing buffer, and the washing times were increased from 6 to 24 to ensure the complete washing of the phages with non-specific interactions.

To elute the bound phages with specific interaction with dexamethasone, mid-log bacteria BLT5403 were used since the commonly employed 1% SDS might increase the solubility of dexamethasone by almost 90 times (as shown in Table 1), which might affect the employment of dexamethasone particles as the solid phase directly. In our experiments, the phages exhibited high affinity to host bacteria BLT5403, thus were easily dissociated from dexamethasone particles. The collected enriched phages from each round screening were amplified, and the resulting library was used for the next round of affinity selection, which was repeated for four times.

3.3. Evaluation of enriched phages by PCR and DNA sequencing

To evaluate the enriched specific phages after each round of screening, cDNAs inserted into the phages were analyzed by PCR with primers flanking the construction site of the T7 phage DNA, and the lysates of phages collected after each round of selection was used as the templates for PCR. The products for the first and second rounds of selection appeared as smears, and those for the third and fourth rounds of selection revealed clear bands of about 400 and 600 bp (as shown in Fig. 3). These results indicated that specific phages were selectively amplified by the affinity selection procedures. After four rounds screening, five random clones of phages with PCR products of 400 bp and six random clones with PCR products of 600 bp were sequenced, and searched in GenBank for highly similar sequences using BLAST program. By Align two sequences (bl2seq) program, two of the five 400 bp PCR products were found with the same sequences, and identical with cytochrome *c* oxidase subunit III (COX III) by Blastx search program. Sequences for the six 600 bp PCR products were found completely identical, and the amino acids sequences were proven the same as that for albumin.

3.4. Interaction between dexamethasone and candidate protein targets

Both albumin and COX III might be the candidate protein targets selected from the cDNA library against dexamethasone. However, since it is too difficult to obtain COX III with high purity, only the interaction between albumin and dexamethasone was performed to validate the feasibility of the screening method.

Albumin is one kind of proteins synthesized by the liver cells, and secreted into plasma, which behaves as the carrier for external drug molecules and internal biological molecules. Therefore, it should exhibit affinity for dexamethasone molecules. To evaluate the interaction between dexamethasone and albumin, FA-CE was employed, which was performed by injecting the pre-equilibrated mixture of dexamethasone and HSA into a capillary filled with phosphate buffer (pH 7.4). The large volume injection resulted in the appearance of two plateaus, representing drug–protein complex and the free dexamethasone respectively, as shown in Fig. 4.

The free dexamethasone concentration was determined by an external calibration procedure. To make the calibration curve, a series of standard dexamethasone solutions with the concentration respectively fixed at 50, 100, 200, 400, 600 and 800 μM were prepared, and the heights of various plateaus were measured. Quite good linear relationship between the plateau heights and dexamethasone concentrations was obtained with the linear regression coefficient of 0.9998. To analyze the mixtures of dexamethasone and HSA, samples were prepared with HSA concentration fixed at 100 μM and dexamethasone concentration at 100, 150, 200, 250, 500 and 750 μM, respectively. By the comparison of free dexamethasone plateau height (h_s) obtained during electrophoresis of dexamethasone-HSA samples to that obtained during electrophoresis of dexamethasone calibration standard (h_c), the free drug concentration [D_f] could be calculated, according to Eq. (1), in which [D_t] represented

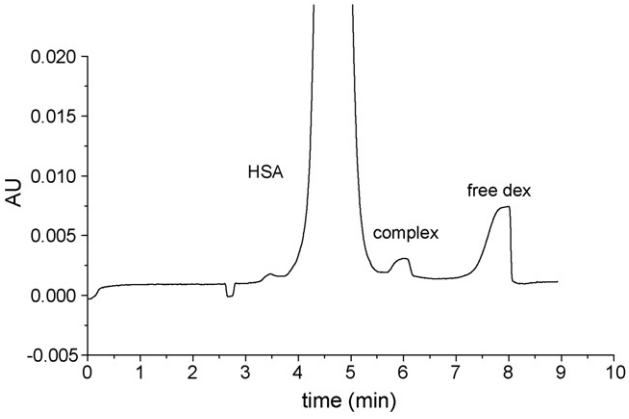

Fig. 4. Electropherogram of dexamethasone sodium phosphate and HSA mixture by FA-CE. The mixture solution contained 100 μM HSA and 750 μM dexamethasone sodium phosphate.

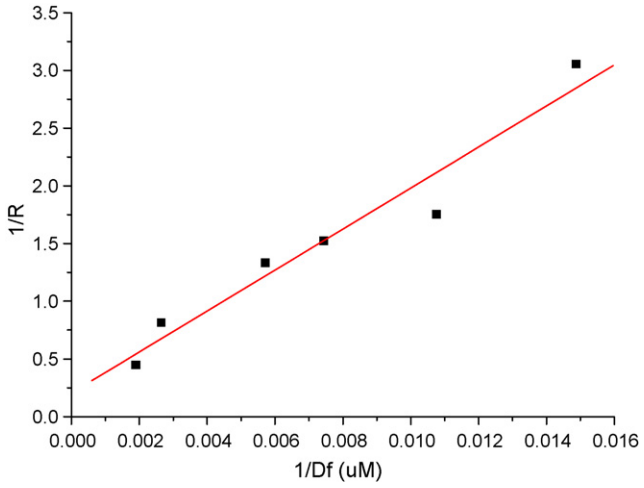

Fig. 5. Linear regression curve of dexamethasone and HSA interaction by FACE.

the starting drug concentration in the mixture. Furthermore, the bound dexamethasone concentrations $[D_b]$ and the r value, which represented the bound drug molecule number per protein was calculated based on Eqs. (2) and (3). According to Klotz equation (Eq. (4)), the curve of $1/r$ versus $1/[D_f]$ was constructed with the linear regression coefficient as 0.9732, as shown in Fig. 5. By linear regression of the curve, the intercept $1/n$ and the slope $1/nk$ values were obtained, and the binding sites (n) for dexamethasone with HSA was found to be 5, and the binding constant (k) was calculated to be $1.153 \times 10^3\ M^{-1}$, which demonstrated that there existed noncovalent interaction between dexamethasone and HSA.

$$[D_f] = \frac{h_s}{h_c}[D_t] \quad (1)$$

$$[D_b] = [D_t] - [D_f] \quad (2)$$

$$r = \frac{[D_b]}{[P_t]} = \sum_{i=1}^{m} ni \frac{K_i[D_f]}{1 + K_i[D_f]} \quad (3)$$

$$\frac{1}{r} = \frac{1}{n} + \frac{1}{nk[D_f]} \quad (4)$$

4. Conclusions

With the insoluble small molecule drug particle as the screening solid phase directly, a new method to screen the candidate drug targets from phage displayed cDNA library was proposed, by which the screening procedure could be significantly simplified since the modification and immobilization of drugs could be avoided. Furthermore, all functional groups of the drug were kept free, so that the interaction between the drug and the targets was complete enough. All the experimental results demonstrated that such a method might pave a simple way to screen the drug targets from phage displayed cDNA library.

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