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Research Paper

Selection of DNA aptamer that specific binding human carcinoembryonic antigen *in vitro*☆

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Abstract

Objective: To select the specific aptamer of carcinoembryonic antigen (CEA), one of the most attractive molecule for cancer target therapy and imaging. **Methods:** Seven rounds *in vitro* selection were performed against the purified CEA protein. Ligand-mediated target purification and Co-immunoprecipitation were adopted to verify the specific binding of the aptamer to the purified and native protein separately. **Results:**The CEA-specific aptamer which can bind both the purified and native protein with the high specificity was obtained. **Conclusion:**This is the first time the CEA specific apatmer was produced. The results in this study provides the preliminary evidence for further investigation and application of CEA-aptamer in the future.

Keywords: aptamer; carcinoembryonic antigen; tumor targeting

INTRODUCTION

Malignant disease is one of the leading causes of death in the world. Due to the limited efficiency and serious side effects of chemotherapy, it is urgent to further develop tumor targeting therapy and diagnosis. As one of the most widely studied human tumor-associated antigens (TAAs), carcinoembryonic antigen(CEA) has an extensive clinical application in multiple type of cancer[1,2]. Because of its expression pattern and the possible role in tumorigenesis[3], CEA has attracted considerable attention, not only as a tumor marker but also as a targeted molecule for cancer target therapy and imaging. Most, if not all, the applications related with CEA are based on the antibody, however due to some inherent drawbacks of antibodies: like immunogenicity, poor permeability through a solid tumor and high cost, it is desirable to develop an alternative which may maintain most advantages of antibodies while overcome their shortcomings. Effective aptamer strategy has for these reasons been put foreword as an alternative.

The technique of Systematic Evolution of Ligands by EXponential enrichment(SELEX), which was initially used for obtain of aptamer, was first proposed by Gold L and Szostak JW in 1990[4,5]. In nearly 17 years, this method had been widely applied in diagnosis, therapy, and drug development. Aptamers, original from Latin *aptus* meaning *to fit*, are DNA or RNA oligonucleotides selected from large combinatorial pools by iterative *in vitro* selection experiments(SELEX) [6-8]. Unlike antisense oligonucleotide and ribozyme, aptamer can be folded into a unique tertiary structure through intra-molecular interactions, which make it capable of binding many different targets ranging from small molecules to proteins or nucleic acids with high affinity and specificity[9,10]. Moreover, it is remarkable that researches of aptamer have been stepped into the therapeutic trials after no more than 10 years of the introduction of concept[11]. In December 2004, the approval of the first aptamer drug, Macugen, against age-related macular degeneration by the US Food and Drug Administration[12]

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demonstrated the potential of aptamer for therapeutic and commercial application. Compared to antibodies, aptamers have several unique properties: lower molecular weights(8-15 KDa *vs* 150 KDa), higher tissue penetration rate and faster blood plasma clearance, non-immunogenic, higher specificity, easier to mass production, cheaper and remarkable stability[13-15].

Though aptamers have shown the potential in diagnosis and target therapy of cancer, it is still in an early stage as a cancer medicine. There are only few reports on aptamer against tumor associate antigen[16,17]. Studies that provide further application of aptamer on this field are lacking. In this study, our aim was to develop the specific aptamer against CEA protein, underscoring the potential for the further highly-expressed CEA tumor targeting therapy and diagnosis.

MATERIALS AND MATHODS

Cell culture

LS174T cell line which expressed CEA at high level[18] was purchased from Cell Bank of Chinese Academic of Science. Cells were grown in RPMI1640 (Gibcol) with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37℃ in 5% CO_2.

SELEX library and primers

Single-strand DNA(ssDNA) templates and primers were synthesized and purified by Invitrogen, China. The initial ssDNA library contained a central randomized sequence of 45 nucleotides(nt) flanked by 18-nt primer hybridization sites(5'-GCC TGT TGT GAG CCT CCT-N45-CGC TTA TTC TTG TCT CCC-3'). A 5'-primer U1:5'-GCC TGT TGT GAG CCT CCT-3', a biotinylated 5'-primer U2:5'-Biotin-GCC TGT TGT GAG CCT CCT-3', and a biotinylated 3'-primer D1:5'-Biotin-GGG AGA CAA GAA TAA GCG-3' were used in the PCR reactions for the synthesis of single-labeled or double-labeled, double strands DNA molecules. To regenerate the ssDNA pool, the purified biotinylated PCR product(single-labeled) was incubated for 15 min at room temperature with streptavidin-coated magnetic beads(M-270 streptavidin dynabeads, Dynal), washed and non-biotinylated strand collected after alkaline denaturation. After precipitation, DNA concentration was measured by UV spectroscopy.

To prepare the biotinylated aptamer, the final selected polyclonal aptamer and the primary ssDNA library were re-amplified using the biotinylated up and down stream primers to produce the double-labeled dsDNA. After heating denaturation and cool immediately on ice, the temporarily separated both strands were used for the following procedure [19].

SELEX

Iterative rounds of aptamer selection and amplification were performed as described previously[20]. Briefly, CEA was immobilized to the surface of 96-well plates (C96 Maxisorb 430341, Nalge Nunc, NY) overnight at 4℃(0.01 mol/L PBS, pH7.4). After removing the excess unbound protein and washing the well, the CEA-well together with the empty-well were blocked with the 3% BSA at 37℃ for 2 h. These wells were then washed with PBS six times. The selection was initiated with 2 nmol of ssDNA pool(100 pmol for the 2 to 7 round). DNA suspended in SHMCK buffer (20 mmol/L HEPES pH 7.4, 120 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$) was heated to 95℃ for 5 min and chilled on ice, before transfer to room temperature. DNA was then mixed with yeast tRNA and salmon sperm DNA(used as competitor, Sigma) and incubated in empty well for 1 h at 37℃, the supernatant were transferred to the CEA-containing well where they were incubated for 1 h at 37℃. The supernatant was then removed and discarded, well was washed with washing buffer (SHMCK, 0.05% Tween) to remove the unbound sequences. Well-retained DNA was eluted by adding the elution buffer (7 mol/L urea, 0.5 mol/L NH_4Ac, 1 mmol/L EDTA, 0.2%SDS) at 95℃ for 10 min and isolated by ethanol precipitation. The isolated DNA was amplified by PCR with U1 and D1 primers(5 min denaturation at 95℃; 8 to16 cycles of 30s at 93℃, 30s at 48℃, 1 min at 72℃, followed by 5min at 72℃). The single strand DNA was prepared as described above and used as the input for the next round of selection (***Fig 1***). All together 7 rounds of selection were performed and the final polyclonal oligobodies(R7 aptamer) were able to recognize the target.

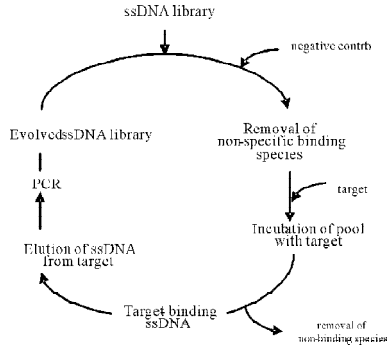


Fig 1 Selection procedure of DNA aptamer binding to CEA

Ligand mediated target recognition[21]

Magnetic streptavidin beads were coated with 200 pmol of R7 aptamer and the same amount beads were coated with 200 pmol of primary ssDNA pool to serve as a control. CEA protein were incubated with the beads-aptamer combination in the binding buffer(SHMCK,

0.1 mg/ml yeast tRNA, 0.1mg/ml salmon sperm DNA) at room temperature for 1 h(gently shaking occasionally). After magnetic separation, the beads were washed three times with SHMCK buffer. Protein was eluted from aptamer-coated beads with the elution buffer (1 mol/L NaCl, 10 mmol/L Tris pH7.5, 0.5 mol/L EDTA, 0.1% TritonX-100) at 4℃ for 30 min. Aptamer-purified proteins were analyzed by SDS-PAGE and silver staining.

Co-immunoprecipitation and Western blot analysis

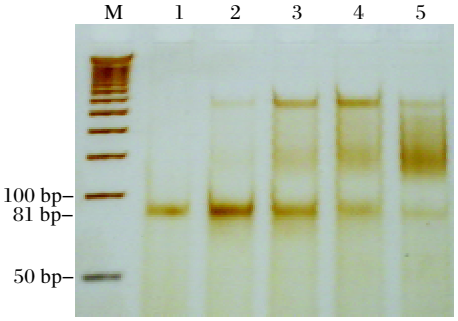
Cultured LS174T cells rinsed twice with cold PBS and cell lysis buffer(0.5 % NP-40, 0.1 mmol/L EDTA, 150 mmol/L NaCl,100 μmol/L Na_3VO_4, 1 mmol/L dithiothreitol, 0.4 mmol/L phenylmethylsulfonyl fluoride, 3 μg/ml aprotinin, 2 μg/ml pepstain and 1 μg/ml leupeptin) was added to the cell monolayer before cells were harvested by scraping. Stripped cells were rotated at 4℃ for 30 min and the supernatants were collected after centrifugation. Protein was measured for concentration by the BCA method and subpackaged, stored at –80℃.

The LS174T extracts(200 μg) were incubated with the biotinylated R7 aptamer for 1 h at room temperature in the binding buffer, the same procedure was done with the unselected biotinylated ssDNA pool to serve as a control. The oligobodies-CEA complex was separated from the unbound proteins by adding the streptavidin coated magnetic beads. After washing with SHMCK buffer, the microbeads-oligobodies-CEA complex was split by heating in the protein loading buffer, electrophoresed for 8% SDS-PAGE and transferred onto nitrocellulose membrane. The target protein was probed by adding the commercial anti-CEA monoclonal antibody(Santa Crutz, US) followed by the peroxidase-conjugated goat anti-mouse IgG(Pirece). The immunoreactive bands were developed and visualized using the ECL reagents kit(Santa Crutz, US) and the blots were exposed to Hyperfilm ECL film(Santa Crutz, US).

RESULTS

Optimization of the selection procedure

At each round of selection, we attempted to elevate the specificity of aptamer by stepping down the amount of inputted ssDNA pool(from 2000 pmol to 100 pmol), increasing the washing frequency and washing time after incubation with the target, and performing negative selection with the empty well before incubation with the target to decrease the non-specific binding. In addition, we controlled the cycles of PCR amplification at each round, to prevent the non-specific amplification of the target(***Fig 2***).

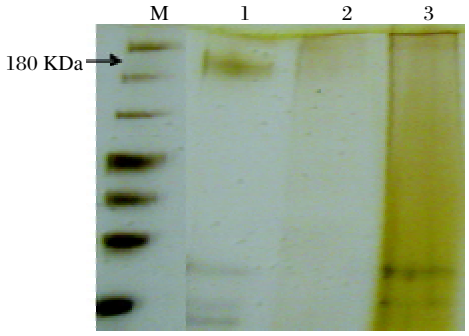


Lane 1-5: 8, 12, 15, 18, 22 cycles by PCR respectively

Fig 2 Polyacrylamide gel electrophoresis of dsDNA products at different cycle of PCR, silver staining

Recognition and binding of polyclonal oligobodies against purified protein

After seven round selection, the beads-conjugated polyclonal oligobodies were found efficiently bound with the purified CEA protein while the primary library showed no binding at all(***Fig 3***).



Lane 1:selected biotinylated-aptamer mediated CEA binding; Lane 2: beads alone; Lane 3: biotinylated unselected ssDNA pool incubated with protein

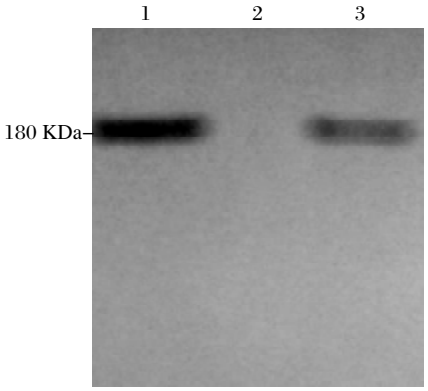
Fig 3 Silver staining SDS-polyacrylamide gel was used to analyze the biotinylated polyclonal oligobody mediated target purification.

Recognition and binding of polyclonal oligobodies against native protein

We further confirmed the binding capacity of the aptamer with the native CEA from the cellular extracts of LS174T. The result of immunoprecipitation demonstrated the specific binding of the biotinylated-aptamer against CEA while the primary ssDNA pool bound to nothing(***Fig 4***).

DISCUSSION

In 1999, it was suggested that aptamer, though still in its early stage, would become a strong competitor against antibody which has been developed for over three decades[22]. On the respect of tumor therapy, besides its inhibitory effects against the target proteins directly, aptamers conjugated with toxin or nanoparticle



Lane 1: CEA protein as the positive control while the Western blot was performed; Lane2: cell extracts incubation with unselected ssDNA pool; Lane3: cell extracts incubation with the selected aptamer

Fig 4 "Immunoprecipitation" of LS174T cell extracts with the biotinylated aptamer.

can facilitate the drug delivery with high efficiency and low toxicity[16,23,24]. Though a few tumor related aptamers have been reported before [16,17], none of them are available for widespread use in multiple types of cancer.

We have several reasons to choose CEA as a target protein in this study. An ideal tumor target antigen is suitable for variety of tumor type, with majority patients in each tumor type and highly-expressed on the cellular membrane while absent from the normal tissues[25]. As a broad-spectrum tumor associated antigen, CEA has been found highly expressed in a variety type of tumors while shows a more limited tissue expression in normal adults tissue. Furthermore, CEA links to the cell membrane through a glycosyl-phosphatidylinositol (GPI) anchor[26], and thus avoids the difficulty in delivering ligand into the cells. All these features made it an attractive molecule target for aptamer binding.

In the present study, the negative selection with the empty well and PCR cycles' control were performed at each round to increase the specificity of aptamer. After 7-rounds *in vitro* selection, the capability of aptamer to bind purified CEA protein was demonstrated by the ligand mediated purification. Considering the protein conformation might be different between the purified and native protein, we further verified the specific recognition and binding native CEA from the cells extracts by Co-immunoprecipitation using the biotinylated-oligobody. In our study we did not test the affinity of aptamer further as we thought a notable specificity of ligands be more significant for the cancer target therapy and diagnosis. This is coincident with a recent result of Carothers JM[27], they thought there was no inevitable correlation between the higher affinity and the more specifically of aptamer and signified the different way should be adopted to gain certain property of aptamer, affinity or specificity. As the present results came from an early work in our research, we will further investigate the sequences and the conserved second structure of selected aptamer in the next step.

In conclusion, this is the first time the CEA-specific aptamer was generated. The results in this study provided the preliminary evidence for further investigation and application of CEA-aptamer in the future. Given the great advantages of aptamer, one can expect a development of TAA-aptamer applications in the future.

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