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Selection of DNA aptamers against epidermal growth factor receptor with high affinity and specificity

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ABSTRACT

Epidermal growth factor receptor (EGFR/HER1/c-ErbB1), is overexpressed in many solid cancers, such as epidermoid carcinomas, malignant gliomas, etc. EGFR plays roles in proliferation, invasion, angiogenesis and metastasis of malignant cancer cells and is the ideal antigen for clinical applications in cancer detection, imaging and therapy. Aptamers, the output of the systematic evolution of ligands by exponential enrichment (SELEX), are DNA/RNA oligonucleotides which can bind protein and other substances with specificity. RNA aptamers are undesirable due to their instability and high cost of production. Conversely, DNA aptamers have aroused researcher's attention because they are easily synthesized, stable, selective, have high binding affinity and are cost-effective to produce. In this study, we have successfully identified DNA aptamers with high binding affinity and selectivity to EGFR. The aptamer named TuTu22 with K_d 56 ± 7.3 nM was chosen from the identified DNA aptamers for further study. Flow cytometry analysis results indicated that the TuTu22 aptamer was able to specifically recognize a variety of cancer cells expressing EGFR but did not bind to the EGFR-negative cells. With all of the aforementioned advantages, the DNA aptamers reported here against cancer biomarker EGFR will facilitate the development of novel targeted cancer detection, imaging and therapy.

1. Introduction

EGFR plays important roles in the physiological processes of cell growth, proliferation and differentiation. Glioblastomas have a certain extent of tolerance to radiation due to EGFR overexpression and increased radio-sensitivity has been observed with EGFR inhibition [1]. Abnormalities in EGFR can be responsible for the occurrence of cancer, diabetes, cardiovascular diseases and immunodeficiency. EGFR is overexpressed in many solid cancers, such as epidermoid carcimomas, malignant gliomas, lung, pancreas and breast cancers making it one of the most sensible targets for therapy.

Currently, most EGFR diagnostic and therapeutic strategies rely on anti-EGFR antibodies. Monoclonal antibodies, such as cetuximab and panitumumab, bind to the extracellular domain of EGFR and small-molecule inhibitors such as gefitinib, erlotinib and lapatinib, compete with ATP for binding to the intracellular tyrosine kinase domain of the receptor [2–5]. Patients receiving these treatments often show primary or acquired resistance to the inhibitors [6–8]. Thus, there is an urgent need to design new EGFR-targeting drugs for more specific and selective cancer therapy and this is why new strategies to overcome tyrosine kinase inhibitor (TKI) resistance are under active exploration.

Recently, an emerging new class of detective and therapeutic molecular probes have been composed of nucleic acid-based aptamers, which are short structured single stranded DNA, RNA, or modified nucleic acids isolated through SELEX [9,10]. Due to unique three-dimensional shapes, aptamers can bind targets such

Abbreviations: EGFR, epidermal growth factor receptor; SELEX, systematic evolution of ligands by exponential enrichment; RPTK, receptor protein tyrosine kinase; NSCLC, non-small-cell lung carcinoma; TKI, tyrosine kinase inhibitors; VEGF, vascular endothelial growth factor; ATCC, American Type Culture Collection; DMEM, Dulbecco's modified Eagle's medium; WB, washing buffer; nt, nucleotides.

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as small molecules, proteins, cells and even tissues with high affinity and selectivity [11–15]. In addition, aptamers possess many advantages over proteins as therapeutic reagents such as high stability, no immunogenicity, convenient synthesis, facile modification, rapid tissue penetration and low toxicity. Furthermore, aptamers can be designed as various chimeras with dyes, drugs and nanomaterials to generate more efficient and diversified molecular probes for sensing, imaging and targeted therapy [16–18]. In recent years, aptamers are being explored as potential therapeutics to target a distinct disease or tissue in a cell-type specific manner [19–21]. There has been only one aptamer (Macugen) against vascular endothelial growth factor approved by the FDA and it is for treatment of age-related macular degeneration [22].

To date, RNA-aptamers have been selected against the purified extracellular domain of EGFR and also used for gold nanoparticle delivery to cancer cells [23]. When this aptamer is applied to EGFR-expressing cancer cells, it inhibits EGFR-mediated signaling pathways and results in selective cell death [24]. Unfortunately, RNA aptamers are easily degraded by nucleases, which limit their application in clinical settings unless expensive modifications, for example, with 2′-F-pyrimidine, are used. Compared with RNA aptamers, DNA aptamers are less expensive and easier to use and store, making them a great candidate for in vitro and in vivo research applications [25].

Herein, we use the purified extracellular domain of EGFR as the target and have generated a group of DNA aptamers recognizing it specifically. Among the aptamers, we chose TuTu22 for the following research. Flow cytometry analysis results indicated that the TuTu22 aptamer was able to recognize EGFR-positive cancer cell lines, U87, U251 and A431 with strong binding affinity and excellent selectivity but did not bind to EGFR-negative Jurkat cells.

2. Materials and methods

2.1. Cell lines and cell culture

Human glioblastoma cell lines U251-MG, U87-MG and human epidermal carcinoma cell line A431 were purchased from American Type Culture Collection (ATCC). All cell lines were cultured at 37 °C in a humid atmosphere with 5% CO_2. The growth medium for U251-MG, U87-MG and A431 cells was composed of Dulbecco's modified Eagle medium (DMEM, Hyclone) with high glucose (4.5 g/L) supplemented with 10% fetal bovine serum and penicillin–streptomycin. Jurkat cells were cultured in RPMI-1640 supplemented with 15% fetal bovine serum (Hyclone) and penicillin–streptomycin. Cells were washed before and after incubation with DNA library during selection with washing buffer.

2.2. Buffers

The washing buffer (WB) contained 5 mM $MgCl_2$ in phosphate buffered saline (PBS, pH = 7.4). The binding buffer was prepared by adding yeast tRNA (0.1 mg/mL) in washing buffer in order to reduce nonspecific binding.

2.3. Primers and SELEX library

Polymerase chain reaction (PCR) for the synthesis of labeled double-stranded DNA sequence utilized a sense primer labeled at the 5′-end with FITC and an antisense primer labeled with biotin at the 5′-end. The sense and antisense primers, 5′-FITC-TAC CAG TGC GAT GCT CAG-3′ and 5′-Biotin-GTC AAC CGA ATG CGT CAG-3′, respectively, were synthesized using standard phosphoramidite chemistry, purified by RP-HPLC to remove truncated DNA fragments and desalted using NAP-5 desalting columns (GE healthcare). The initial library (5′-TAC CAG TGC GAT GCT CAG (N) 40 CTG ACG CAT TCG GTT GAC-3′, 76mer) synthesized by Sangon Biotech (Shanghai) contained a central randomized sequence of 40 nucleotides (nt) flanked by 18 nt primer hybridization sites.

2.4. Protein and preparation

EGFR was purchased from Sino Biological Inc. (Beijing). EGFR was incubated with Ni-beads (GE Healthcare) in binding buffer (phosphate buffer with 40 mM imidazole) for one night at 4 °C followed by three washes with PBS buffer. The Ni-beads complexed with EGFR (EGFR-beads) were kept at 4 °C in PBS buffer before using.

2.5. Protein-SELEX procedure

For the first round of selection, 5 nmol of initial ssDNA library was mixed thoroughly with 500 μL binding buffer, and heated at 95 °C for 5 min and cooled immediately on ice for 10 min. EGFR-beads (roughly 150 pmol of protein) were incubated with the ssDNA library at 37 °C for 30 min on a rotary shaker. After incubation, EGFR-beads containing binding sequences were washed with washing buffer and added to the PCR cocktail to be amplified. In preparative PCR, the optimum cycle of amplification was applied to produce more PCR products for the preparation of ssDNA required for the next round of selection. Negative selection was applied after the second round. Before incubation with EGFR-beads, the ssDNA library was incubated with Ni-beads for 10 min to remove the ssDNA strands which may bind to Ni-beads. The nonbinding ssDNA was collected and incubated with EGFR-beads in the positive selection. In order to acquire sequences with high affinity and specificity, we enhanced wash strength gradually with washing buffer (from 200 to 1000 μL) and the frequency of washes (from 2 to 6 times). Additionally, the negative control (Ni-beads) was also gradually increased from 10 to 40 min to reduce the nonspecific binding. After 11 rounds of selection, the selected ssDNA pool was PCR using unmodified primers for cloning and sequencing by Sangon Biotech (Shanghai).

2.6. Flow cytometry analysis

To monitor the enrichment of aptamers after selection, 1×10^5 EGFR-beads or control Ni-beads were incubated with FITC-labeled ssDNA in binding buffer (C_f = 200 nM, V_f = 200 μL) at 37 °C for 30 min. The fluorescence intensity of the beads was monitored with a FACS Aria cytometer by counting 10,000 events. The FITC-labeled unselected ssDNA library acted as the negative control. To evaluate the binding affinities of aptamers toward cells, U251 cells or other positive cells (1×10^5 cells) were incubated with various concentrations of FITC-labeled aptamers in 200 μL of binding buffer at 37 °C for 30 min. Cells were washed twice, suspended in 200 μL binding buffer and analyzed by flow cytometry. The FITC-labeled initial library acted as negative control to determine the background binding. All binding assays were performed three times. Using SigmaPlot software 12.5 (Jandel Scientific), the K_d of the aptamer-cell interaction was obtained by using the equation:

$$Y = B_{max}X/(K_d + X)$$

To investigate the specificity of aptamers for molecular recognition, EGFR-positive cell lines, U251, U87, A431 and Jurkats as control were applied for binding specificity assays with FITC-labeled aptamer candidates and unselected initial library or random sequence by flow cytometry.

3. Results

3.1. Selection of DNA aptamers to recognize EGFR

To identify DNA aptamers that can recognize EGFR, the C terminus of EGFR was fused with Ni-beads to facilitate the attachment of the protein to solid supports. The EGFR-beads were used as the positive target in SELEX while the Ni-beads served as a negative control to remove the non-specific surface binding sequence. The progress of the selection process was monitored by flow cytometry [26,27]. DNA probes were gradually enriched with better binding affinity to targets as the number of selection cycles increased (Fig. 1). After 11 rounds of selection, an evident increase in fluorescence intensity was observed in flow cytometry in the presence of EGFR-beads, but no significant change in fluorescence intensity was observed with EGFR-free Ni-beads (Fig. 2). These results suggest DNA aptamers specifically recognizing EGFR were enriched during the selection process.

3.2. Identification of selected aptamer for EGFR

After 11 rounds of selection, 22 oligonucleotides were analyzed by DNA sequencing. The data were analyzed by sequencing analysis software Clustal X 2.0.3 [28].To confirm whether these sequences were indeed aptamers, a series of experiments were performed to investigate their binding affinity and specificity toward EGFR. Four sequences (TuTu22, TuTu21, TuTu20 and TuTu19) from different sequence families were chosen (Table 1) and chemically synthesized on a DNA synthesizer for further characterization. TuTu22 was selected for further research. Flow

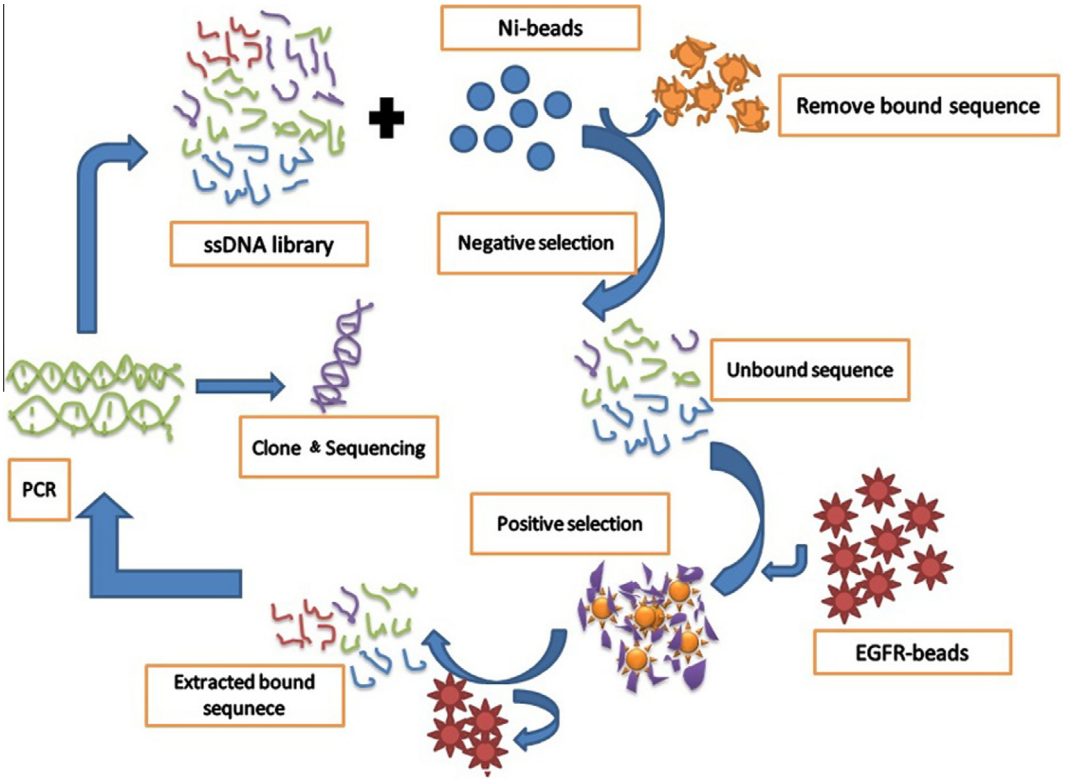

Fig. 1. Schematic of systematic evolution of DNA aptamers against EGFR.

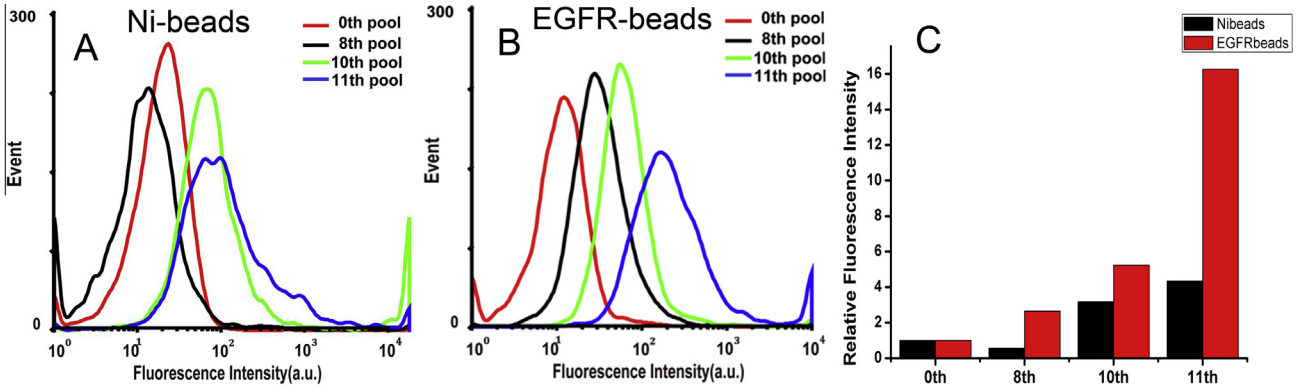

Fig. 2. Enrichment of DNA sequences that bind to EGFR. (A) Flow cytometry assay to monitor Ni-beads (control beads), (B) binding of enriched pools with EGFR-beads (target beads) and (C) the quantity of sequences in the 11th pool had an obvious difference between EGFR-beads and Ni-beads.

Table 1
Sequences of aptamers identified.

Name	Sequence	K_d (nM)
TuTu22	5′-TACCAGTGCGATGCTCAGTGCCGTTTCTTCTCTTTCGCTT TTTTTGCTTTTGAGC A TGCTGACGCATTCGGTTGAC-3′	56 ± 7.3
TuTu21	5′-TACCAGTGCGATGCTCAGCCTACACGCCACCTTTTCTTTT CTATCCTTCTTTTACGTGCTGACGCATTCGGTTGAC-3′	78 ± 4.9
TuTu20	5′-TACCAGTGCGATGCTCAGTCCGTATGCTCAGTCTTACTATA TACTTCTCTGCTCGTTGCTGACGCATTCGGTTGAC-3′	85 ± 9.4
TuTu19	5′-TACCAGTGCGATGCTCAGACCAGTTTCAACATCCTCTCTT TTTCTTTCTTCTCTTTCGCTGACGCATTCGGTTGAC-3′	74 ± 6.6

cytometry results revealed that TuTu22 was able to bind to EGFR with high affinity and not to Ni-beads, confirming the chosen sequence was an aptamer against EGFR.

In order to test whether the selected EGFR aptamer was able to bind to EGFR on the surface of human cells from different histopathological origins, we used flow cytometry to test the interaction of the aptamer with EGFR-positive cell lines. We chose the epidermoid carcinoma cell line A431, human glioblastoma cell lines U251 and U87, EGFR-positive cell lines and Jurkats as EGFR-negative (control) cell line (Fig. 3). TuTu22 was able to bind the two positive cell lines, but not Jurkats. These results indicate the aptamer is selective against recombinant EGFR and can also recognize the native EGFR protein on live cell membranes.

4. Discussion

EGFR, a member of the ErbB/HER family of Type I transmembrane tyrosine kinase receptors, has been implicated in numerous cancers, including breast cancer, lung cancer, colorectal cancer, and head and neck squamous cell cancers [29–33]. Overexpression of ErbB receptors, especially of EGFR, has been shown to cause uncontrolled cell proliferation, leading to malignant tumors [29]. In addition, increased activation of EGFR has been linked to decreased apoptosis, increased metastasis and increased angiogenesis, all hallmarks of cancerous cells [30]. Thus, anti-EGFR therapy is a major area of research for anti-cancer therapeutics. Current anti-EGFR agents include monoclonal antibodies against EGFR (Cetuximab) and tyrosine kinase inhibitors (Gefitinib) [29,30,33]. Unfortunately, antibodies have large size and high immunogenicity affecting their efficacy. EGFR RNA aptamers face disadvantages of instability and high cost of modification. Thus, it is urgent to develop alternative, stable, cheap and efficient ligands against EGFR for cancer imaging, diagnosis and therapy. Recently, DNA aptamers of EGFRVIII, which is one of the mutations of EGFR, were selectively targeted in the human glioblastoma cell line U87-MG

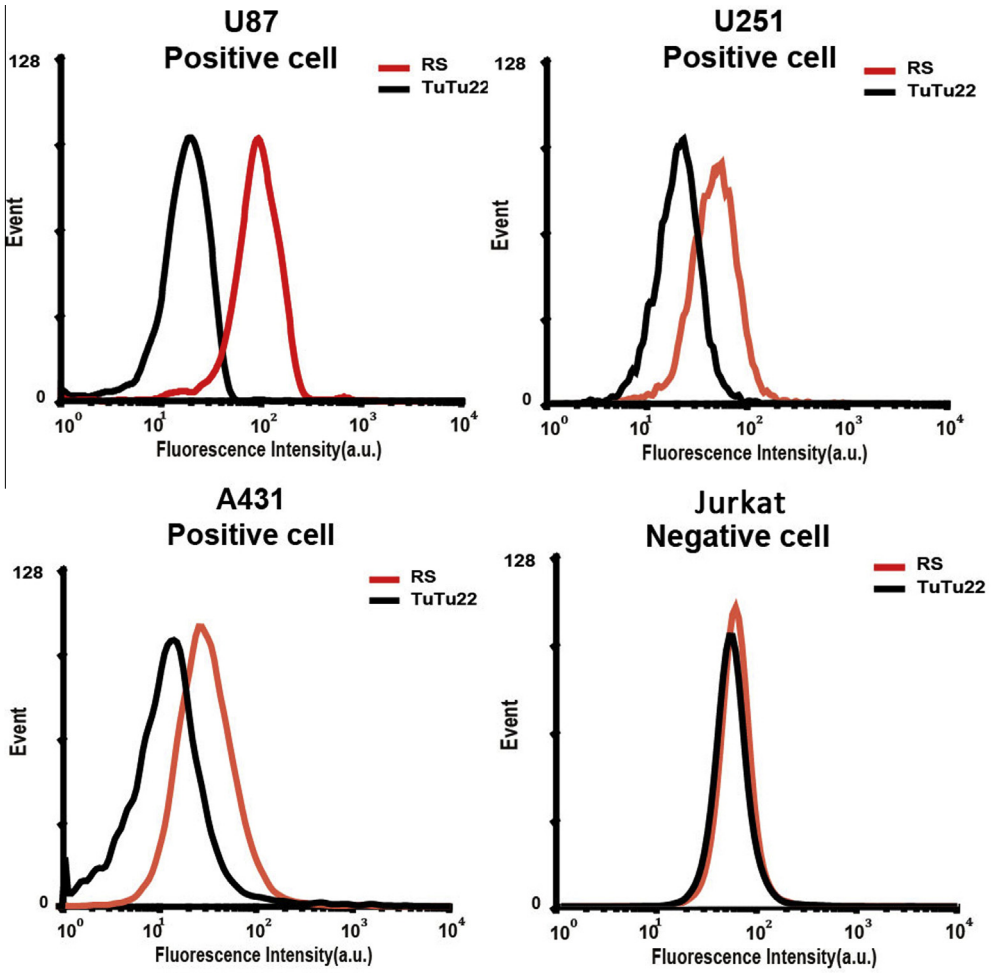

Fig. 3. Binding of the TuTu22 aptamer or random sequence (RS) with different cell lines, including three EGFR-positive cell lines, U87, U251, A431 and EGFR-negative cells, Jurkats.

[34]. The overexpression of EGFRVIII can lead to sustained activation of EGFR and activate abnormal signaling pathways. The role of EGFRVIII in cancers may depend on EGFR itself. In this study, we utilize an effective and convenient way to determine molecular probes that recognize EGFR. First, we obtained DNA aptamers that can bind EGFR after the 11th cycle of selection with K_d of 56 ± 7.3 nM. Our selected aptamers can also bind living cells overexpressing EGFR. Therefore, these data suggest that the EGFR aptamers indeed bind specifically to EGFR on the cancer cell surface but not to other molecules present on the cell surface.

In summary, our results indicate we can obtain selective, high affinity DNA aptamers that can bind membrane protein. It is simpler and more convenient to evolve specific cell binding aptamers as compared with cell-SELEX. Using this strategy, we have successfully generated a series of DNA aptamers against cancer biomarker EGFR. With the advantage of small size, easy synthesis, good stability, high binding affinity and selectivity, this DNA aptamer against the universal cancer biomarker EGFR will facilitate the development of novel targeted cancer detection, imaging and therapy.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgments

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