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RESEARCH ARTICLE

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Selection and identification of an ssDNA aptamer to NB4 cell

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Abstract

This study was to find the aptamers with high affinity and specificity binding to acute promyelocytic leukemia (APL) NB4 cell line.

These aptamers targeted NB4 cells were selected from a random single-stranded DNA (ssDNA) library of systematic evolution of ligands by exponential enrichment (CELL-SELEX). The binding rate of FITC-ssDNA library and NB4 cells was monitored using flow cytometry and fluorescence microscope. After cloned and sequenced, the structure, specificity, and affinity of these candidate aptamers were further analyzed.

After a total of 19 rounds of selection, the ssDNA library was enriched and the BR (19.9%) of the 16th round was 12 times of the first round (1.6%). Three enriched aptamers were obtained from 21 positive clones of the 16th round, and the predicted secondary structures of these aptamers were mainly stem-loop. The aptamer CX9 had the highest affinity, and the equilibrium dissociation constant (Kd) was 16.2 nM. The fluorescence intensity of CX9 binding to NB4 cells was stronger than HL60 and K562 cells under fluorescence microscopy.

The study indicates that aptamer CX9 exhibits high affinity and specificity with NB4 cells and lay a foundation for the rapid diagnostic method to detect APL with fluorescence-labeled aptamer.

KEYWORDS
APL, aptamer, CELL-SELEX, leukemia, NB4 cell

1 | INTRODUCTION

Acute promyelocytic leukemia (APL) is a unique subtype of acute myeloid leukemia and accounts for 10%–15% of all acute myeloid leukemia.[1] APL is characterized by 15;17 chromosome translocation with breakpoints within promyelocytic leukemia (PML) gene on 15 and the retinoic acid alpha receptor gene (RARa) on 17. The translocation leads to the formation of PML-RARA fusion protein, which interference the structure of POD and block myeloid differentiation.[2] In the past three decades, the treatment of all-trans retinoic acid and (ATRA) and arsenic trioxide (ATO) has been expected to cure the majority of APL patients and improved the long-term survival to 80–90% or higher.[3,4] However, early death with hemorrhage and the risk of relapse remain to be a challenge.[5]

Early detection and diagnosis of new and recurring patients are vital. Currently, immunophenotyping via immunohistochemistry and flow cytometric analysis plays a pivotal role in the detection and diagnosis of APL. However, it is nonspecific for the surface biomarkers used for immunophenotyping APL. And it is difficult to determine the disease status, when there are only a few leukemic cells with similar immunophenotypic to normal cells in relapsed patients. In addition, fluorescent labeled antibodies are hard to preserve and expensive, which limit their application for the primary laboratory. Therefore, it is necessary to establish a new strategy to detect APL and discover new biomarkers.

Aptamers, which are synthetic single strand oligonucleotides, can bind their target with high specificity and affinity. They are screened from large oligonucleotide pools by the SELEX technology. Their characteristics are superior to antibodies in many aspects. They may substitute antibodies to determine the levels and inhibit the bioactivities of their target. There are broad application prospects of aptamers in the treatment of varied diseases. Cell-SELEX uses intact living cells as a screening target. Compared to SELEX based on known targets, its major advantage is that it can simultaneously target a variety of membrane proteins with natural conformations as target molecules during the screening process. And it is not necessary to know the types of cell surface proteins and their expression levels in advance. Aptamers screening for tumor cells has brought new ideas and methods for drug screening, clinical diagnosis, disease treatment, and basic medical research.

In previous studies, we have selected aptamers targets staphylococcal enterotoxin A (SEA) and B (SEB), which have been proven to bind these toxins and inhibit their biological activity in vivo and in vitro. In this study, NB4 cells were used as the target cell and specific aptamers were selected by cell-SELEX to provide new molecule candidates for the targeted diagnosis and treatment of APL.

2 | MATERIAL AND METHODS

2.1 | SELEX library and primers

The ssDNA library consisted of a random sequence of 40nt as a central region and two fixed 18nt as primer hybridization regions. All oligonucleotides were synthesized and purified by Invitrogen (Guangzhou, China). The sequences of library and primers are as follows:

Library:5′-ATACCAGCTTATTCAATT–N40–AGATAGTAAGTGCAATCT-3′.

"P1" forward primer: 5′-ATACCAGCTTA TTCAATT-3′.

"P2" reverse primer: 5′-AGATTGCACTTACTATCT-3′.

"P3" forward primer: 5′-FITC-ATACCAGCTTATTCAATT-3′.

"P4" reverse primer: 5′-Biotin-AGATTGCACTTACTATCT-3′.

"P5" forward primer: 5′-TAMRA-ATACCAGCTTATTCAATT-3′.

2.2 | Cells and cell culture

Acute myeloid leukemia (AML) cell lines NB4 cells, HL60 cells, and CML K562 cells provided by the blood institute of Union Hospital of Fujian Medical University. All cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS and 100 units/ml penicillin-streptomycin (Invitrogen). Cells were washed with phosphate-buffered saline (Invitrogen) three times and then resuspended in the selection buffer. The selection buffer was prepared by adding 4.5 g/L glucoses, 5 mM MgCl2, 0.1 mg/ml yeast tRNA (Invitrogen), and 1 mg/ml bovine serum albumin (BSA) (Amresco) into PBS. All cells were incubated in an incubator with 37℃ and 5% CO_2.

2.3 | Cell-SELEX process

The selection processes were performed similarly as described before.[1319] The initial ssDNA pool (2000 pmol) was dissolved in 1 ml of the selection buffer, denatured by heating at 95℃ for 5 min, and placed on ice for 10 min immediately.

Firstly, 4× 106K562 and HL60 cells were incubated with the initial DNA pool at 37℃ for 30 min with shaking. Next, the supernatant was used for the first round selection, which was incubated with 6×10^6 NB4 cells at 37℃ for 60 min with shaking. After the incubation, unbound ssDNA supernatant was removed and the cells were washed with washing buffer (4.5 g/L glucose, 5 mM MgCl2, PH 7.5) 2–5 times. The washing time was increased with selection round. Finally, cells with bound ssDNA were resuspended with 500 μl ddH_2O, followed by incubation at 94℃ for 10 min. Then, the bound ssDNA was used as templates for amplified with P3 and P4 primers (5 min at 94°C, 15 to 30 cycles of 30S at 94°C, 30S at 57°C, and 30S at 72°C, followed by 5 min at 72°C). DNA products of FITC-labeled sense and biotin-labeled antisense were synthesized. All PCR products were purified using a Biospin gel extraction kit (Bioer Technology, Hangzhou, China). The M-280 streptavidin Dynabeads (Dynal) was used to separate the biotinylated strands from the FITC-labeled strands based on the manufacturer's protocol. The FITC-labeled ssDNA was used for binding assay and the next round library.

In order to obtain aptamers with high affinity, the selective pressure was increased by reducing the target cell number, ssDNA concentration, and incubation time, and by increasing washing times. To increase the specificity of aptamers, the mixed liquor of K562 and HL60 was used for counter selection.

2.4 | Cloning, sequencing, and bioinformatics analysis

After rounds of cell-SELEX selection, the affinity analysis showed the library was enriched. The enriched ssDNA was amplified using unlabeled P1 and P2 primers and then were purified and cloned into pGEM-T vector (Promega, Madison, WI, USA).

After being transformed into Escherichia coli DH5, the cultured colonies were chosen randomly and sequenced by Huada Genomics Institute (Beijing, China). The aptamer sequences were analyzed by Chromas software, and the secondary structure was predicted by Dnaman.

2.5 | Flow cytometric analysis of aptamer binding to target cells

Flow cytometric analysis was used to monitor the enrichment of the ssDNA library every three rounds. FITC-labeled ssDNA library (50 pmol) or selected individual aptamers (50 pmol) was then incubated with 1×10^6 cells in 500 μ L of binding buffer at 37°C for 30 mins. After

incubation, the cells were washed three times with the washing buffer. The fluorescence intensity was determined with a FACS Aria SORP flow cytometer (BD Bioscience, USA) by counting 10,000 events. The FITC-labeled initial library was used as a negative control.

The affinity of the selected individual aptamers was also analyzed as described above but with varying concentration of ssDNA aptamer (10 to 150 nM/L) and a constant amount of NB4 cells (6×10^6). The GraphPad Prism 6.0 software was used to calculate the Kd value via nonlinear regression analysis.

2.6 | Fluorescence microscope assays for aptamers

Fluorescence microscope IX81 (Olympus, Japan) assay was performed to determine the specificity of each selected aptamer visually. The best candidate aptamer with TAMRA-labeled was incubated with NB4 cells (1×10^6) and the mixture of HL60 and K562 cell (each 0.5×10^6), respectively, in 500 μl binding buffer at 37℃ for 30 min. After washing three times with washing buffer, the cells were observed by fluorescence microscope.

3 | RESULTS

3.1 | The enrichment of ssDNA library

The enrichment of the ssDNA library was monitored by flow cytometric every three rounds. The binding rate of ssDNA libraries was gradually increased with the number of screening rounds until the 16th round. As shown in Figure 1, there was no further increase after additional 3 rounds selection. The result indicated that the oligonucleotides with high binding affinity to NB4 cells had already been enriched at the 16th round.

3.2 | Cloning, sequencing and secondary structure of selected Aptamers

The enriched library of the 16^{th} round was chosen to be amplified and cloned. Two positive clones were selected and sequenced randomly. The result showed three enriched aptamers. There were two CX1, three CX5, and sixteen CX9 sequences, respectively. Details of three selected aptamers are shown in Table 1. The predicted secondary structures of three aptamers are mainly stem-loop (Figure 2).

3.3 | Binding rate of these selected aptamers

Flow cytometric analysis was also performed to determine the binding affinity of selected aptamers to NB4 cell. The analysis revealed that the BRs of CX1, CX5, and CX9 reached 9.7%, 12.6%, and 17.2%, respectively.

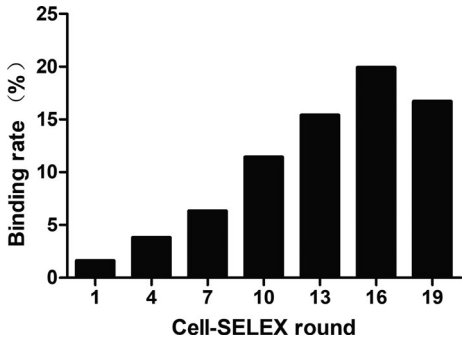

FIGURE 1 The binding rate of aptamer pool in the SELEX experiment

3.4 | Affinity and specificity of CX9 aptamer

The best candidate aptamer CX9 was used for further analysis. The FITC-labeled CX9 aptamer was diluted with the concentration range of 0–150 nm. Then, a fixed amount of live NB4 cells (1×10^6) was combined with the various concentrations aptamers in the binding buffer, and the fluorescence intensity was measured by flow analysis. These data were obtained and analyzed by GraphPad.Prism. V4.03 software, and the saturation combination curve was obtained, as shown in Figure 3. It could be seen that the dissociation constant Kd value of the aptamer CX9 is 16.2 ± 3.22 nM.

TAMRA-labeled aptamer CX9 was used to verify the specificity with NB4 cells. As show in Figure 4, the scattered, light, red fluorescence could be seen under fluorescence microscopy when aptamers incubated with NB4 cells. While incubating with HL60 and K562 cells, there was no obvious red fluorescence. The result showed that CX9 aptamer could recognize the target cells specifically.

4 | DISCUSSION

Acute promyelocytic leukemia is distinguished by a large number of hyper granular promyelocytes in the blood and bone marrow of patients. Although most of the APL patients have been induced complete remission, it is still urgent to detect rare tumor cells for early diagnosis. The NB4 cell line, which is characterized by t (15;17) translocation and highly sensitive to the differentiation induced by ATRA and ATO, is an ideal cell for studying the proliferation and differentiation of APL. In this study, we identified three aptamers and their structure targets NB4 cells. Further analysis revealed that the aptamer CX9 could bind NB4 with high sensitivity and specificity.

Aptamers are short ssDNA or ssRNA, which bind extensive targets including metal ions, bacteria, proteins, and cells with their three-dimensional structure.[6-8] RNA could form abundant 3D structures and was used as the initial oligonucleotide pools previously.[9] Currently, due to their high stability, DNA pools are more widely used for the selection of new aptamers. In this study, a total length of 76nt ssDNA library was chemically synthesized, consisting of a random middle 40nt sequence flanked by a 18nt fixed sequence. The

TABLE 1 The sequence, dG, BR, and Kd of clone aptamers

Aptamer	Random sequence of the selected aptamers	dG kcal/mol	BR%	Kd nM
CX1	CGTCGTGCTATAATGTCTAACCCCGGCAAGTTTTCCATCG	−0.62	9.7	n.d.
CX5	CGCAAATGGGTTTTTTATTCTTTTTGGATTTTGGGTGTGG	−0.32	12.6	n.d.
CX9	CCATATGTGAGTTTCTTTTTTTGTTTCTCTTGGGCGGGCG	−1.33	17.2	16.2

Abbreviations: dG, free energy; BR, binding rate; Kd, dissociation constant; n.d., not determined.

FIGURE 2 The predicted secondary structures of three enriched aptamers

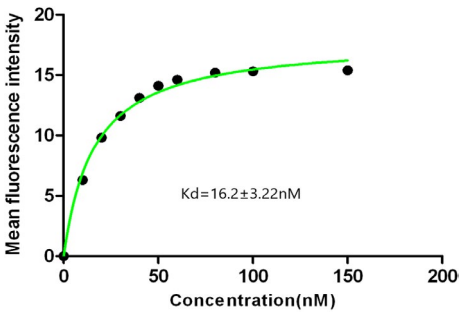

FIGURE 3 Binding affinity of the aptamer CX9 with NB4 cells

library, with approximately 10^{14}–10^{16} random ssDNA, was applied to generate aptamers against SEA in our previous study successfully.

CELL-SELEX technology, which is used for developing aptamers specific to whole cells, has been performed to screen aptamers binding to diverse tumor cells, such as prostate cancer,[10] breast cancer,[11,12] ovarian cancer cell,[13] gastric cancer,[14] and liver cancer.[15] Cell-SELEX is carried out in the native state of the target cell without any prior knowledge of the membrane protein, which not only enhances the success of screening, but also has the potential to discover new cell biomarkers. Besides, the optimization of screening process plays a crucial role to success.[16] Herein, with the selection rounds, we reduced the pool concentration, the number of PCR cycles and incubation time and increased the washing times to enhance the pressure and save time.

In order to reduce the nonspecific ssDNA, k562 and HL6 cells were used for counter selection since the second round. The cells status is also vital to enrich target-specific aptamers efficiently. More than 5% dead cells may lead to the failure of the experiment.[16] Therefore, during the selection process, the experimental environment was strictly controlled to ensure state the cells. The living cells were guaranteed to free from pollution and maintain a stable state by using a low speed and low temperature centrifugation to separate the bound or unbound ssDNA with NB4 cells.

Nucleic acid not only takes biological genetic information, but also has the structure information. ssRNA or ssDNA can form structures with high thermal stability like stem ring, convex ring, fake knot, G-four polymers to embed the crack of target molecules, and bind to target cells. In our study, a total of 22 clones from the 16th round were chosen to identify the enriched specific sequences and predict their secondary structure. The sequencing results showed that one sequence had bimodal, which might be due to two or more T-vectors transformed to one bacterium. Other candidates were classified into three families based on the same DNA sequences and named by the debut candidates. Dnaman software was used to predict the secondary structure of three aptamers. Simulated diagram displayed that these secondary structures of the three aptamers were mainly circle and convex ring, which might be the spot of aptamers combined with target cells. Each aptamer contained an unclosed semicircle with different size and position, which was due to the common fixed sequence on both sides.

These BRs of the three aptamers with NB4 living cells were further analyzed by flow cytometry. It showed that CX9 had the highest binding rate of 17.2%. In general, the secondary structure with lower free energy was more stable. Besides, the number of repeats was considered important in preselecting potential aptamers. CX9 with the lowest free energy and high BR also had the absolute

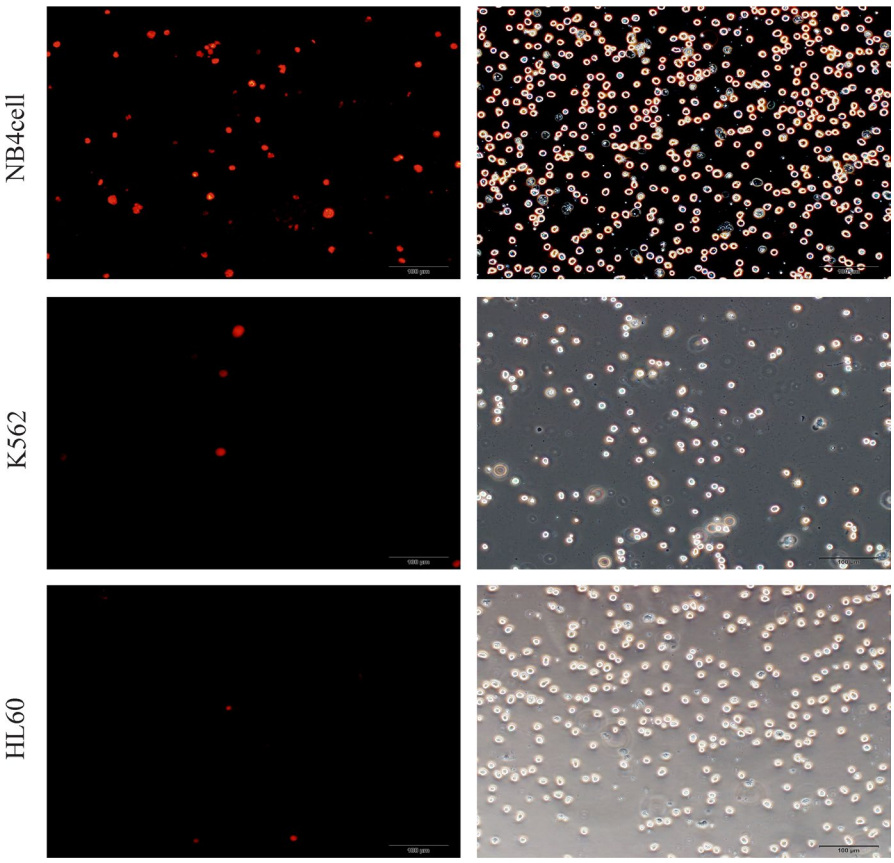

FIGURE 4 Specificity analysis of TAMRA-labeled aptamer CX9 with NB4 cell, K562, and HL60

predominance in the number of repeats, suggesting that CX9 might be the ideal candidate. Therefore, it was chosen for further characterization of its affinity and specificity with NB4 cell. The dissociation constant Kd value of the aptamer CX9 was as low as 16.2 nM, indicating a high affinity CX9 with NB4 cells.

FITC-labeled ssDNA was used in the SELEX process, and fluorescence microscope was also performed to observe the combination of the ssDNA pools and NB4 living cells. However, there were more cluster nonspecific green light with lower brightness under the fluorescence microscope, which was called spontaneous fluorescence.[17] Therefore, TAMRA was chosen as an alternative fluorescence to avoid cell spontaneous fluorescence when we analyzed the specificity of CX9 with NB4 cells. The results showed CX9 could recognize the NB4 cells specifically and rarely binding to K562 cell and HL60 cell.

With the advanced in aptamers selection and modification, a number of aptamers have been on the road to clinical application. Based on their unique characteristics, such as lack of immunogenicity, small molecular weight, chemical synthesis, and easily modification, high thermal stability, and better tissue penetration, aptamers hold great potential for cancer imaging, diagnosis, and therapy. In conclusion, in the study, we successfully select an aptamer CX9 targeting NB4 cell with high affinity and specificity and lay a foundation for the rapid diagnostic method to detect APL with fluorescence-labeled aptamer. Future studies are still needed to validate the target molecule and function of CX9 to NB4 cell.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All the data that related to this study are available from the corresponding author upon reasonable request.

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