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Original Article

Identification of a novel molecular probe for recognition of human osteosarcoma cell using the cell-SELEX method

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Abstract: Osteosarcoma is the most common primary malignant bone tumor in adolescents and young adults. The lack of specific probes for human osteosarcoma hinders the early diagnosis and treatment of metastatic disease. In the present study, we have designed a novel aptamer using the cell-based Systematic Evolution of Ligands Exponential Enrichment (cell-SELEX) technique that specifically recognizes the U-2 OS human osteosarcoma cell line. Candidate aptamer families were identified through nine rounds of selection followed by sequence analysis and fluorescent labeling in addition to specific binding to U-2 OS cells. We identified one aptamer that showed high affinity and specificity to target cells, but did not recognize non-osteosarcoma negative control tumor cell lines. Moreover, we show that the selected aptamer can effectively be used as a molecular probe for specific recognition of clinical osteosarcoma samples. The generation of aptamer libraries can be used not only for the specific diagnosis of osteosarcoma, but also to build a platform for developing probe-carrier-antitumor drugs complexes and targeted therapies for osteosarcoma.

Keywords: Cell-SELEX, osteosarcoma U-2 OS, aptamer

Introduction

Osteosarcoma is the most common malignant tumor of bone, which develops from mesenchymal stem cells through endochondral ossification. Osteosarcoma is the most prevalent aggressive pediatric bone cancer, occurring in 5% of children and adolescents [1]. As a highly aggressive tumor, the prognosis of osteosarcoma is extremely poor, with the possibility of pulmonary metastasis occurring within several months. Amputation of the affected limb is not always curative, and the 3-5 year survival rate after amputation is only 5-20% [2, 3]. Currently, clinical diagnosis of osteosarcoma relies primarily on morphological evaluation in combination with serology and iconography. Increasing evidence has demonstrated that advances in the diagnosis and treatment of osteosarcoma can improve the clinical outcome in OS patients, however, the overall survival of patients with osteosarcoma remains unsatisfactory [4, 5]. Therefore, it is imperative to identify novel biomarkers for predicting osteosarcoma prognosis that can accurately identify the biological characteristics of tumors, improve therapeutic strategies, and accurately predict clinical outcomes [6, 7].

Novel molecular probes, classified as aptamers, have emerged as molecular biomarkers for diagnosis and treatment of multiple diseases. Aptamers are single-stranded DNA or RNA oligonucleotides that bind to various targets ranging from small organic molecules to proteins [8, 9]. Aptamers are synthesized using molecular biology methodologies, namely Systematic Evolution of Ligands Exponential Enrichment (SELEX) [10]. Single-stranded oligonucleotide libraries can theoretically form three-dimensional structures that themselves can combine with various types of target molecules. A high-

flux (10^{15}-10^{18}) oligonucleotide library contains all possible spatial sequences. Cell-SELEX is based on an aptamer selection process that generates cell-specific aptamers using whole cells as targets by exploiting the differences among two given cell lines to enable differentiation between them at the molecular level [11, 12].

The high affinity and specificity achieved by the combination of aptamer and target material reflect their ability to spontaneously curl to form a 3D shape or conformation as well as to undergo chemical modification *in vitro*. The advantages of aptamers include easy synthesis and preservation of chemical modifications that is retained throughout repeated cycles of denaturation and renaturation. These properties make aptamers versatile for both preparation and application in the field of diagnostics and treatment of illnesses as well for rationale design of novel therapies [13, 14]. At present, there are no reports on the development or use of biological probes for osteosarcoma.

In this study, human osteosarcoma cells U-2 OS were used as target cells, and randomly sequenced DNAs derived from *in-vitro* synthesis were assigned as the selected single-stranded DNA library. Cell-SELEX technology was applied to select for aptamers that could bind with high specificity to U-2 OS osteosarcoma cells in order to generate an aptamer library suitable for diagnosing osteosarcoma with high specificity.

Materials and methods

In vitro culture of tumor cells

Three tumor cell lines were maintained in liquid nitrogen until thawed for *in vitro* studies. The U-2 OS human osteosarcoma cell line was cultured in McCoy's 5A medium containing 10% fetal bovine serum (FBS) (Invitrogen, USA). The SGC701 gastric cancer cell line and MCF-7 mammary cancer cells were cultured in RPM1-1640. Cells were plated into 10 mm × 10 mm dishes and were grown for 24 hrs to a density of 5 × 10^6 cells/dish prior to the experiment. The human osteosarcoma U-2 OS cell line was used as a positive control, while the gastric cancer cell line SGC7901, MCF-7 breast cancer cell line and the HT-1080 human fibrosarcoma cell line served as negative controls.

Preparation of DNA oligonucleotide library

A single-stranded random oligonucleotide library (ssDNA) was synthesized by Sangon Biotech (Shanghai) Co., Ltd. The starting DNA library consisted of 40 nucleotides of random sequences, which were flanked by constant sequences in the following sequence context: 5'-ATC CAG AGT GAC GCA GCA 40 (nt) TGG ACA CGG TGG CTT AGT-3'; A fluorescein isothiocyanate (FITC)-labeled 5'-primer (5'-FITC-ATCCAG-AGTGACGCAGCA-3') and a biotinylated (Bio) -labeled 3'-primer (5'-Biotin-ACTAAGCCACCGT-GTCCA-3') were used for PCR synthesis of double-stranded DNA molecular probes in order to separate single ssDNA. The DNA pools were combined with binding buffer [1 L Dulbecco's phosphate buffered saline (DPBS) containing 4.5 g of glucose, 1 g BSA (Sigma), 100 mg yeast tRNA (Invitrogen) and 5 ml of 1 M $MgCl_2$] heated to 95°C for 5 minutes, and cooled to room temperature during every round of selection.

In vitro elutriation of DNA aptamers of human osteosarcoma U-2 OS cells

The cell-based systematic evolution of ligands by exponential enrichment (c-SELEX) was carried out as previously described [15]. Briefly, U-2 OS osteosarcoma cells were cultured to a density of 5 × 10^6 cells/dish (first round selection), washed three times with wash buffer [1 L DPBS containing 4.5 g of glucose and 5 ml of 1 M $MgCl_2$], and incubated with the cold ssDNA library pool (10 nmol of ssDNA library for the first round of selection, and 1000 nmol for subsequent rounds of selection) in 1 ml of binding buffer at 37°C with shaking at 50 rpm for 1 h. The cells were then rinsed three times each with wash buffer and DNAse-free water. Cells were collected from the culture dish and transferred into 1.5 ml tubes, which were boiled at 95°C for 10 min and centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant was transferred to an Eppendorf tube, and oligonucleotide ssDNAs contained in the supernatant were PCR amplified. SGC7901 gastric cancer cells and MCF-7 breast cancer cells were cultured to a density of 3 × 10^6 cells in DMEM containing 10% fetal bovine serum. Both negative control cell lines were incubated with cold oligonucleotides ssDNA library pools obtained from positive selection (1000 nM final concentration) in 600 µl of binding buffer with shaking at 50 rpm for 40 min at 37°C. After incubation,

the binding buffer, which contains only sequences of ssDNA capable of recognizing and binding to U-2 OS cells, was collected from the culture dish and used for PCR amplification to generate a new ssDNA pool for the next round of positive selection. Negative selections were completed by the fifth and eighth round of selections.

Optimization of PCR condition

It was necessary to optimize PCR conditions in each round of selection in order to determine optimal repeating times and template potential. Briefly, 4 PCR reactions containing 50 μl 2 × Taq MasterMix (Invitrogen, USA) and 0.5 μM concentration of each primer were prepared. Increasing volumes of ssDNA templates (5 μl, 10 μl, 15 μl and 20 μl) were added to the four PCR reactions and double distilled water was added to a final volume of 100 μl. Reactions were mixed thoroughly and subjected to 6, 8, 10 or 12 cycles of PCR with denaturing at 95°C for 30 s, annealing at 56°C for 30 s, and elongation at 72°C for 30 s, followed by a final extension at 72°C for 3 min. After amplification, optimal PCR conditions were confirmed by agarose electrophoresis of 15 μl of PCR products (3% agarose gel, 100 V). The double-stranded DNA oligonucleotides (dsDNA) from the supernatant of PCR amplification were used to prepare highly purified double-stranded DNA (dsDNA), which was then purified by phenol-chloroform extraction.

Aptamer-based fluorescence assay

In the 6^{th}, 9^{th}, 11^{th}, 12^{th}, and 13^{th} round of screenings, fluorescent monitoring was used to filter oligonucleotides during the enrichment process. U-2 OS cells (3×10^6) were plated in 60 mm × 15 mm culture dishes and grown for 24 hours in McCoy's 5A medium supplemented with 10% fetal bovine serum. The cells were collected, rinsed with wash buffer, and incubated with prechilled ssDNA (1 μM in binding buffer) at 37°C for 40 minutes with shaking at 50 rpm. After washing with binding buffer, cells were collected with a cell scraper and transferred to 1.5 mL centrifuge tubes. Cells were heated at 95°C for 10 minutes and centrifuged at 13,000 rpm at 4°C for 5 minutes. The supernatant was collected, a portion of which was used to prepare the following round of ssDNA library screening. The remainder of the cell supernatant was subjected to PCR amplification using FITC-conjugated upstream and Biotin-conjugated downstream primers.

Using streptavidin-conjugated sepharose (GE Healthcare Life Science, USA), FITC-labeled ssDNA was enriched by separation from FITC and Biotin tagged amplified products. Aptamer enrichment was monitored by fluoremetric analysis. Timing of the termination of the screening process was determined by comparison of fluorescence intensity between positive and negative controls. Screening was terminated when there was an unequivocal difference between positive and negative control and fluorescent signals exhibited no significant signs of drifting among positive samples.

Cloning and sequencing of the aptamer library

The PCR products obtained from the 13^{th} round of selection were cloned using a pGM-T cloning kit according to the manufacturer's instruction (TIAgen). Twenty white colonies were subsequently picked and grown in 3 ml LB broth for 18 hours; plasmid DNA was extracted from bacterial cultures using a plasmid DNA extraction and purification kit (Promega, USA). Plasmids containing the 76 bp insert were verified by PCR. Plasmids identified as positive by PCR were confirmed by DNA sequencing on an ABI Prism 377 DNA sequencer.

Validation of the aptamer to mammalian cells by binding assay

A series of sequenced aptamers were randomly selected to verify the specificity using clinical samples of osteosarcoma cells. Seven tumor tissues were obtained from patients with osteosarcoma (diagnosed by a senior pathologist). Tumor samples were used for isolation of cells, which were maintained in culture. Clinical samples of human fibrosarcoma cells and breast cancer cells were prepared in parallel as negative controls. Twenty-four hours prior to the assay, 3×10^6 cells were plated into 60 × 15 mm culture dishes and incubated in the appropriate culture medium.

An aptamer was selected through the previously described method of separation after amplification of the inserted sequence (cloning plasmid No. 6). The aptamer was diluted in 600 μl of binding buffer to a final working concentra-

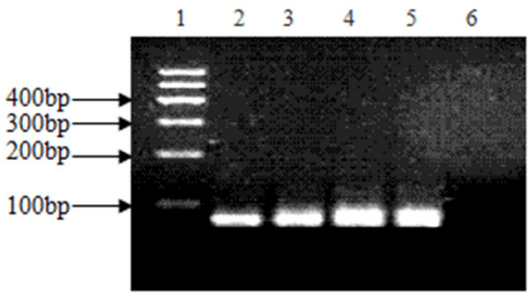

Figure 1. Agarose gel electrophoresis of dsDNA from PCR products in different screening of cycles for PCR conditions with 15% template. Lane 1: marker; lane 2~5: 6, 8, 10 and 12 cycles respectively; lane 6: negative control.

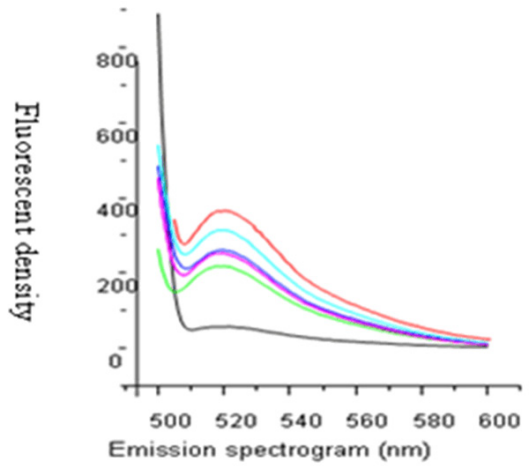

Figure 2. Fluorescence spectrometry assay to assess the binding of selected ssDNA pools to osteosarcoma cells. Black: negative control; red: round 3; blue: round 6; green: round 9; pink: round 11; purple: round 13.

tion of 1 μM. After 24 hrs in culture, all cell lines included in the study were rinsed with wash buffer prior to incubation with the aptamer at 37°C for 40 minutes with shaking (50 rpm). After elutriation, aptamers bound to the cell surface were retrieved for use as templates by heating followed by PCR amplification.

Results

Optimization of the PCR conditions

The results from the PCR optimization indicated that a template concentration of 15% and 8 or 10 rounds of amplification was sufficient to generate large amounts of PCR product. Agarose gel electrophoresis after 8 rounds of PCR indicated the presence of only specific products, with no non-specific amplification products observed. Therefore, a template concentration of 15% and 8 rounds of PCR was optimal to produce sufficient dsDNA to generate a single-stranded oligonucleotide library for the subsequent round of screening. In addition, the PCR products generated after 6, 8, or 10 rounds of amplification with a template concentration of 10%, 15%, or 20%, respectively, demonstrated optimal amplification (**Figure 1**). Also, the high yield of dsDNA could be extracted using phenol-chloroform method to generate input material of high purity (data not shown).

The screening of specific oligonucleotides under fluorescent monitoring

The fluorescence curves after 11 and 13 rounds of PCR were nearly overlapping, suggesting that enrichment of the oligonucleotides had plateaued and that the filtering process could be terminated (**Figure 2**).

Cloning and sequencing

Cloning was performed on the oligonucleotide library from the final screenings based on the fluorescence spectrometry results. The presence of correct insert sequence was evaluated in the randomly picked 20 cloning plasmids, 12 of which were found to contain the correct 76 bp oligonucleotide insert as determined by DNA sequencing (**Figure 3**).

A database including the homologous sequences of GGTGGGGG, GGCGTTATTTG, TT (T/G) TTGG and their aptamers were listed as No. 6, 9, 11, 13, 67, and 68; as well as AAC (A/G) CCCC, CTT (G/C) TT, CCCTGC and their aptamers as No. 14, 21, 33, 37, 69, and 70. For example, the aptamer sequence of No. 6 was TGGTGGGGGTTTTGGCGTTATTTGTGCTTTTTT-GGCGGGG, which was selected for further validation using clinical samples.

Recognition of tumor-derived osteocarcoma cells by specific aptamers

While ssDNA aptamer No. 6 failed to recognize or bind to either breast cancer cells or fibrosarcoma cells isolated from primary tumors, aptamers 6 and 7 showed positive reactions with osteosarcoma-derived cells (**Figure 4**). Since the case of osteosarcoma identified by a

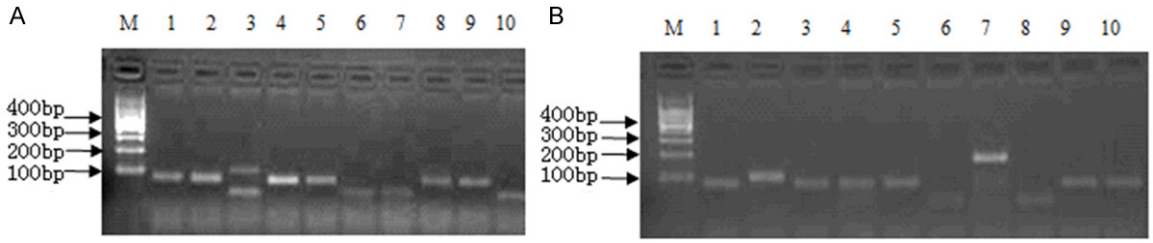

Figure 3. Agarose gel electrophoresis of PCR products from the randomly picked 20 plasmids insert in 76 bp oligonucleotides sequence. A. M: marker; lane 1~10: the randomly picked 10 plasmids owned the 76 bp oligonucleotides sequence; B. M: marker; lane 1~10: the randomly picked 10 plasmids owned the 76 bp oligonucleotides sequence.

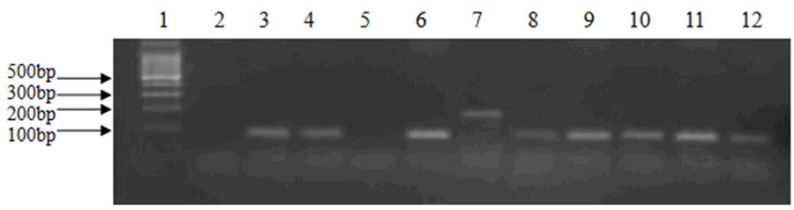

Figure 4. Agarose gel electrophoresis of aptamer No. 6 affinity and specificity to osteosarcoma cells from clinical cases. Lane 1: marker; lane 2: mammary cancer cells; lane 3~4: positive controls; lane 5: human fibroma sarcomatosum cells, lane 6~12 human osteosarcoma cells from clinic.

senior pathologist was initially misdiagnosed, we conclude that the aptamer designed and validated in this study exhibited greater specificity for the diagnosis of osteosarcoma.

Discussion

In the majority of patients, early stage osteosarcoma manifests as relatively indolent pain however by the time of diagnosis, metastasis is a frequent occurrence [16]. Chemotherapy and aggressive surgical techniques have increased osteosarcoma survival, however, early metastasis and chemoresistance are critical hurdles that must be overcome to control the clinical progression of osteosarcoma [17]. Therefore, it is imperative to develop more effective anti-osteosarcoma agents in addition to suitable molecular probes or biomarkers. Until recently, no oligonucleotide aptamer with validated specificity for osteosarcoma cells had been reported.

In this study we describe for the first time the application of the cell-SELEX method to design and generate molecular probes for specific recognition of human osteosarcoma cells. A panel of aptamers that bound specifically to the target U-2 OS osteosarcoma cell line was identified without prior knowledge of the exact membrane binding site. During the multiple rounds of enrichment, the presence of viable cells ensured that the targets remained in their native formation on the membrane in order to maintain aptamer specificity and affinity.

During the process of specific aptamer filtering, the ratio between ssDNA library and target cell concentration was critical for successful SELEX screening. In the first few rounds of filtering, both inputs were necessarily large to ensure capture of the oligonucleotide aptamers by U-2 OS cells. During the later rounds of selection, the number of cells could be reduced due to enrichment of specific aptamers at this time, which is reflected by the reduction in cell number from 5×10^6 to 3×10^5. From 6^{th} round onward, the binding reaction time was also decreased from 40 minutes to 25 minutes to prevent further non-specific binding, consistent with other reported protocols [18-20].

PCR amplification is an essential phase of the SELEX protocol. Since the ssDNA derived from the cell-ssDNA complex produced in every round of PCR has to be transformed into double-stranded DNA (dsDNA), the optimization of cycle numbers and template concentration was performed in each round of PCR in order to acquire the PCR product representing dsDNA without any non-specific products. Phenochloroform extraction was used to purify the dsDNAs from the reaction, which enabled us to obtain a large yield of purified dsDNAs. While the enormous dsDNAs were produced, the reverse biotin-labeled primer was induced to the PCR amplification, as biotin has strong

affinity for streptavidin. In fact, when dsDNAs flew through the chromatographic media of streptavidin sepharose, the biotin-labeled negative stranded oligonucleotide DNA was tightly absorbed, while the positive stranded DNAs were eluted and retained as the library for the next round of screening. This represents the best method developed thus far to prepare a secondary library, and is consistent with previous reports in the literature [21-23].

The screening process was monitored every other round using a fluorescent spectrophotometer. Increased fluorescence intensity on the target cell surface after positive cells with the selected DNA pools, indicated enrichment of aptamers binding to target cells. This method of monitoring during the cell enrichment process is easier than enrichment using flow cytomtery.

In the present study, the SELEX method was applied using whole cells as targets, therefore the range of target molecules available for screening broadened. In the filtering process, one random oligonucleotide library was used to screen multiple target molecules simultaneously, therefore the specific aglycone for each molecule could be identified concurrently. This synchronous screening method could significantly improve the efficiency and reduce the duration of the filtering period [24].

One of the advantages of screening using whole cells as target molecules is that cellular proteins are maintained in their native state, compared to structural changes that occur during isolation and purification. Therefore, as a type of recognition molecule, aptamers have this advantage of high affinity and specificity [25]. Aptamers are capable of recognizing slight differences, such as discriminating between methyl and oxhydryl groups in the target molecular structure. Therefore the screening period could be shortened by automating the process; in addition, aptamers exhibit great stability which enables long term storage and transportation at ambient temperatures [26, 27].

Using osteosarcoma U-2 OS cell lines as target cells on which the specific oligonucleotides were screened with SELEX technology, we successfully developed a molecular probe for diagnosing osteosarcoma with high specificity. These osteosarcoma cells specifically bound to aptamers obtained through screening, which could be used directly in diagnosis and guided treatments without knowing the precise differences between normal and tumor cells. Importantly, the proteins or other constituents to which these aptamers bind specifically represent the differences between healthy and cancerous cells. Therefore, new molecular markers await discovery and have the potential to be specific tumor markers through purifying, validating and testing of these molecular differences.

Although our results are promising, we must acknowledge limitations of this study. In this cell-SELEX based approach, the numbers of human osteosarcoma cell lines included in the study were not sufficiently high enough to conclusively determine the predictive power of this aptamer family. Further large-scale validation studies are needed to explore their value as predictive biomarkers for osteosarcoma.

In conclusion, a novel aptamer recognizing U-2 OS human osteosarcoma cells was successfully generated and validated through screening of a DNA library, and the selected aptamer bound specifically to human osteosarcoma cells. Despite the need for further validation of the aptamer specificity, this study provides evidence for the use of aptamers for rapid detection of the human osteosarcoma, and for their potential use in therapeutic applications.

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Disclosure of conflict of interest

None.

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