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Research paper

Screening and characterization of an Annexin A2 binding aptamer that inhibits the proliferation of myeloma cells

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ABSTRACT

Multiple myeloma (MM) is a malignant plasma cell disease and is considered incurable. Annexin A2 (ANXA2) is closely related to the proliferation and adhesion of MM. Using protein-SELEX, we performed a screen for aptamers that bind GST-ANXA2 from a library, and GST protein was used for negative selection. The enrichment of the ssDNA pool was monitored by filter-binding assay during selection. After nine rounds of screening and high-throughput sequencing, we obtained six candidate aptamers that bind to the ANXA2 protein. The affinities of the candidate aptamers for ANXA2 were determined by ELONA. Binding of aptamer wh6 to the ANXA2 protein and to the MM cell was verified by aptamer pulldown experiment and flow cytometry, respectively. Aptamer wh6 binds the ANXA2 protein with good stability and has a dissociation constant in the nanomolar range. The binding specificity of aptamer wh6 was confirmed in vivo in nude mouse xenografts with MM cells and with MM bone marrow aspirates. Furthermore, aptamer wh6 can block MM cell adhesion to ANXA2 and block the proliferation of MM cells induced by ANXA2. In summary, wh6 can be considered a promising candidate tool for MM diagnosis and treatment.

1. Introduction

Multiple myeloma (MM) is a malignant clonal proliferative disease of the bone marrow (BM) [1,2]. Recent studies have defined the importance of interactions between the MM cells and their BM microenvironment for tumor cell growth and survival, and the development of resistance to therapy [3]. Although the continuous development of MM therapies, such as the immunomodulator thalidomide or lenalidomide or the proteasome inhibitor bortezomib, have improved the survival rate of patients with MM, MM is still an incurable disease [4,5]. Thus, it is necessary to develop new treatment methods and strategies.

Annexin A2 (ANXA2), also known as Annexin II, is a protein superfamily member with Ca^{2+}-mediated phospholipid-binding properties that has three domains: a N-terminal domain, a C-terminal domain, and a core domain [6]. ANXA2 has received increasing attention because of the abnormal expression of ANXA2 observed in various types of tumors [7,8], which is closely related to the occurrence, proliferation, invasion and metastasis of malignant tumors [9–13]. The expression of ANXA2 on the plasma membrane of cells of myeloma patients was significantly higher than that of healthy cells. In addition, there was a significant negative correlation between ANXA2 expression and disease-free survival and overall survival in multiple myeloma patients [14]. Knockdown of ANXA2 can inhibit MM cell proliferation and induce the apoptosis of MM cells [15]. More recently, ANXA2 was showed to play an important role in supporting MM adhesion and growth in the bone marrow microenvironment [16]. ANXA2 expression levels also increased in the bone marrow microenvironment of MM patients [17]. The above studies suggest that ANXA2 is a potential diagnostic and therapeutic target for MM.

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Nucleic acid aptamers are single chains of DNA or RNA that bind specifically to a target molecule with little immunogenicity, no biotoxicity, strong tissue penetration, easy synthesis and labeling, and other characteristics [18,19]. Nucleic acid aptamers could inhibit target protein functional activity and block intracellular signal transduction [20]. Because of their many advantages and characteristics, nucleic acid aptamers show considerable clinical application prospects in the treatment of diseases [21–23]. A thrombin-specific aptamer can inhibit thrombin binding to heparin and block thrombin activity for the treatment of coagulopathy [24]. Macugen [25] an aptamer targeting VEGF had been endorsed in clinical applications and many aptamers are currently undergoing clinical trials [26]. In addition to being able to treat diseases directly as a drug application, nucleic acid aptamers can increase their selectivity and transport capacity by binding to drugs [19,27,28].

Nucleic acid aptamers are screened by the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) method. Many nucleic acid aptamers targeting functional proteins, including PD-L1 [29], VEGF165 [30], CD38 [31], and prostatic acid phosphatase [32], have been screened by SELEX. Using the recombinant SHP2-GST protein as the target protein and GST as the negative screening protein, a nucleic acid aptamer that specifically binds to SHP2 was selected [33]. Magnetic bead-based SELEX is a classical and common method used in screening.

In this study, we screened and characterized aptamers that specifically target the ANXA2 protein with a low nanomolar range of dissociation constant. Furthermore, we find that aptamer wh6 has good binding ability with regard to ANXA2 in clinical patient samples and in mouse MM nodes. We also show that aptamer wh6 can inhibit the adhesion and proliferation of MM cell lines. In summary, wh6 is a promising candidate for use as a tool in MM diagnosis and treatment.

2. Methods and material

2.1. Cell culture

MM.1 R, MM.1 S, ARP-1 and ANBL-6 cell lines were obtained from the Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Science & Peking Union Medical College, Tianjin, China. The NCI-H929 cell line was obtained from the American Type Culture Collection (ATCC). Human peripheral B lymphocytes (B-cells) were obtained from the State Key Laboratory of Medical Genetics. All cell lines were cultured in RPMI 1640 and were supplemented with 10 % (v/v) fetal bovine serum (FBS, Invitrogen), 100 U/mL penicillin and 100 μg/mL streptomycin (Cellgro) in 5% CO_2 at 37 °C. Surplus MM patient bone marrow samples were obtained from Xiangya Hospital of Central South University. This study was carried out after approval by the Ethics Committee of the Xiangya Hospital and obtaining informed consent from all subjects. The methods in treating tissues were carried out strictly in accordance with institutional policies and approved guidelines of experiment operations.

2.2. Protein extraction and western blot

Proteins were extracted using RIPA protein lysate (Beyotime). Total protein was collected from ANBL-6 and NCI-H929 cells, and the protein concentration was measured by a BCA Kit (Promega). The proteins were resolved by using gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked with the 1% BSA and then incubated with ANXA2 antibody (Santa Cruz) or HSP90 antibody (Santa Cruz) at a dilution ratio of 1:1000 overnight at 4 °C. After the membrane was washed three times, an HRP-labeled secondary antibody was used for colorimetric staining.

2.3. Protein expression and purification

The ANXA2 gene was inserted into the pET-42a vector with a ClonExpress II One Step Cloning Kit (Vazyme Biotech). BL21(DE3) bacteria expressing GST-ANXA2 or GST were lysed for 30 min at 4 °C, and the bacterial lysate was centrifuged at 10,000 × g for 30 min at 4 °C. The protein supernatant was obtained. The recombinant GST-ANXA2 and GST proteins were purified by a MagneGST™ Protein Purification System Kit (Promega). The purified proteins were dissolved in PBS and stored in a refrigerator at −80 °C. The purified protein concentrations were determined by a BCA Kit (Thermo Fisher Scientific).

2.4. Protein-SELEX

A library with candidates 80 nt long was constructed (synthesized by Sangon Biotech). The specific nucleotide sequence is as follows: 5′-FITC-ACCGAC CGT GCT GGA CTC A (N)42 A CTA TGA GCGAGC CTG GCG-3'. Both ends are the fixed sequence, the middle (N)42 represents 42 random nucleotides. A random oligo-ssDNA library (DNA-lib) was then amplified with the following primers: F: 5′-FITC-ACC GACCGT GCT GGA CTC A-3′ and R: 5′-biotin-CGC CAG GCT CGC TCA TAG T-3'.

GST-ANXA2 protein was mixed with MagneGST™ particles in a total volume of 500 μL binding buffer (40 mmol/L HEPES pH 7.2, 100 mmol/L NaCl, 3 mmol/L $MgCl_2$, 2.5 mmol/L KCl, 1% BSA, 10 μg/mL tRNA), incubated for 60 min at 4 °C, and washed three times with washing buffer (40 mmol/L HEPES, pH 7.2, 100 mmol/L NaCl, 3 mmol/L $MgCl_2$, 2.5 mmol/L KCl). A randomized ssDNA library (5000 pmol) was dissolved in binding buffer, denatured at 95 °C for 10 min, and placed on ice for 10 min. The ssDNA was added to the GST-ANXA2 protein-bead complex and incubated for 60 min at 4 °C. The unbound ssDNA was washed with washing buffer three times. Then, 200 μL of deionized water was added to the GST-ANXA2 protein-bead complex and heated at 95 °C for 10 min, and the supernatant was used as a template for PCR amplification.

The PCR system conditions were as follows: template, 2 μL; the upstream primer F (10 μM), 2 μL; the downstream primer R (10 μM), 2 μL; dNTP (2.5 mM each), 4 μL; PCR buffer, 5 μL; Champagne Taq DNA polymerase (vazyme, 5 U/μL), 0.25 μL; and ddH_2O, 36.75 μL (total volume, 50 μL). PCR amplification was performed according to the following conditions: initial predenaturation at 95 °C for 3 min; followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s; and a final extension at 72 °C for 5 min. The PCR product was isolated by alkaline denaturation to obtain a single strand. An NAP-5 desalting column (Thermo Fisher Scientific) was used to obtain the next DNA single-stranded library for screening. From the second round, GST protein was used to remove non-specific ssDNA binding to GST protein or magnetic beads, and the cycle was repeated 9 times. In the screening process, the positive incubation time was reduced from 60 min to 30 min as the number of selection rounds increased. At the same time, negative incubation time was gradually increased from 30 min to 60 min (Supplementary Table S1).

2.5. Filter-binding assay

To evaluate the affinities of the enriched library, we conducted filter-binding assay as previously described [30]. The GST-ANXA2 (1 μg), the GST (1 μg) and BSA protein (1 μg) were spotted on a nitrocellulose membrane, and then, the nitrocellulose membrane was blocked with 1% milk. The 9 t h round of screening products