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Aptamers Associated with Nasopharyngeal Carcinoma Metastasis

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KEYWORDS

Aptamer;
Nasopharyngeal Carcinoma;
Metastasis;
Cell-SELEX Technology

ABSTRACT

Nasopharyngeal carcinoma (NPC) has high metastasis, high recurrence and poorly differentiated clinical characters. There has not been a biomarker for predicting NPC metastasis. Searching for molecular biomarkers linked with NPC metastasis and forecasting NPC metastasis is urgently need to be addressed. This study showed a specific Aptamer (Seq4 and Seq5) to link to NPC metastasis, and may be a potential biomarker for NPC metastasis. In the experiment study, NPC cells 5-8F with high metastatic ability were served as target cells for forward selection and 6-10B cells without metastasis as a reverse selection, cell-SELEX technology was used to screen NPC metastasis specific aptamers. Then, the screening products were sequenced using high-throughput sequencing, some DNA sequences that are associated with NPC metastasis were obtained. Further, these DNA sequences were screened with target cells 5-8F and control cells 6-10B. Finally, the specific combination with NPC cells 5-8F and non-combination with the control cell 6-10B were used to verify the specificity of the Aptamer seq4/seq5. Aptamer seq4/seq5 may be a specific nuclear aptamer for NPC metastasis. This provides a novel molecular marker for early diagnosis and target therapy of NPC metastasis.

Introduction

Nasopharyngeal carcinoma (NPC) is a common malignant tumor originating from the epithelial cells of the nasopharynx [1]. NPC has a certain tendency towards familial clustering and regional distribution characteristics. From a global distribution perspective, NPC is mainly concentrated in Southeast Asia, with high incidence in Hong Kong and Malaysia. In southern China, especially in Guangdong, Guangxi, Fujian, and Hunan regions, it is a high-risk area [2]. The incidence rate of nasopharyngeal carcinoma in Chinese population (50-100/100000) is significantly higher than the world average (1.2/100000), of which the incidence rate of male is higher than that of female, and there is a trend that the incidence rate gradually increases with the age increase [2]. The etiology of NPC is related to factors such as diet, environment, genetics, latent infection of EB virus, and epigenetics [3]. Among them, the viral tumor gene of Epstein-Barr (EB) virus can alter the biological behavior of NPC cells, enhance the invasion and metastasis ability of NPC cells[4], and is closely related to the clinical characteristics of high metastasis and high recurrence of NPC [4].

At present, the clinical diagnosis of NPC includes EB virus serological testing, nasal endoscopic examination, and cancer mass biopsy, as well as imaging examinations such as CT, MRI, PCT, etc. However, the early symptoms of NPC are not obvious and the primary site is concealed. Early distant metastasis and high malignancy have brought great difficulties to the diagnosis and treatment of NPC [5]. Previous studies have shown that the 5-year survival rate of early diagnosed NPC patients can exceed 90%, while the 5-year survival rate of NPC patients at middle to late stage is only 50% [6]. Therefore, early detection, diagnosis, and treatment are of great significance for the effective treatment of NPC pa-

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tients. However, the pathogenesis of NPC is not yet fully understood, and the diagnosis is relatively single and lacks specificity. The epigenetic studies no miRNA regulation, DNA methylation, and histone modification in NPC have attracted great attention, and specific expression chips for the mechanism of NPC have provided a certain value for early diagnosis and specific treatment of NPC. But, the invasion and distant metastasis of NPC is a complex process, and it is particularly important to search for specific molecular markers and therapeutic targets for targeting localization of distant metastasis. Based on the high metastasis and recurrence clinical characteristics of NPC, efforts should be made to search for new molecular markers and therapeutic targets for NPC invasion and metastasis, which have extremely important clinical significance in NPC' diagnosis and therapy.

Aptamer is a type of single stranded DNA or RNA with high affinity, and has an ability to specifically recognize target molecule. In 1990, Tuerk and Gold successively screened nucleic acid aptamers through extracorporeal circulation, and proposed the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) technology [7,8]. The targets of aptamer include small molecules, metal ions, proteins, peptides, bacteria, viruses, and cells, among which cells are the most widely used targets for screening applications [9-14]. The apdamter has a structurally stable three-dimensional spatial structure, and it binds to the target through van der Waals forces, hydrogen bonds, electrostatic interactions, and hydrophobic bonds [15-18]. The dissociation constant of the aptamters is between pmol and nmol, with strong affinity. The chemically modified aptampters have higher stability[19,20]. Aptamers have biological characteristics with high specificity and affinity, they are also called "chemical antibodies" [21,22]. Aptamers have stronger penetration into cells and tissues, and thus they are easily cleared by the body. Aptamers are beneficial for the diagnosis and treatment of living organisms in the field of oncology. In the present study, cell SELEX technology was used to perform forward screening on highly metastatic NPC cells 5-8F as target cells, and reverse screening on non metastatic NPC cells 6-10B, as control cells. High affinity aptamers Seq4 and Seq5 were obtained, they may be potential molecular markers for predicting NPC metastasis, and these findings provide experimental and theoretic evidences for further investigating NPC metastatic biomarker.

Material and Methods

Cell Lines

NPC cell lines, 5-8F and 6-10B cells, were derived from the Affiliated Cancer Hospital of Sun Yat sen University. 5-8F is a high metastatic and high tumorigenic NPC cell line, while 6-10B is a low metastatic NPC cell line. Both cell lines have been identified by short tandem repeat (STR) detection.

Preparation of ssDNA Library

10 OD ssDNA library dry powder was taken and centrifuged at high speed. ssDNA dry powder was dissolved in 100 μl of binding buffer, and then denatured at 95 ℃ for 10 minutes. This denature treatment also stretched ssDNA chains into their natural structure. Then the ssDNA solution was quickly cooled in ice for 10 minutes. The initial amount of library used is 10 nmol, and the subsequent screening quantity was the enriched PCR product from the previous round.

Forward Direction Screening

NPC cell line 5-8F cells used for forward screening were washed with pre-cooled washing buffer, and then were incubated at pre-treated initial library at 4 ℃ on a horizontal shaker (100rpm) for 2 hours. After incubation, the cells were washed with washing buffer to remove ssDNA that has not bound to 5-8F cells or is not firmly bound. The cells were collected in an eppendorf (EP) tube, and then denatured in a 95 ℃ water bath for 10 minutes. The cells were cooled on ice for 10 minutes, then centrifuged at 4 ℃ and 5500 rpm for 5 minutes, and transferred to a new EP tube after centrifugation.

Reverse Directions Screening

Starting from the fourth round of screening, reverse screening was performed, followed by forward screening in each subsequent round of screening. The human NPC cell line 6-10B cells were used as a control cell for reverse screening. The cells were washed with pre-cooled washing buffer, and the enriched library from the previous round was incubated for 30 minutes. After incubation, the supernatant was collected and added to 5-8F cells for incubation. The subsequent forward screening process was the same with the forward screening process described above.

PCR Amplification

To prevent the loss of single copy nucleic acid aptamers and ensure sufficient ssDNA library for the next round of screening, the supernatant obtained from each round of screening was subjected to amplification with 8 cycles, with a reaction system of 100 μL and 5 parallel tubes. The PCR reaction system consisted of 28 μL of H_2O, 5' terminal primer FAM 1 μL, 3' terminal primer Biotin1 μL, 2× PCR-mixture 50 μL, and ssDNA 20 μL. The PCR reaction is denaturation at 95℃ for 5 minutes, denaturation at 95 ℃ for 30 seconds, annealing at 58 ℃ for 30 seconds, extension at 72 ℃ for 30 seconds, and amplification for 8 cycles; Finally, extend at 72 ℃ for 5 minutes.

Flow Cytometry Detection of Binding Strength

The amplified product of PCR during the screening process was dsDNA. Due to the reverse primer labeling with biotin, the agarose beads labeled with streptavidin interacted with biotin, which caused the antisense strand to dissociate and become ssDNA, leaving behind the sense strand. The sense strand was labeled with fluorescein FAM, the fluorescence intensity was detected by Flow Cytometry. Through continuous screening and enrichment, the fluorescence signal gradually increased. The enriched ssDNA library was detected to be highly bound to the target cell, and when it almost did not bind to the control cell, the screening reached saturation, which was the final enriched product.

Preparation of ssDNA Library and Flow Cytometry Analysis

The screening products from the initial library, rounds 8, 12, 14, and 15 were detected. 50 pmol of the dried product was dissolved in 45 μL of binding buffer (BB). The solution was placed in a 95 °C water bath for 10 minutes, cooled on ice for 10 minutes, and added 150 μL BB to each EP tube to

adjust the final concentration to 250 nM. After being incubated with target cells on ice for 1 hour, the solution was centrifuged at 4 °C and 1000 rpm for 3 minutes, the supernatant was discarded. The pellet was washed with 1mL of washing buffer (WB) for three times, the solution of the pellet was centrifuged at 1000 rpm and 4 °C for 3 minutes for each washing. And finally, the pellet was resuspended in 400 μL of BB. The fluorescence intensity on the cell surface was measured using a flow cytometer.

High-Throughput Sequencing Validation

When the saturation of binding with the target cells was detected by the flow cytometer, and almost no binding with the control, the final enriched library was used for high-throughput sequencing, which uses fluorescently labeled primers for amplification. High throughput sequencing was completed by Shanghai Shenggong Bioengineering Co., Ltd.

ssDNA Library Validation

Based on the high-throughput sequencing results, 5 sequences with the highest frequency of occurrence were obtained, and these 5 ssDNAs with FAM were also synthesized by Shanghai Shenggong for further validation. The synthesized ssDNA labeled with FAM was dissolved in BB at a concentration of 1uM. The synthesized ssDNA was denatured at 95 ℃ for 10 minutes, and immediately mixed on ice for 10 minutes. When the target cells and control cells were validated to grow well and to 90% fusion, the cells were digested with trypsin, and then harvested and centrifuged at 1000rpm for 3 minutes. The supernatant was discarded, 1mL of WB was added to the cells, the cells were washed for 3 times. $3 \times 10_5$ cell were incubated with the preprocessed ssDNA, and placed on ice for 1 hour. The cell solution was centrifuged at 1000 rpm for 3 minutes at 4 ℃, the supernatant was discarded, the pellet was washed with 1 mL of linking buffer (LB) for three times, and finally was resuspended in 400 μl of BB for flow cytometry detection. Among them, the binding of cells 6-10B in the control was also detected, and the operation was the same as above.

Results

Optimization of PCR Experiment

In order to determine the optimal annealing temperature, the annealing temperature within the range of 55-62 ℃ was used to PCR amplified, and then agarose gel electrophoresis and gel imaging were used to observe PCR results, the band with the best brightness, clearest and unspecific amplification was determined to be the optimal annealing temperature. According to gel imaging, 59 ℃ was selected as the optimal annealing temperature.

In each round of screening, to ensure the output and purity of each round of screening products, the number of cycles needs to be optimized fro PCR amplification. Similarly, PCR products amplified with different cycles (4, 6, 8, 10, 12, 14, 16) were verified by agar gel electrophoresis. Finally, 8 cycles was a appropriate cycle times for PCR amplification.

Enrichment of Screening Library

To calculate the input amount for the next round screening, the ssDNA solution collected from each screening round needed to be tested for UV absorption value. The input amount was calculated based on the UV absorption value. The input amount for first round was 10 nmol, followed by 200 pmol, 100 pmol, 80 pmol, 70 pmol, 50 pmol, etc. The UV absorption values of ssDNA aqueous solution at 260nm were all greater than 0.1 (**Table 1**), indicating a high concentration and good enrichment of the screened library.

Monitoring of Enriched Libraries

During the entire screening process, the fluorescence intensity of the screened product binding with target cells 5-8F and control cells 6-10B was detected by flow cytometry. The

Table 1 | Product concentration and absorbance values

Screening turns	Product concentration (μg/μl)	Absorption values (A260)
1	26.6	0.7
2	20.5	0.61
3	18.3	0.55
4	12.5	0.51
5	19.2	0.58
6	20.4	0.61
7	19.9	0.6
8	17	0.5
9	17.3	0.52
10	17.1	0.51
11	11.09	0.33
12	9.7	0.26
13	9.8	0.27
14	10.7	0.32
15	11.7	0.35

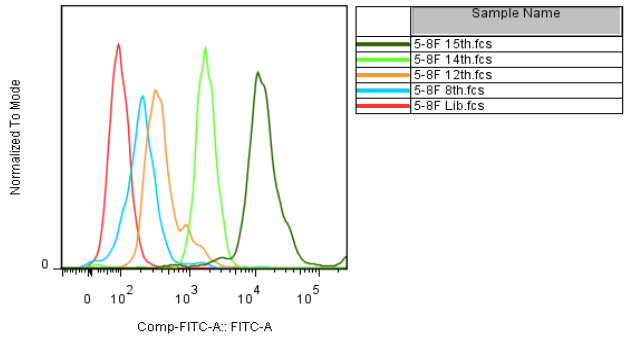

Figure 1 | Flow cytometry fluorescence intensity validation for target cell 5-8F screening

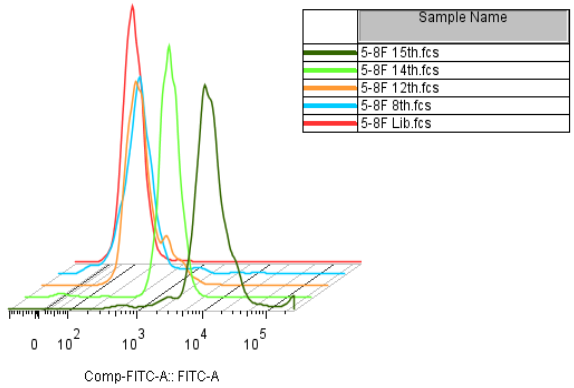

Figure 2 | Three dimensional validation plot of flow cytometry fluorescence intensity for target cells 5-8F screening

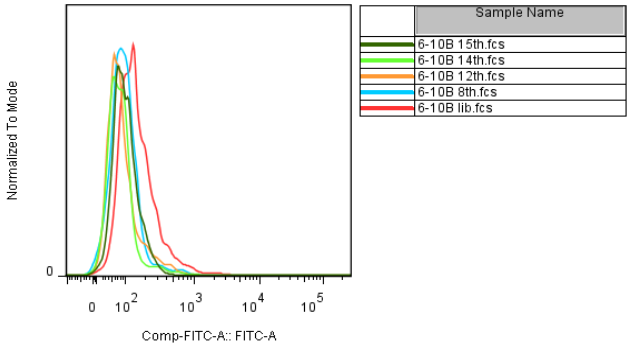

Figure 3 | Flow cytometry fluorescence intensity validation for control cell 6-10B screening

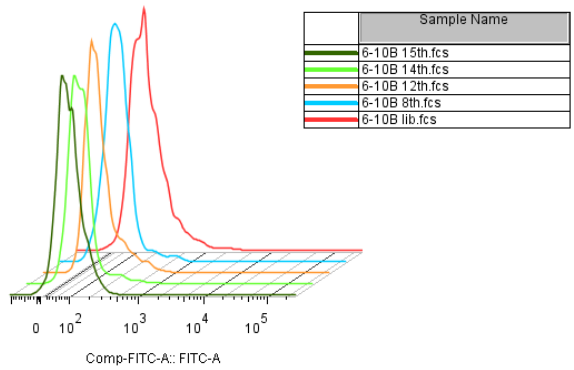

Figure 4 | Three dimensional validation plot of flow cytometry fluorescence intensity for control cells 6-10 screening

Table 2 | DNA sequence of screening products at fifteenth screening

No	5'to 3' (mid sequence)	Ratio in DNA library
1	CAAGCGTTTCCAAAATGAGTGTTATGTGGAAGCTTTAACC	17.23%
2	AGGTTGTGCGTACTAATTCTACCTTCCTTCAGGAAACTTA	8.91%
3	AGTATGGCTGTGTTCCTCTAACTACTTTCCAAGCATTTTA	7.94%
4	CTTGAAGGCCCAACGTCGGCGAAATTAATAGGTTGCTTCA	7.85%
5	GCCGTACCGCCGGCGAAATTAATAGGGTACGGTAAAACTA	7.21%
6	CAGCGACCAATGCAGGCGAAATTAATAGATTAGTTGCAAT	5.67%
7	AGAACGCATATGGACAGATCGTGACCTTCAGTTTAGAAAG	5.21%
8	GGTAAGACAACGCCGGAAGACGGAACGTACTCCACGGTTC	4.84%
9	ATCGGCTCCGATCGCGACAGAGCGTCGGAACCTCTTTTAC	3.42%
10	TCGGTCCCTGACTCATGCAGGAAGTCTTAATAACGGTCAA	2.1%
11	CAGCGACCAAGGCAGGCGAAATTAATAGATTAGTTGCAAT	1.61%
12	TTTGGCAGACTGAAGAGACTTTCTCAG	1.6%
13	AGGTTGTGCGTATTAATTCTACCTTCCTTCAGGAAACTTA	1.54%
14	TAAGTATACTCGCGATCTGGGTGAGCTAATCTACAACGA	1.53%
15	TCGGTCCCTGACTCATGCAGGATGTCTTAATAACGGTCAA	1.40%
16	ACCTAGCTAGCTTTGTCAAGTCCTGATTGTTAGGAATTGC	0.81%
17	AACCAGCTAGACAGCCGTGACTTGAAGATTGCTCTGTTTA	0.66%
18	GCACATGTACATGGTAAGTTAGTCAAATCTACAGTCGCTT	0.65%
19	ATTGAAGGCCCAACGTCGGCGAAATTAATAGGTTGCTTCA	0.59%
20	AGAATATGCACCTGAACTGTGCCGAAGCTGCCTATCATGA	0.59%

products from initial library, eighth, twelfth, fourteenth, and fifteenth round screening were tested, respectively. The stronger the fluorescence intensity, the stronger the binding ability. The flow cytometry results were shown in the **Figure 1**. The x-axis of flow cytometry represents the fluorescence value, and the y-axis represents the number of cells. The Figure 1 showed that, the fifteenth round was the strongest, and the fluorescence value of the fourteenth round was not much different from that of the fifteenth round. These indicated that the ssDNA library has reached saturation with the target cells (**Figure 1** and **Figure 2**), at the same time, there was no significant change in the fluorescence values of the control cells and the products screened (**Figure 3** and **Figure 4**), which was basically consistent with the initial library and ssDNA library did not bind to the control cells. The above results indicated that, during the fifteenth round of screening, ssDNA showed well enrichment with target cells 5-8F, while there was almost no enrichment with control cells 6-10B.

Homology Analysis on the Sequences From High-Throughput Sequencing

The flow cytometry analysis results showed that the enrichment level of screening products at the 15_{th} round was highest. Therefore, the screening products at 15_{th} round were sent to high-throughput sequencing (Shanghai Shenggong, Shangsha, China). The corresponding sequences were obtained according to the repeat rate (**Table 2**). The first 5 repeat rate sequences accounted for 47% of the entire secondary library at15_{th} round screening.

Next, the top 20 sequences were ranked by the proportion of repetitive sequences, DNAMAN software was used to perform homology analysis on these top 20 sequences. The 20 sequences were arranged according to their proportion. And based on homology comparison analysis, the 20 sequences were mainly divided into three families (**Figure 5**). The first family includes Seq1, Seq9, Seq4, Seq19, Seq6, Seq11, Seq12, Seq5, and Seq17. The second one includes Seq2, Seq13, Seq3, Seq7, Seq18, Seq20, Seq14, and Seq8. The third part includes Seq10, Seq15, and Seq16.

Analysis on the Binding Ability of Different Family Sequences to Target Cells

By using DNAMAN software to perform homology classification of the primary structure of the 20 sequences ranked by the proportion of repeatability, the sequencing results were divided into three families. The sequences with higher repeatability were selected as representatives, Seq1 and Seq2 were distributed in different families and had a higher proportion. The Seq1 and Seq2 were selected for binding ability analysis with target cells. Seq10, Seq15, and Seq16 sequences in the third family had a lower proportion, and could not be used for sequence analysis. Among them, Seq3 and Seq4 had high homology with Seq1 and Seq2 and accounted for a high proportion, which were also included in the analysis of binding ability with target cells. Finally, the sequences for binding ability validation with target cells (5-8F cells) (**Figure 6 and Figure 7**) and control cells (6-10b cells) (**Figure 8 and Figure 9**) were seq1, seq2, seq3, seq4, and seq5, respectively. Through flow cytometry verification, it was found that seq4 and seq5 sequences have a certain binding ability. According to the homology comparison analysis, seq4 and seq5 belonged to the same family, simultaneously, the fluorescence intensity of seq4, seq5 binding with 6-10B remained basically unchanged. Based on the analysis of the above results, nucleic acid aptamers seq4 and seq5 may have a specificity for recognizing metastatic NPC cells 5-8F.

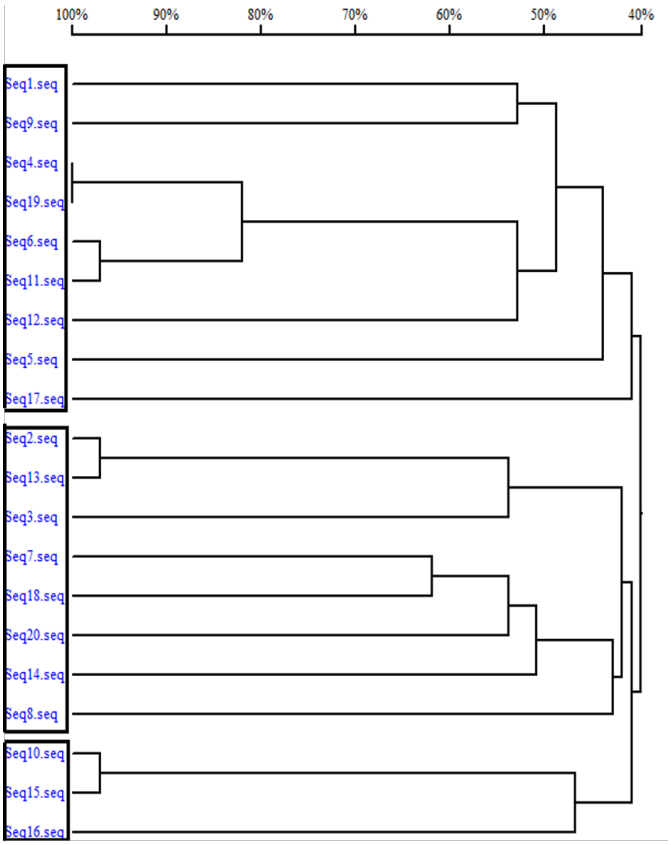

Figure 5 | Homology comparative analysis of synthesized sequences. 20 sequences are mainly divided into three families

Discussion

Aptamer is single stranded DNA or RNA screened from oligonucleotide libraries in vitro using SELEX technology. The cell SELEX technology is characterized by targeting live cells to screen for nucleic acid aptamers that specifically bind to surface molecular proteins of live cells. This study utilized cell SELEX technology to select metastasis relative aptamers, highly tumorigenic and metastatic NPC cell 5-8F acted as forward screening cells and low tumorigenic and non metastatic NPC cell 6-10B acted as reverse screening cells. Through repeated cyclic screening, specific binding aptamers for metastasis were identified. The enrichment and optimization process in each round of screening is an important factor to determine the success or failure of the entire screening process. In the process of screening and enrichment, the amplification efficiency of PCR is used to test screening resutls. Agarose electrophoresis was used to find the optimal annealing temperature, the annealing temperature of 59 ℃ was the optimal temperature. The number of amplification cycles was optimized for the PCR amplification process to each round of screening, 8 cycles was optima PCR amplification cycle. This laid a good foundation for the next round of screening and the selection of highly affinity aptamers.

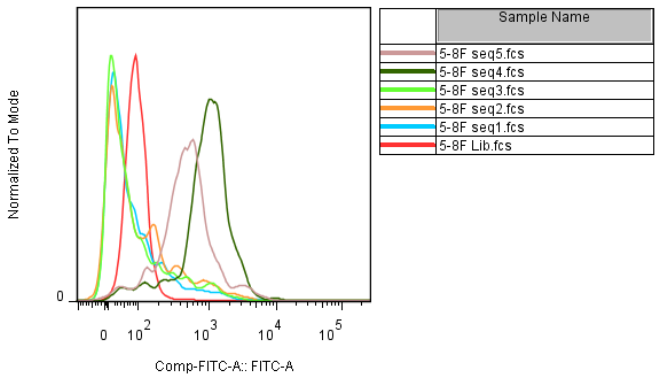

Figure 6 | Flow cytometry fluorescence intensity validation of seq1, seq2, seq3, seq4, seq5 binding to target cells 5-8F

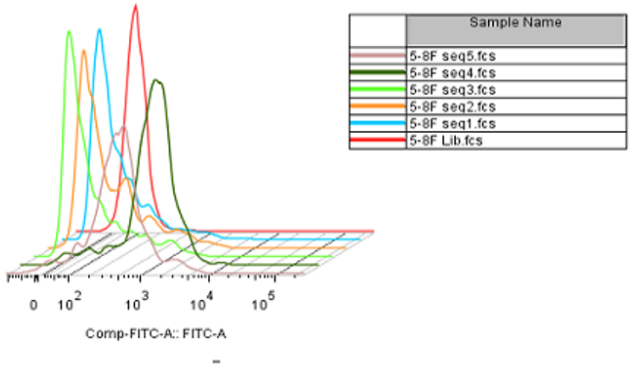

Figure 7 | Three dimensional validation of flow cytometry fluorescence intensity for the binding of seq1, seq2, seq3, seq4, seq5 to target cells 5-8F

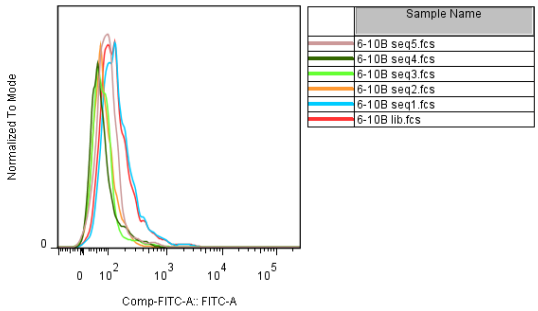

Figure 8 | Flow cytometry fluorescence intensity validation of seq1, seq2, seq3, seq4, seq5 binding to control cell 6-10B

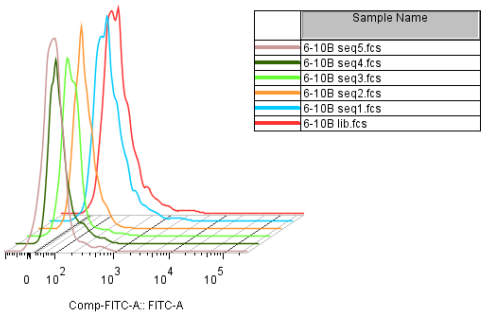

Figure 9 | Three dimensional validation of flow cytometry fluorescence intensity for the binding of seq1, seq2, seq3, seq4, seq5 to control cells 6-10B

In cell SELEX technology, besides 5-8F cells were used as target cells, and 6-10B cells were used as control cells, the key factors for successful screening also included adjusting screening pressure conditions. Firstly, the incubation time and the proportion of initial library with target cells gradually decreased, while the incubation time and the proportion of control cells gradually increased. Secondly, the volume and frequency of washing buffer were gradually adjusted accordingly to screened aptamers that bound highly specifically to target cells. Finally, after 15 screening rounds, the aptamers that specifically bind to metastatic NPC cells were obtained. In the screening process, the amount of screening input in each round and the final saturation of screening mainly include the following two aspects. On the one hand, during the entire screening process, the absorbance value of ssDNA is detected. These values are used to calculate the concentration of the product and determine the input amount for the next round screening; On the other hand, the binding of oligonucleotides to target cells is mainly achieved through flow cytometry, which the fluorescence intensity of the target cell surface is measured and its affinity with the target cell is determined. In this study, fluorescence values on the surface of target cells were monitored at the initial library, 8th, 12th, 14th, and 15th rounds. As the screening gradually increased, it can be found from the results that the fluorescence value of 5-8F binding to target cells gradually increases, at the 14th round of screening, the fluorescence value did not increase significantly compared to the 15th round. The binding force may basically reached saturation in the 15th round, indicating a high degree of binding to target cells at the 15th round of screening. Under the condition of the same number of cycles, the fluorescence values of control cells 6-10B were observed. As the number of screening rounds increased, the fluorescence values on the surface of control cells 6-10B remained basically unchanged. The selected aptamers can recognize metastatic cells with high specificity, but cannot recognize non metastatic cells.

The products from the fifteenth round screening were sequenced. The sequencing results showed that the top 5 sequences with high repetition rates accounted for 47% of the entire random sequence. The higher the repetition rate, the higher the enrichment level. In theory, the higher the enrichment level, the stronger the binding ability with target cells. Among sequencing data, the top 20 sequences in terms of repeatability were subjected to homology comparison analysis, and these sequences could be divided into 3 families. The top 5 sequences with the best enrichment were synthesized, and their binding ability with target cells and control cells was further verified by flow cytometry. The results showed that Seq4 and Seq5 sequences have a certain binding ability with target cells 5-8F. According to homologous comparative analysis, Seq4 and Seq5 belonged to the same family, with

poor recognition ability compared to control cells. This study utilized cell SELEX technology and performed 15 rounds of screening to identify the largest enriched ssDNA library. This library can recognize metastatic NPC cells and does not bind to non metastatic cells. Based on the proportion of repeated sequences in the enriched library and homology comparison analysis, 5 highly enriched libraries were selected for synthesis. Their affinity for target cell 5-8F and control cell 6-10B was verified by flow cytometry. Nucleic acid aptamers Seq4 and Seq5 can specifically bind to metastatic NPC cells, and provide new diagnostic and therapeutic basis for biomarkers of predicting NPC metastasis.

Conclusion

Cell SELEX technology has been successfully used for screening nucleic acid aptamers related to NPC metastasis. The NPC metastasis specific nucleic acid aptamers Seq4 and Seq5 have been obtained. The sequences of nucleic acid aptamers seq4 and seq5 have been confirmed to specifically bind to 5-8F cells. Seq4 and Seq5 sequences may be specific nucleic acid aptamers for NPC metastasis.

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