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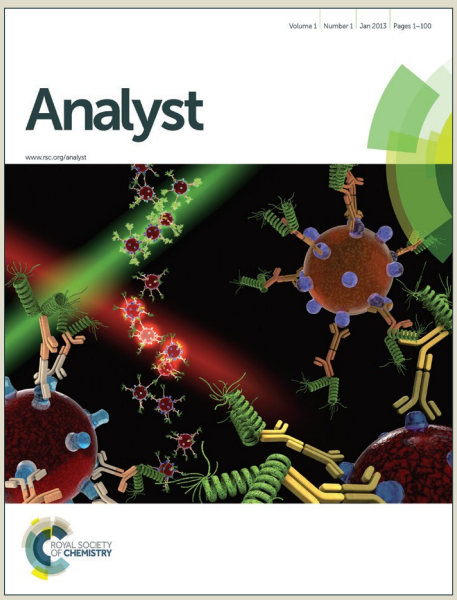

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ARTICLE

Cell-SELEX based selection and optimization of DNA aptamers for specific recognition of human cholangiocarcinoma QBC-939 cells

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Cholangiocarcinoma (CCA) is a very aggressive biliary tract malignancy with no efficient early diagnosis and therapeutics available, calling for effective molecular probes. Herein, we performed cell-based systematic evolution of ligands by exponential enrichment (cell-SELEX) to obtain aptamer for specific recognition of human cholangiocarcinoma QBC-939 cells. By coordinating sequence homology analysis and secondary structure analysis, we successfully obtained two aptamers with dissociation constant (K_d) in the low nanomolar range. A 23-nt truncated sequence was identified after further elaborative analysis on the secondary structure. More importantly, because hepatocellular carcinoma SMMC-7721 cells were employed as control in the counter selection, the achieved aptamers demonstrated excellent specificity to target cells, especially no binding to several hepatocellular carcinoma cell lines was observed. Moreover, the aptamers were preliminarily identified to recognize membrane proteins, endowing them great potential of facilitating biomarker discovery. These newly generated aptamers may play a key role in the early diagnosis and clinical treatment of CCA.

Introduction

CCA is the most common biliary tract malignancy and the second most common primary hepatic carcinoma. The worldwide incidence and mortality rates had significantly increased throughout the last decades, and 5-year survival rates is dismal[1, 2]. Due to CCA cells' remarkable resistance to common chemotherapeutic drugs and radiation, the clinical benefits of traditional treatment is very poor[3]. Surgical resection is the only potentially curative treatment for CCA, unfortunately the majority of CCA patients present at an advanced stage where surgical therapy is not feasible[4, 5]. Worse still, existing screening methods such as magnetic resonance imaging and carbohydrate antigen 19-9 (CA 19-9) only have limited accuracy, especially on patients with other biliary diseases[6, 7]. As a result, it's very urgent to develop novel probes with high sensitivity and specificity to promote the early diagnosis and therapy of CCA.

In the last decades, aptamer has arisen as a promising recognition reagent. Aptamers are single-stranded oligonucleotides (ssDNA or RNA) capable of binding to target molecules with high affinity and specificity by folding into unique tertiary structures[8-10]. Their attractive features such as low molecular weight, quick and reproducible synthesis, easy modification, good stability, low toxicity, low immunogenicity, and fast tissue penetration ability make them promising alternative to conventional antibody[11, 12].

Aptamers can be selected through SELEX against different targets ranging from small molecules to complex objects. A recently developed new strategy termed cell-SELEX can even screen aptamers recognizing whole living cells[13]. Cell-SELEX has obvious advantages over protein-SELEX in cancer research. Firstly, prior knowledge of potential target molecules of cancer cells is not required before the selection, and the obtained aptamers can even recognize different potential biomarkers on cell surface[14]. Moreover, in cell-SELEX, the target molecules presented on cell surface are in the native state, while protein-SELEXs use purified proteins as target[15]. Up to now, there have been many reports recording aptamers targeting various cancer cell lines, including leukemia[16], lung cancer[17-19], colon cancer[20], hepatocellular carcinoma[21, 22], ovarian cancer[23], prostate cancer[24], nasopharyngeal carcinoma[25] , and gastric cancer[26-28]. Tremendous efforts have also been made to expand aptamers' application in biomedical research, including cancer cell detection, cell capture, imaging, targeted therapy, and biomarker discovery, and the results are promising[29]. Therefore, aptamers holds great potential to facilitate the development of cancer early diagnosis and targeted therapy.

In the past few years, our group has successfully achieved a number of aptamers against different targets, including several hepatocellular carcinoma cell lines. Clinically, CCA is easily misdiagnosed as hepatocellular carcinoma (HCC) in imaging tests because their pathogenic site is very close. Therefore, it could be of great value to achieve aptamers that could distinguish these two cancers. Herein, by employing cholangiocarcinoma cell line QBC-939 as target and hepatocellular carcinoma cell line SMMC-7721 as control, we have successfully identified two aptamers with equilibrium dissociation constant (K_d) in the nanomolar range.

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† Electronics supplementary information (ESI) available: homology analysis of the sequences; cell recognition with other sequences; Secondary structure analysis of the sequences; temperature's effect on aptamers' recognition ability.

Furthermore, the obtained aptamers and a 23-nt truncated sequence were further evaluated for binding specificity, determination of target type, and effect of temperature on their recognition of target cells via Flow cytometric analysis or confocal imaging.

Experimental

Cell lines and cell culture.

All cell lines used in this paper are human derived. cholangiocarcinoma cell line QBC-939 (target cell) and nasopharyngeal carcinoma cell line CNE2 were maintained at our laboratory. Hepatocellular carcinoma cell line HepG2, cervical cancer cell line HeLa and adenocarcinoma cell line A549 were purchased from American Type Culture Collection. Hepatocellular carcinoma cell lines SMMC-7721 (control cell) and Bel-7404 were purchased from the Shanghai Institute of Cell Biology of the Chinese Academy of Science. All cells were cultured in RPMI medium 1640, containing 10% FBS (GIBCO) and 100 U/mL penicillins-treptomycin (Cellgro), and maintained in an incubator at 37°C with 5% CO_2 atmosphere.

SELEX library and primers.

The initial library and primers were designed by ourselves and then synthesized by Sangon Biotech (Shanghai). The library comprised a randomized region of 40 nucleotides (N40) flanked on both sides by two constant regions for PCR amplification. The forward primer was labelled at the 5' end with FAM (5'-FAM-AGAAGGATGGAGAGGAGCAC-3') to monitor the enrichment of the selected pools by flow cytometry, and the reverse primer was labelled at the 5' end with biotin (5'-Biotin-AGTAGGAAGGAGACGAGGAC-3') to isolate the sense strands from antisense strands with streptavidin-coated sepharose beads (GE Healthcare, USA) for subsequent selection rounds.

SELEX procedures.

For the first selection round, the ssDNA library (10 nmol) dissolved in binding buffer (BB, 1 mg/ml BSA and 0.1 mg/ml yeast tRNA in washing buffer) was denatured by heating at 95 ^{o}C for 5 min, cooled on ice for 10 min, and then incubated with 1×10^6 QBC-939 cells on ice in a rotary shaker for 1 h. In addition, 20% FBS and excess of random DNA were added into the incubation solution to reduce the nonspecific adsorption of cell and the container. After incubation, the cells were washed three times using washing buffer (WB, 4.5 g/L glucose and 5 mM $MgCl_2$ in PBS) to remove the unbound sequences. The cells were then collected and heated at 95 ^{o}C in WB for 10 min to elute the bound ssDNA. The supernatant was used to prepare pool for the next selection round by PCR. After isolation by streptavidin-coated sepharose beads, the enriched FAM-labelled ssDNA pool was collected.

For the second and subsequent rounds, the ssDNA pool was first subjected to a counter selection: control cells SMMC-7721 (2-fold excess than QBC-939 cells) were incubated with the ssDNA pool on ice for 1 h, and the supernatant was then subjected to positive selection. To acquire aptamers with high affinity and specificity, the selection pressure was gradually enhanced by decreasing the amount of the ssDNA pool (from 200 pmol to 30 pmol) and the incubation time for the target cells (from 60 min to 30 min), meanwhile the number of the control cells (from 2×10^6 to 1×10^7) and the number of washes (from three to six) were gradually increased. After enrichment of the selection pool, the obtained ssDNA pool was sent to Sangon Biotech (Shanghai) for cloning and sequencing.

Monitoring the enrichment of the selection.

FAM-labeled ssDNA pools at a final concentration of 250 nM were incubated with 2×10^5 target cells or control cells on ice for 30 min. After washing with 500 μL of washing buffer three times, cells were resuspended in 200 μL of washing buffer. The fluorescence intensity was recorded by the flow cytometer (FACScalibur, BD Bioscience) by counting 10 000 events. The FAM-labeled unselected ssDNA library or a random sequence were used as the negative control.

Evaluating the binding affinity and selectivity of the aptamers.

The binding affinities of aptamers were determined by incubating QBC-939 cells (1×10^6) with a series of concentrations of FAM-labeled aptamers on ice for 30 min in the dark, and then subjected to flow cytometric analysis after three times of washing with 500μL of WB. FAM-labeled initial DNA library was used as negative control. The mean fluorescence intensity was recorded and the K_d value was calculated by fitting the dependence of fluorescence intensity of cell-aptamer complex on aptamer concentration with the equation $Y = B_{max}X/(K_d + X)$ using SigmaPlot software (Jandel Scientific).

To determine the binding selectivity of the selected aptamers, several other human carcinoma cell lines were subjected to the aptamer binding assay using flow cytometry as described above.

Imaging of cells with aptamers.

Laser scanning confocal microscope (Olympus, Japan) was used to further confirm the specificity binding of obtained aptamers. QBC-939 and SMMC-7721 cells were cultured in dish for 24h and then washed with WB three times. The cells were incubated with FAM-labeled aptamers or control ssDNA (250 nM) in BB on ice for 60 min. Then it was washed twice with WB before imaging. FAM was excited by a 488-nm laser and the fluorescence signals were collected by a 40× objective.

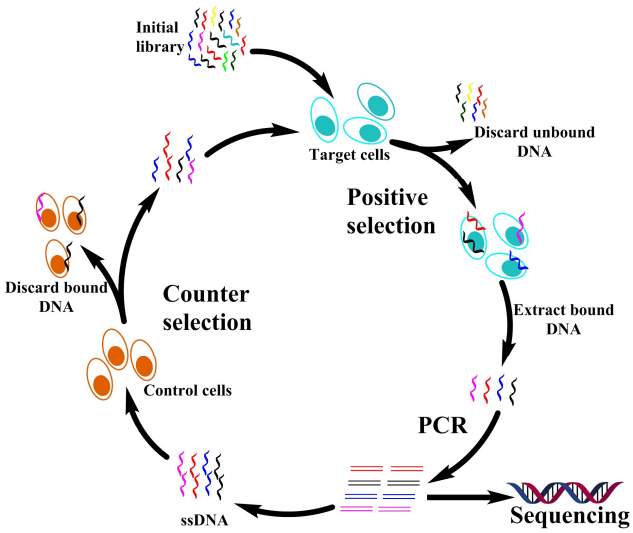
Proteinase Treatment.

QBC-939 cells (1×10^6) were harvested and treated with 0.1 mg/mL proteinase K at 37 ^{o}C for 10 min. After washing three times with WB, they were then subjected to the binding assay using flow cytometry.

Results and discussion

Selection of DNA aptamer against cholangiocarcinoma cell line QBC-939.

To generate aptamers specific for cholangiocarcinoma cells, human cholangiocarcinoma cell line QBC-939 was used as target and hepatocellular carcinoma cell line SMMC-7721 as control. The cell-SELEX process is schematically shown in Scheme 1.



Scheme 1. Schematic representation of systematic enrichment of aptamers against human cholangiocarcinoma cell line QBC-939.

An 80 nt ssDNA library were designed, which contained two 20-nt primer regions and a 40-nt random region. The target cells then were incubated with the ssDNA library. After the first round, the DNA library was firstly incubated with negative SMMC-7721 cells to remove nonspecific sequences. The unbound ssDNA was collected and then incubated with target QBC-939 cells for positive selection. After washing, the bound ssDNA was eluted and amplified for next-round selection.

Monitoring the enrichment of SELEX

The enrichment of the aptamer selection process was monitored using flow cytometry. The fluorescence intensity of the labeled cells represented the binding capability of FAM-labeled ssDNA pools towards target QBC-939 cells. As shown in Fig. 1, obvious increase of fluorescence intensity on target QBC-939 cells was observed with selected pool comparing to the unselected random sequences, and a saturated fluorescence intensity was observed after the thirteenth round of selection. In contrast, almost no increase in fluorescence signal was observed with the control SMMC-7721 cells.

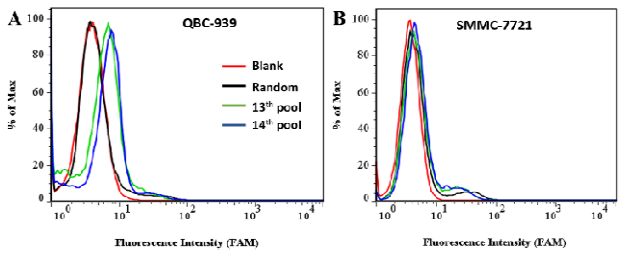


Fig. 1 Flow cytometric assay of selected pools with target QBC-939 cells and control SMMC-7721 cells. Significant increase in fluorescence intensity were detected on QBC-939 cells when incubated with evolved pools (A), but not on SMMC-7721 (B), suggesting the enrichment of QBC-939 specific aptamers.

These results suggested that DNA sequences with specific recognition ability to target cell QBC-939 had been enriched.

Aptamer identification based on homology analysis and structure comparison

After cloning and sequencing, the obtained 23 sequences were analyzed using Clustal X[30]. The sequences were mainly grouped into five families based on the homology of the DNA sequences of individual clones. Sequences with the highest abundance in each family, yl1, yl2, yl4, yl7 and yl8, were chosen as representatives and synthesized for further analysis. Unfortunately, flow cytometric analysis revealed that this five sequences exhibited negligible or only very weak binding ability to the target cells (Fig. S2, ESI). This result unveiled the high indeterminacy of SELEX that the sequences with highest copies in the evolved pool may not certainly possess good target binding ability. Similar results have also appeared in many other SELEX investigations conducted by our group and others and the cause was commonly presumed to be PCR bias[31, 32].

Despite the above undesirable result, the recognition ability increment of the thirteen pool demonstrated that aptamer candidates with good recognition ability certainly exist in the pool. It is well established that specific motifs in aptamers' structure, including hairpin, pseudoknot and bulge, play a very important role in target binding. Hence, we further analyzed the secondary structure of the sequences and divided them into six groups based on their presented motifs (Fig. S3, ESI). Interestingly, two groups were found to be totally uninspected previously, so yl19 and yl23 were picked out and further tested (sequences listed in Table 1). Flow cytometric assay illustrated that yl19 and yl23 exhibited good binding ability to target QBC-939 cells, while showed no obvious binding to control SMMC-7721 cells (Fig. 2A and Fig. 2B). The equilibrium dissociation constant (K_d) values of yl19 and yl23 were in the low nanomolar range, 42.44±11.82 nM and 87.45±15.50 nM respectively (Fig. 2D and Fig. 2E). Confocal imaging result also indicated that yl19 and yl23 specifically bound to target cells on the surface, rather than the control cells (Fig. 2C).

The fact that the sequences with highest copies in the evolved pool may not possess good target binding ability has caused many frustration and consequently failure of selection. The dramatic analyzing process in this work inspire us that structure analysis may offer alternative guidance to identify aptamer candidates.

Sequence optimization of aptamer yl19

Besides the characterization of aptamer's binding ability, further investigation into the function of different motifs in target binding could also be of great importance for their future application. It has been reported that shorter ssDNA owned better tissue penetration ability. Meanwhile, proper truncation on aptamer could remove the useless nucleotide sequences that may hinder target binding[33-36].

Table 1 Sequences and their affinity to target QBC-939 cells.

Name	Sequence 5'-3'	Length(nt)	K_d (nM)
yl15	AGAAGGATGGAGAGGAGCACATGGCGGTACCGTGTGGTGGACGCAGGCGAAATTAATAGGTCCTCGTCTCCTTCCTACT	79	-
yl15a	AGAAGGATGGAGAGGAGCACATGGCGGTACCGTGTGGTGGACGCAGGCGAAATTAATAGGTCCTGCGTCTCCTTCCTACT	80	-
yl19	AGAAGGATGGAGAGGAGCACACAATTGGGCGGGGTGTTGACGGGGGAGTTTAAAGAAGTGGTCCTCGTCTCCTTCCTACT	80	42.44±11.82
yl19a	ACACACAATTGGGCGGGGTGTGT	23	82.49±23.22
yl23	AGAAGGATGGAGAGGAGCACCTAAGCGGGGTTCGAAGTATTCTTATCTTTGCCTGGATTGTCCTCGTCTCCTTCCTACT	79	87.45±15.50

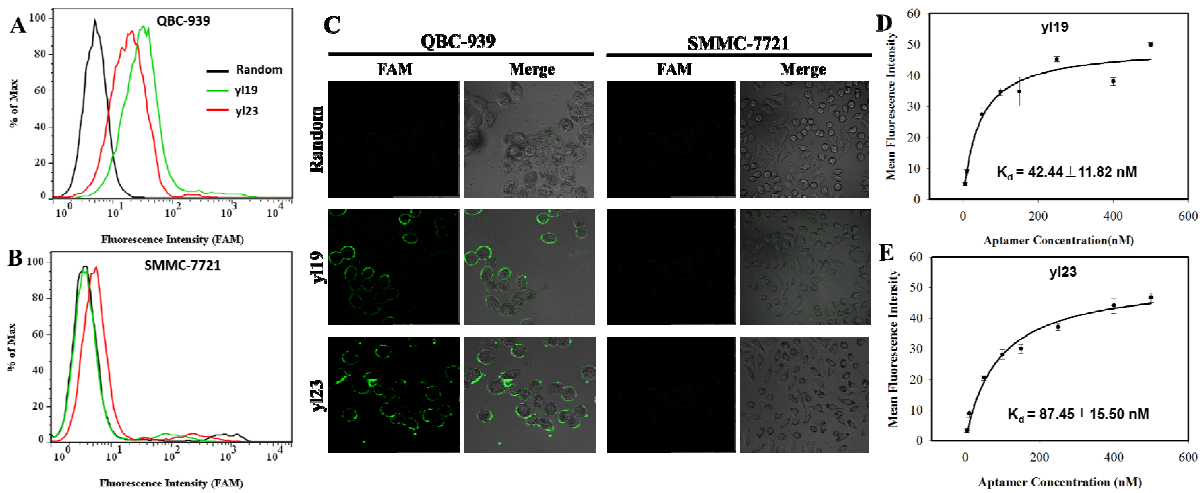


Fig. 2 Recognition of the QBC-939 cells with aptamers. Flow cytometric assay of selected aptamers binding to target QBC-939 cells (A) and negative control SMMC-7721 cells (B), Blank is the background fluorescence of untreated cells, Random is FAM-labeled unselected DNA library as negative control. (C) Confocal imaging of cells stained by the FAM-labeled aptamers or random sequences, and the aptamers had obvious cell membrane binding ability. (D, E) Flow cytometric assay to determine the binding affinity of the FAM-labeled aptamers yl19, yl23 to QBC-939 cells, respectively.

while we comparing the simulated secondary structures, yl15 and yl19 attracted our attention, because they had very similar structure but exhibited totally opposite target binding ability. These two sequences both have two hairpins on a big loop, among which the structure of hairpins Ⅱ and Ⅳ is highly resembled, while hairpins Ⅰ and Ⅲ have huge difference (Fig. 3A).

Therefore, we firstly reconstructed hairpin Ⅱ in yl15 to mimic hairpin Ⅳ, the resulting yl15a still hardly bound to target cells (Fig. 3B), implying that the structural difference between hairpin Ⅱ and Ⅳ isn't the key factor that caused yl15's poor target binding ability. Meanwhile, yl19a was designed on the basis of hairpin Ⅲ to testify the role of hairpin Ⅲ played in target recognition. As expected, yl19a is comparable to yl19 in terms of fluorescence intensity and K_d value (Fig. 3B and Fig. 3C). What's more exciting was that yl19a only consists of 23 nucleotides and has a very simple hairpin structure, these unique properties giving it tremendous advantages in synthesis and design for the future application.

Investigation on the specificity of aptamers

To inspect the selectivity of the selected aptamers, FAM-labeled aptamers were tested with different human cancer cell lines, including cervical cancer cell line HeLa; hepatocellular carcinoma cell lines HepG2, Bel-7404 and SMMC-7721; nasopharyngeal carcinoma cell line CNE2 and adenocarcinoma cell line A549. As shown in Fig. 4, aptamers yl19, yl19a, yl23 all showed strong binding to the target QBC-939 cells but presented no significant recognition on other cancer cells, demonstrating their excellent specificity. It is worth noting that all the three liver cancer cell lines cannot be recognized by the selected aptamers, which is probably because hepatocellular carcinoma SMMC-7721 cells were employed in the counter selection process. The excellent specificity of these aptamers is of great value for their future application in diagnosis and targeted therapy.

Identification of the aptamers' target type

Because the endocytosis activity of cells was suppressed at 4 °C during the selection, the aptamers are most likely to target molecules on cell surface. In order to investigate the molecule type of aptamers' target, QBC-939 cells were treated with proteinase K before they were incubated with FAM labelled aptamers and analyzed using flow cytometry. As presented in Fig.5, after treatment with proteinase K for 10 min, the fluorescence intensity on target cells with aptamers yl19, yl19a and yl23 all showed a conspicuous decrease compared with no treatment, indicating that the enzyme degraded the target molecules on the cell surface. Hence, the target molecule is most likely to be membrane protein. Therefore, these aptamers hold great promise for promoting

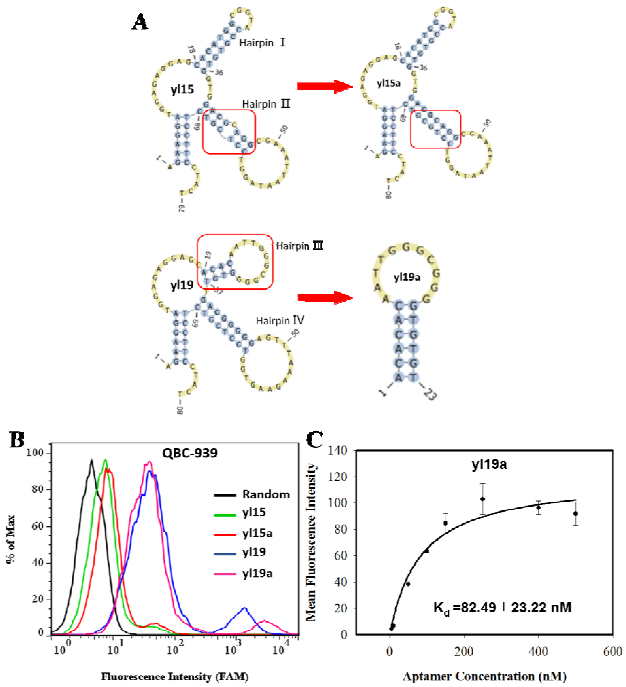


Fig. 3 Sequence optimization of aptamer yl19. (A) Prediction and comparison of the secondary structure of yl15 and yl19. Yl15a and yl19a were further constructed after structure analysis. (B) Flow cytometric assay to compare the target binding ability between the optimized sequences (yl15a and yl19a) and original sequences (yl15 and yl19). (C) The determination of yl19a's binding affinity.

cholangiocarcinoma biomarker identification and consequently facilitating our understanding of this disease.

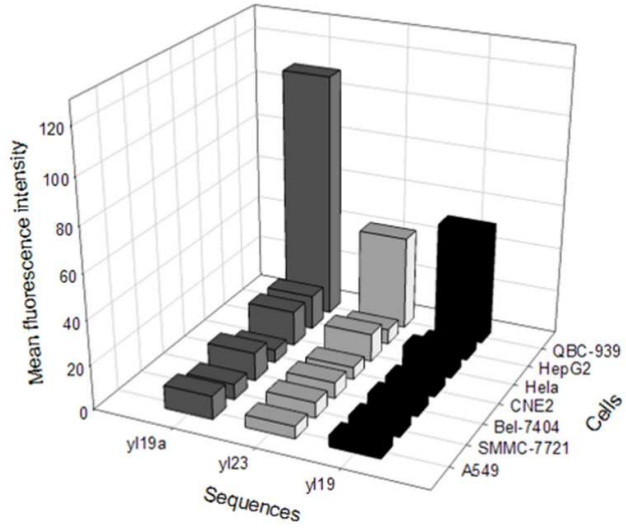


Fig. 4 Binding specificity study of aptamers yl19, yl19a, and yl23 to different cancer cell lines. The fluorescence was recorded by flow cytometry using the same method mentioned above.

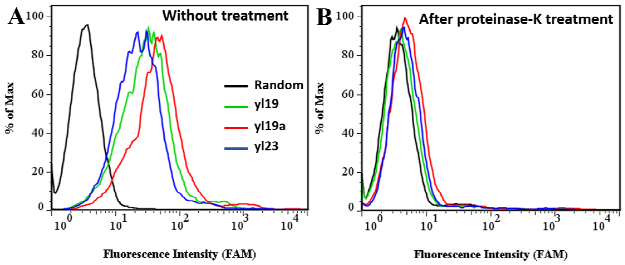


Fig.5 The binding of selected aptamers to QBC-939 cells pre-treated with proteinase-K. After the cells were treated with proteinase-K, the fluorescence intensity showed a conspicuous decrease, indicating the loss of target molecule on the surface of target cells.

Conclusions

In summary, we have successfully performed cell-SELEX against cholangiocarcinoma cell line QBC-939. After a dramatic analyzing process combining sequence homology analysis and secondary structure analysis together, aptamers with high affinity were successfully identified. The K_d values of these newly developed aptamers are in the low nanomolar range, and a 23-nt truncated sequence was further obtained by aborative analysis on their secondary structures. What's more, because hepatocellular carcinoma SMMC-7721 cells were employed in the counter selection, the achieved aptamers demonstrated excellent specificity to target QBC-939 cells, especially no binding to several hepatocellular carcinoma cell lines was observed. Moreover, the target of these aptamers was preliminarily identified to be membrane protein. Taking all these together, we have achieved a panel of aptamers with potential of promoting cholangiocarcinoma early diagnosis and therapeutics.

Acknowledgements

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Graphical abstract

Aptamers recognizing human cholangiocarcinoma QBC-939 cells with high affinity and specificity were successfully generated via cell-SELEX.