Acta Biochim Biophys Sin 2014, 46: 128–135 | © The Author 2013. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmt130.
Advance Access Publication 2 December 2013

Original Article

Screening and verification of ssDNA aptamers targeting human hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is one of the most malignant cancers in the world. Molecular probes that can recognize biomarkers specific for HCC are urgently needed to improve the sensitivity and specificity of early diagnosis. A recent study has applied the method of systematic evolution of ligands by exponential enrichment and produced several aptamers that can bind specifically to mouse liver cancer cells and tissues. However, the binding affinity to human liver cancer has not been fully identified. Using human-derived hepatoma cell line HepG2 as positive target cell line and normal hepatocyte cell line HL-7702 as negative one, we obtained an aptamer HA09 that could specifically bind to human liver cancer cells with K_d in the nanomolar range and recognize paraffin-embedded human HCC tissues. This aptamer may facilitate the discovery of novel biomarkers and serve as an ideal molecular probe for intracellular delivery with both diagnostic and therapeutic implications.

Keywords DNA aptamer; cell imaging; liver cancer; drug delivery

Received: August 22, 2013 Accepted: October 18, 2013

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent cancers with high morbidity and high mortality [1]. Viral hepatitis is the major risk factor for HCC worldwide. The development of HCC in chronic viral hepatitis is a long-term process, which provides possibilities for early diagnosis. Alpha-fetoprotein determination and ultrasound have been employed in screening for HCC at an early stage. Nevertheless, the sensitivity and specificity are low in surveillance and early diagnosis of HCC [2,3]. Because of the lack of accurate diagnostic methods, most HCC cases are detected at intermediate or advanced stages, and the therapeutic efficacy is unsatisfactory. In this context, identification of novel cancer-specific biomarkers is essential to the detection of small HCC and to the improvement of survival rates.

Aptamers, which are selected by an *in vitro* process known as systematic evolution of ligands by exponential enrichment (SELEX), are single-stranded DNA (ssDNA) or RNA oligonucleotides that can bind to metal ions, polypeptides, proteins, or even whole cells with high selectivity, affinity, and stability [4]. In contrast to antibodies, aptamers have many advantages, including wide range of targets, quickness and reproducibility in synthesis, non-toxicity and lack of immunogenicity, and easiness and controllability in modification [5]. In tumor research, aptamer technology has turned out to be of great value. There have been some breakthroughs in the research of tumor-specific aptamer screening, biomarker discovery, and targeted imaging and therapy [6,7].

To cancer cells with unknown molecular biomarkers, it has been proven to be difficult to systematically produce a panel of antibodies for molecular profiling [8]. Using cell-SELEX, which starts with a random library of 10^{13}–10^{16} ssDNA molecules and is followed by SELEX targeting a whole live cell, it can generate multiple aptamers for specific cell recognition [9]. Importantly, with this method, aptamers can be isolated without prior knowledge of numbers or types of membrane proteins in their native conformation [8]. Based on cell-SELEX, Shangguan *et al*. [10] screened out and validated several liver cancer-specific aptamers. However, both the target and control cell lines of the selection were derived from BALB/cJ mice, not human, and

the binding affinity of these aptamers to human liver cancer cells was unsatisfactory.

Herein, we used two human-derived cell lines, one hepatoma cell line HepG2 and one normal hepatocyte cell line HL-7702, to obtain and validate aptamers that specifically recognized human liver cancer, hoping to get an ideal tool for the study of cancer recognition, targeted drug delivery, or even molecular mechanism of liver carcinogenesis.

Materials and Methods

Cell lines and reagents

HepG2, Huh7, QGY-7703, HL-7702, HGC-27, AGS, GES-1, and BEAS-2B were cultured in Dulbecco's modified Eagle's medium with high glucose (DMEM-h) (Gibco, Gaithersburg, USA) supplemented with 10% fetal bovine serum (FBS; Gibco). SW116, SW480, and H1299 were cultured in RPMI 1640 (Gibco) supplemented with 10% FBS. Before and after the incubation in SELEX, cells were washed with washing buffer [4.5 g/l glucose and 5 mM $MgCl_2$ in Dulbecco's phosphate-buffered saline (PBS) (Gibco)]. During the incubation, the binding buffer [0.2 mg/ml yeast tRNA (Roche, Basel, Switzerland) and 2 mg/ml bovine serum albumin (BSA; Sangon, Shanghai, China) in washing buffer] was used to reduce background binding.

SELEX library and primers

The high-performance liquid chromatography-purified ssDNA library contained a central randomized sequence of 40 nucleotides flanked by two 20 nucleotide primer hybridization sites (5′-ACGCTCGGATGCCACTACAG-N40-CTC ATGGACGTGCTGGTGAC-3′). A Carboxyfluorescein (FAM)-labeled 5′ primer (5′-FAM-ACGCTCGGATGCCA CTACAG-3′), and a biotin-labeled 3′ primer (5′-biotin-GTCACCAGCACGTCCATGAG-3′) were used in the polymerase chain reaction (PCR) for the synthesis of double-labeled, double-stranded DNA molecules.

SELEX procedures

The cell-SELEX was carried out based on the previous report [11]. Approximately 250 pmol (10 nmol for the first round) of ssDNA library/pool was dissolved in 400 μl washing buffer, denatured at 95°C for 5 min and quickly cooled on ice for 10 min. HL-7702 cells (5×10^6) were washed once with washing buffer and then suspended in 500 μl binding buffer with an addition of 100 μl FBS. They were further incubated with the ssDNA pool in an orbital shaker for 1 h at 4°C. The incubation time increased with 5 min per round of SELEX, until 1.5 h. After the incubation, the supernatant was absorbed and centrifuged at 8000 *g* for 10 min at 4°C. The supernatant containing the ssDNA sequences was incubated with 5×10^6 HepG2 cells in an orbital shaker for 1 h at 4°C. The incubation time decreased with 5 min per round of SELEX, until 30 min. After that, the cells were washed three times to remove unbound ssDNA sequences. The bound DNA sequences were eluted with 1 ml washing buffer by heating at 95°C for 5 min and then centrifuged. The bound sequences were amplified by PCR using FAM- and biotin-labeled primers. The selected sense ssDNA strands were separated from the biotinylated antisense ssDNA by alkaline denaturation (0.2 M NaOH) after affinity purification with streptavidin-coated Sepharose beads (GE Healthcare, Wisconsin, USA). After desalination by NAP-5 columns (GE Healthcare), the pool of ssDNA strands was centrifugal dried (DNA 120 SpeedVac Concentrator; Thermo Scientific, Waltham, USA) for the next round of selection. The entire selection procedure was repeated according to the extent of enrichment. The enriched pool was amplified by PCR using unlabeled primers. The PCR product was cloned into *Escherichia coli* DH5α using pMD19 T-vector (TaKaRa, Dalian, China) and the positive clones were sequenced and aligned by the software of MegAlign.

Monitoring the enrichment of aptamer pools by real-time PCR

Real-time PCR was carried out using SYBR green PCR master mix (10 μl reaction volume) according to the manufacturer's protocol in an Applied Biosystems 7500 sequence detection system (Applied Biosystems, Foster City, USA). The melting curve was obtained from 7500 software v2.0.1 and used as the indicator of the enrichment of the aptamer pools.

Monitoring the enrichment of aptamer pools by flow cytometry

FAM-labeled ssDNA pool (25 pmol) was dissolved in 50 μl washing buffer and then incubated with 3×10^5 target or negative control cells in 50 μl binding buffer containing 20% FBS for 30 min at 4°C. The cells were washed and resuspended in 300 μl washing buffer. The fluorescence intensity was determined with a FACScan cytometer (Beckman, Pasadena, USA) by counting 10,000 events. The unselected library was used as a blank control.

Determination of aptamers' affinity and specificity by flow cytometry

To determine the binding affinity and specificity of aptamers, cells (3×10^5) were incubated with FAM-labeled aptamers at varying concentrations in a mixture of washing buffer (50 μl), binding buffer (40 μl), and FBS (10 μl) for 30 min at 4°C. Then, cells were washed and resuspended in 500 μl washing buffer and analyzed by flow cytometry. The unselected library was used as a blank control. The mean fluorescence intensity of target cells was used to describe the specific binding of the labeled aptamers. The equilibrium dissociation constant of the aptamer–cell interaction was

obtained by fitting the dependence of intensity of specific binding on the concentration of the aptamers to the equation $Y = B \max X/(K_d + X)$, using the software of Sigma Plot.

Confocal imaging

Target or negative control cells were incubated with a mixture of FAM-labeled aptamer in 80 μl washing buffer and 120 μl binding buffer containing 20% FBS for 30 min at 4°C. The final concentration of aptamer was 500 nM. The cells were then washed twice with washing buffer and fixed with 4% paraformaldehyde for 30 min. After two washes with PBS, they were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Beyotime, Haimen, China) for 30 min. After another three washes with PBS, they were mounted with glycerin. Cell imaging was performed with a Leica TCS SP5 Confocal microscope (Solms, Germany).

Aptamer-based histochemistry staining

The formalin-fixed and paraffin-embedded tissue sections (patient specimens with hepatocellular, gastric, or colon carcinoma) were obtained from Huashan Hospital according to an Institutional Review Board approved protocol. The aptamer-based histochemistry staining (AptHS) procedure is as follows: following microwave pretreatment in citrate-buffered saline buffer (pH 6.0), sections were blocked with PBS containing 10% BSA, 1 μg/μl yeast tRNA, and 1 μg/μl salmon sperm DNA [both purchased from Sigma-Aldrich (St Louis, USA) and pretreated following the manufacturer's instructions] for 60 min at 4°C. After that, sections were probed with 100 μl FAM-labeled aptamer or unselected library (500 nM in binding buffer) and incubated for 2 h at 4°C. Visualization was achieved using a fluorescence microscope (Nikon ECLIPSE 80i, Tokyo, Japan).

Results

Cell-SELEX and monitoring of the enrichment of the aptamer pools

Hepatoma cell line HepG2 was used as target because of its predominance in the studies of liver cancer. HL-7702, a human normal hepatocyte cell line, was used as negative control to eliminate the collection of DNA sequences that could bind to common surface molecules presenting in both types of cells.

Flow cytometric analysis was carried out to monitor the selection process. As only the FAM-labeled oligos bound to HepG2 were collected, it was observed that the mean fluorescence intensity was increased as selection proceeded in target cells but not in control cells (**Fig. 1A**). There was a clear fluorescence signal shift in the 11th round in HepG2 cells, but was not in HL-7702 cells, indicating that the DNA pool with better binding affinity to the target cells was enriched after 11 rounds of selection.

As an alternative approach to monitor the SELEX procedure, real-time PCR was carried out to confirm the enrichment of the aptamer pool. The melting curves of the products from the ssDNA pools (**Fig. 1B**) showed that the melting temperature rose as selection rounds increased until the 11th round and after that it gradually decreased. It suggested that the enriched aptamer pool was finally achieved after 11 rounds of selection, which was consistent with what we had observed in flow cytometric analysis.

Binding ability of the candidate aptamers

After 11 rounds of selection, the enriched aptamer pool was cloned and 50 randomly selected clones were sequenced. The sequences were grouped based on their homology.

Three sequences that repeated most in clones were selected as candidates for further characterization (**Table 1**). Among them, HA05 repeated four times and the other two repeated three times. The binding affinities of the aptamer candidates to HepG2 cells and HL-7702 cells were evaluated by flow cytometric analysis. All of these three sequences showed apparent binding ability to HepG2 cells, but did not to HL-7702 cells (**Fig. 2A**). We also calculated the equilibrium dissociation constant [11] of the aptamer–HepG2 interaction to measure their binding ability quantitatively. HA09 showed the highest affinity with the K_d in the nanomolar range ($K_d = 103.61 \pm 35.05$ nM) (**Fig. 2B**). Furthermore, HA09 was found to have a good binding ability to other two human hepatoma cell lines: QGY-7703 ($K_d = 35.8 \pm 31.0$ nM) and Huh7 ($K_d = 190.2 \pm 50.9$ nM) (**Fig. 2B**). Thus, it was identified as the target aptamer for further studies.

Specificity of the selected aptamer

We observed the specificity of HA09 by flow cytometry and Confocal imaging. Several other types of cell lines were incubated with FAM-labeled HA09 and the mean fluorescence intensity was measured by flow cytometry. No increased fluorescence was observed in these cells after incubation (**Table 2**), implying that HA09 was highly specific to liver cancer cells.

Confocal imaging was used to visualize the specific binding sites of HA09 on cell surface. HepG2, Huh7, and QGY-7703 incubated with FAM-labeled HA09 displayed obvious fluorescence, while HL-7702 did not (**Fig. 3**). None of these four types of cells showed significant fluorescence after incubation with FAM-labeled unselected library.

Recognition of the HCC tissue sections with the selected aptamer

To validate whether the selected aptamer HA09 could recognize human HCC tissues, the formalin-fixed and paraffin-embedded sections of human liver cancerous and pre-cancerous tissues, gastric cancerous tissues, and colon cancerous tissues were stained with the FAM-labeled HA09.

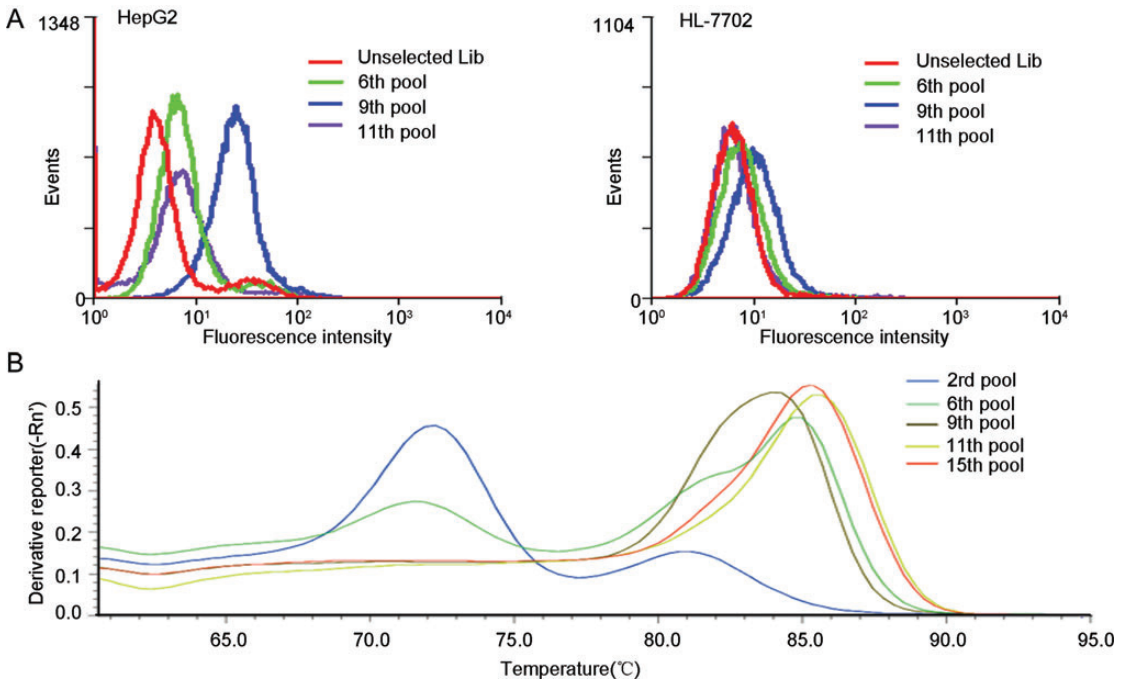

Figure 1. Aptamer pools are enriched after 11 rounds' selection (A) Binding ability of the selected pools to HepG2 and HL-7702 cells was tested by flow cytometric assay. The final concentration of aptamer is 250 nM. (B) Melting curves of real-time PCR products of the ssDNA pools were obtained to evaluate the diversity of selected pools.

Table 1 Sequences of the aptamer candidates

Name	Sequence
HA05	ACG CTC GGA TGC CAC TAC AGG CCC CAA CTA GGT TAA AAG TAT CAC ATT CGA GGT GCT ATG CTC ATG GAC GTG CTG GTG AC
HA09	ACG CTC GGA TGC CAC TAC AGG ACA GGG CAC CAC CAT AGT ACC TGT ACT ACC CAG CCG AGG CTC ATG GAC GTG CTG GTG AC
HA15	ACG CTC GGA TGC CAC TAC AGC TGG GCA CCA TGC AGG TTT GTC ACG TTT ATC GCG TAA TGG CTC ATG GAC GTG CTG GTG AC

HA09 showed apparent stronger fluorescence in liver cancerous tissue sections than that in pre-cancerous tissue sections, gastric cancerous tissue sections, or colon cancerous tissue sections (**Fig. 4**). Very weak fluorescence was observed when all tissue sections were stained with FAM-labeled unselected library as control.

Discussion

Liver cancer is one of the most malignant cancers in the world. Most of HCC patients were diagnosed at a later stage for the lack of effective approaches of early detection. Novel biomarkers are urgently needed to improve the sensitivity and specificity of early diagnosis of HCC.

In recent years, several biomarkers of HCC, such as Golgi protein 73 [12] and heat-shock protein 27 [13], have been identified by 2D gel electrophoresis and mass spectrometry. But the specificity and sensitivity of these biomarkers are relatively low [14,15], partially due to the fact that their discovery is based on proteins aberrantly expressed in HCC that are obtained after cell lysis along with the destruction of protein conformation. Traditional methods to seek for biomarkers always need rough treatments with cells resulting in the loss of some membrane proteins [16]. In fact, the change of membrane proteins is associated with carcinogenesis [17,18]. Abnormal membrane proteins are proven to be ideal biomarkers in tumor diagnosis, treatments, and prognosis. Hence, it is indispensable to adapt new strategies that can keep membrane proteins in their native conformations to explore biomarkers that can be recognized by molecular probes.

In this study, we applied an approach named cell-SELEX and generated molecular probes effectively recognizing unknown biomarkers on the surface of human liver cancer cells. By enriching ssDNA oligonucleotides that only bind to target cells, this technology has prompted the progress of molecular recognition and biomarker identification. For instance, Shi *et al*. [19] designed an activatable aptamer probe, which targeted the cell membrane protein tyrosine kinase-7 of living cancer cells and achieved contrast-enhanced cancer visualization inside mice. Berezovski *et al*. [20] introduced a new technology termed aptamer-facilitated biomarker

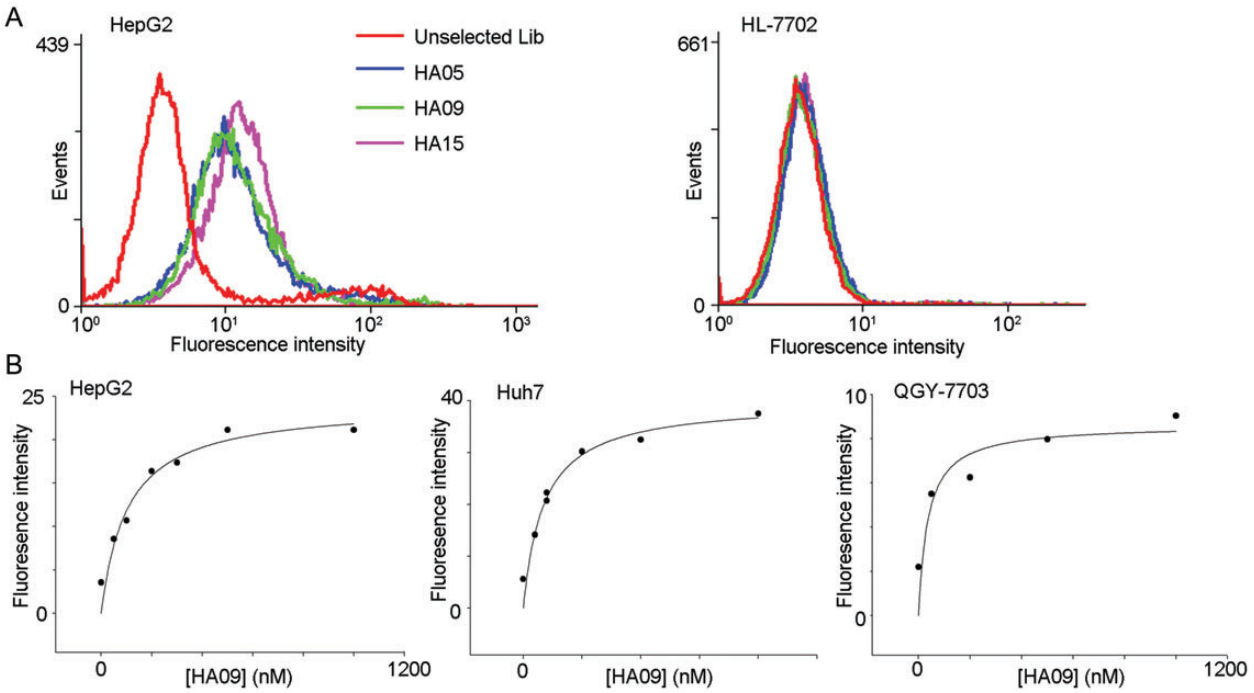

Figure 2. The selected aptamers can bind to human liver cancer cells with high affinity and specificity (A) Binding ability of HA05, HA09, and HA15 to HepG2 (target cells), HL-7702 (control cells). The final concentration of aptamer is 200 nM. (B) Binding curves of HA09 to HepG2, Huh7, and QGY-7703 cells.

Table 2 Aptamers binding to multiple types of cell lines

Cell line	HA09
HepG2 (target cell)	+++[a]
HL-7702 (control cell)	−
Huh7 (liver cancer cell)	++
QGY-7703 (liver cancer cell)	+
AGS (gastric cancer)	−
HGC-27 (gastric cancer)	−
GES-1 (normal gastric epithelial cell)	−
SW116 (colon cancer cell)	−
SW480 (colon cancer cell)	−
H1299 (lung cancer cell)	−
BEAS-2B (normal lung epithelial cell)	−

[a]A threshold based on fluorescence intensity in the flow cytometric analysis was set so that 95% of cells incubated with the FAM-labeled unselected library would have fluorescence intensity below it. The percentage of cells incubated with FAM-labeled aptamer above the set threshold was used to assess the binding ability of the aptamer to the cells: −, <10%; +, 10%–35%; ++, 35%–60%; +++, 60%–85%; ++++, >85% [10]. The final concentration of aptamer is 200 nM.

discovery and discovered surface biomarkers distinguishing mature and immature dendritic cells. Hence, our findings may help to achieve the target imaging of HCC and to discover novel cancer biomarkers.

In a previous study, Shangguan *et al.* [10] used a pair of murine cell lines to produce aptamers specific for liver cancer cells. Despite that only about 300 genes of both the human and mice genomes appear to be unique to one species or the other [21], there are significant differences between the mice and the humans in tumor transformation, development, and responses to drugs. In their study, only one human hepatoma cell line was used to test the binding ability of these aptamers and most of them displayed poor affinities. It is unclear whether these aptamers could recognize human liver cancer tissue. In addition, most of the cell lines used in the specificity test were suspension cells that are quite different from solid tumor cells, making the results not so convincing. Consequently, it is of great necessity to screen aptamers directly against human-derived liver cancer cells. Thus, we chose human-derived cells HepG2 and HL-7702 to carry out the selection process, which ensured a high probability of acquiring human liver cancer-specific aptamers. Besides, multiple types of human cell lines were included in the evaluation of specificity to bring a more holistic assessment of the selected aptamer.

Our results clearly showed that the DNA pool was enriched after 11 rounds of selection, which was supported both by flow cytometric analysis and real-time PCR. Flow cytometric analysis is seen as an ideal way to monitor the enrichment process because of high reproducibility, good statistical precision, convenient detection, and the quantitative nature of the analysis [10]. However, its procedure is time-consuming. Meanwhile, according to our experience, its outcome is also quite unstable. It is even altered when flow rate is changed,

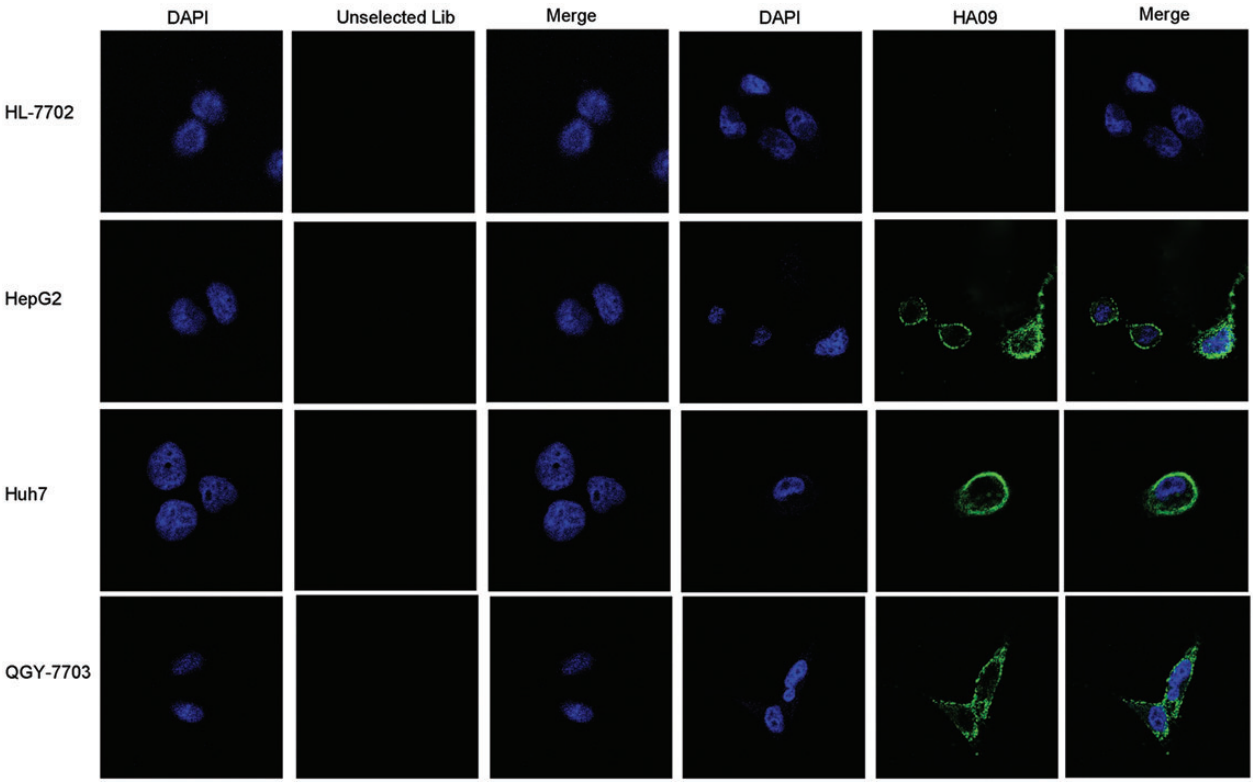

Figure 3. HA09 can bind to the membrane surface of liver cancer cells Fluorescence Confocal imaging of HL-7702 cells, HepG2 cells, Huh7 cells, and QGY-7703 cells stained by FAM-labeled unselected ssDNA library and FAM-labeled HA09, respectively. DAPI was used to label the cell nucleus (blue). The final concentration of aptamer is 500 nM.

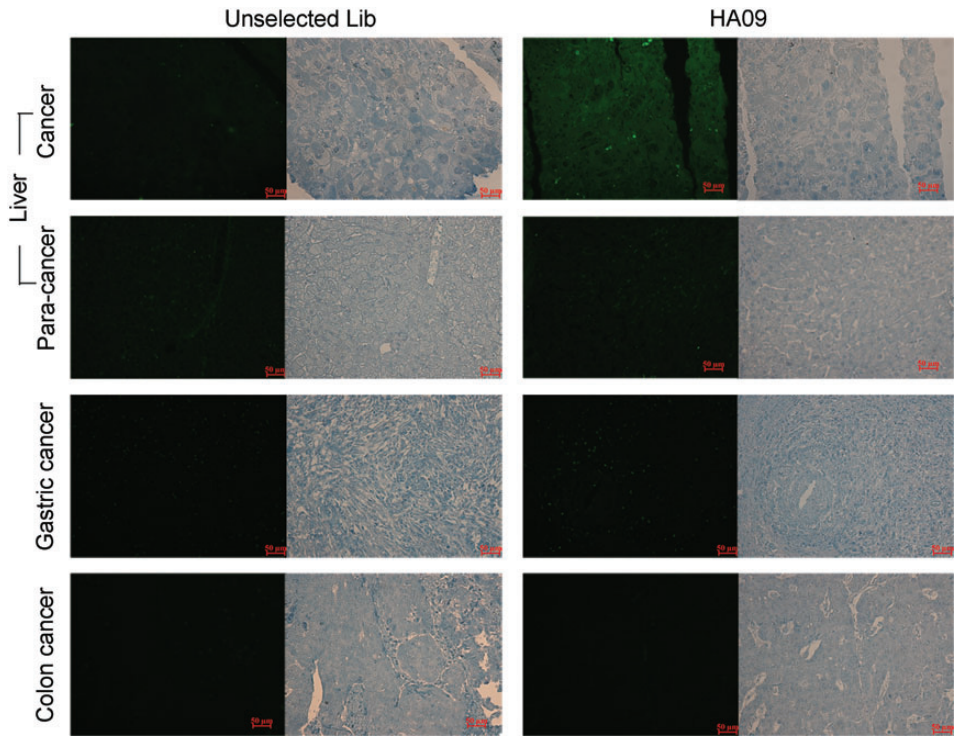

Figure 4. HA09 can recognize human liver cancerous tissue specifically Use of HA09 to recognize human liver cancerous tissue (top) and para-cancerous tissue, gastric cancer tissue, and colon cancer tissue (bottom). The tissue sections of each row were from the same patient. The final concentration of aptamer is 500 nM.

thus the result is somehow questionable. It was reported that DNA templates with different complexity had different dissociation curves [22]. The more specific the template, the sharper the dissociation peak, or in other words, the higher the melting temperature. Since the melting temperature can be viewed as the indicator of sequence complexity that decreases as the aptamer selection proceeds, it is believed that real-time PCR used to detect the dissociation curves of aptamer pools is feasible to monitor the selection, as the procedure is much easier, clearer, and cost-efficient. In this study, we applied this new method and found that the melting temperature reached its highest in the 11th round, suggesting that the enrichment of aptamer pools was accomplished after 11 rounds of selection. This result was consistent with what we had observed by flow cytometric analysis, indicating that real-time PCR could be an alternative monitoring approach.

Besides the 11th round, we also cloned and sequenced the DNA pool of the 21th round and then grouped the DNA sequences on the basis of the homology. There was one sequence with at most eight repeats from the DNA pool of the 21th round, compared with that with four repeats from the 11th round. The sequence that repeated eight times, however, could not recognize liver cancer cells (data not shown). The reason was believed to be amplification bias. During the SELEX, sequences achieved in each round of selection were amplified by PCR. Of these sequences, some are more easily amplified while others are not. With the processing of SELEX rounds, sequences with higher binding affinity may be lost because of amplification bias, leading to the failure of selection.

Aptamers can be chemically synthesized, easily labeled with fluorescent molecules, and directly used in the detection of the target cell. Confocal imaging of liver cancer cells incubated with FAM-labeled aptamers showed that HA09 could bind to cell surface, which accorded with the selection strategy in which membrane proteins were expected to be the target. Hence, cell-SELEX is a good way to screen aptamers for cell imaging, drug delivery, and biomarkers discovery, while further efforts are needed to focus on the identification of the target of HA09.

Immunohistochemistry staining, also called antibody-based histochemistry staining, is an effective and reliable method to diagnose diseases, especially cancers. Nowadays, scientists have begun to seek for new probes that are easier to obtain to serve as a supplementary or even major diagnostic tool. Zeng *et al*. [23] developed an RNA-based CD30 aptamer for the staining of formalin-fixed and paraffin-embedded lymphoma tissues and they demonstrated a nearly similar specificity of both the CD30 aptamer and the CD30 antibody to $CD30^{+}$ lymphoma cells. In this study, we evaluated the potential of HA09 in the clinical recognition of HCC tissue samples by AptHS and we observed positive staining within tumor sites. The negative staining of the liver pre-cancerous tissue, gastric cancerous tissue, and colon cancerous tissue further proved the liver cancer specificity of the aptamer, promising its prospect as a molecular probe. When the target of the aptamer is validated, we may compare the sensitivity and specificity of the aptamer with that of the antibody.

In conclusion, in this study, we obtained one aptamer targeting human liver cancer based on the cell-SELEX method. The aptamer HA09 showed excellent binding affinity to both liver cancer cells and tissues, but did not to other kinds of cancers. Therefore, as a novel molecular tool, HA09 can be used to facilitate the discovery of new biomarkers and also to deliver agents of interest such as fluorescent molecules or chemotherapeutic drugs for target imaging or therapy. Further study is still needed to boost the application of HA09 in basic research as well as clinical diagnosis and therapy of liver cancer.

Acknowledgements

We thank Drs Li Xu and Xiaohong Fang (from Institute of Chemistry, Academy of Sciences, China) for their help in the designing of SELEX protocol. We thank Drs Shaohua Li and Ningsheng Shao (from the Academy of Military Medical Sciences, China) for their presenting of the protocol of AptHS. We also thank Dr Zhaofeng Luo (from University of Science and Technology of China) and Dr Eli Gilboa (from University of Miami) for their ideal suggestion in the analysis and interpretation of the results.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (Nos. 81125001, 91129702, and 81161120431), the Ministry of Science and Technology of China (No. 2010CB732405), and the Science and Technology Commission of Shanghai Municipality (Nos. 09JC1403000 and 10XD1400800).

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