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Recognition and capture of metastatic hepatocellular carcinoma cells using aptamer-conjugated quantum dots and magnetic particles

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ABSTRACT

Metastatic recurrence is the most important biological behavior of hepatocellular carcinoma (HCC) and the main cause of treatment failure. Early prediction of metastasis is currently impossible due to the lack of specific molecular probes to recognize metastatic HCC cells. Aptamers have recently emerged as promising potential molecular probes for biomedical applications. Two well-matched HCC cell lines including HCCLM9 with high metastatic potential and MHCC97-L with low metastatic potential, were used to select aptamers for HCC metastasis. With a whole-cell-SELEX strategy, in which HCCLM9 cells were used as target cells and MHCC97-L cells as subtractive cell, 6 potential aptamers had been generated. Detailed study on selected aptamer LY-1 revealed that it could bind metastatic HCC cells with high affinity and specificity, not only in cells culture and animal models of HCC metastasis, but also in clinical HCC specimens. Moreover, the aptamer LY-1 and magnetic particles conjugates could efficiently capture the HCC cells from complex mixture whole blood. These studies demonstrated that this HCC specific aptamer LY-1 could be a promising molecular probe to recognize metastatic HCC cells.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common and highly malignant tumors worldwide, accounting for about 6% of all cancers [1,2]. The disease is particularly common in China, which accounts for 53% of the global HCC cases. Although significant progress has been made in basic researches in HCC, the treatment efficacy remains poor, with 5-year survival rate around 5% [2,3]. For patients with early HCC with tumor nodule < 5 cm in diameter, the best treatment is resection with curative intent. Even for such patients, however, the 5-year recurrence rate is as high as 40–50% [3]. Post-operative recurrence and metastasis has long been the most difficult obstacle to durable treatment efficacy and long-term survival.

Currently, medical imaging exams and serum detection of alpha-fetoprotein (AFP) levels are major techniques to monitor HCC recurrence and metastasis. But, AFP-positive HCC only accounts for 70–80% of HCC and ultrasound cannot detect tumor < 1 cm in diameter [4]. Therefore, there is an urgent need for new tumor markers for clinical application.

So far, both genomics and proteomics approaches have been used to search for HCC metastasis-related markers [5,6], resulting in deep insights into the mechanism of HCC recurrence and metastasis.

It has been suggested that HCC metastasis may occur at the early stage of cancer development [3], and it involves multiple factors and through many stages, including tumor cell proliferation, adhesion, invasion, extracellular matrix degradation, angiogenesis and cell motility. In general, HCC metastasis is influenced by the constant interactions between HCC cells and the tumor microenvironment, resulting in particular tumor cell molecular phenotypes and biological characteristics favoring metastatic progression [7,8]. Indeed recent studies have shown the significance of tumor cell surface molecules in HCC metastasis, such as E-cadherin, β-catenin, laminin, CD44 and ICAM-1, which are important for tumor cell adhesion, invasion and movement [9–11].

Human HCC cell lines MHCC97-L with low metastatic potential and HCCLM9 with high metastatic potential are ideal cell models for the comparative study of HCC metastasis [12]. Both cell lines

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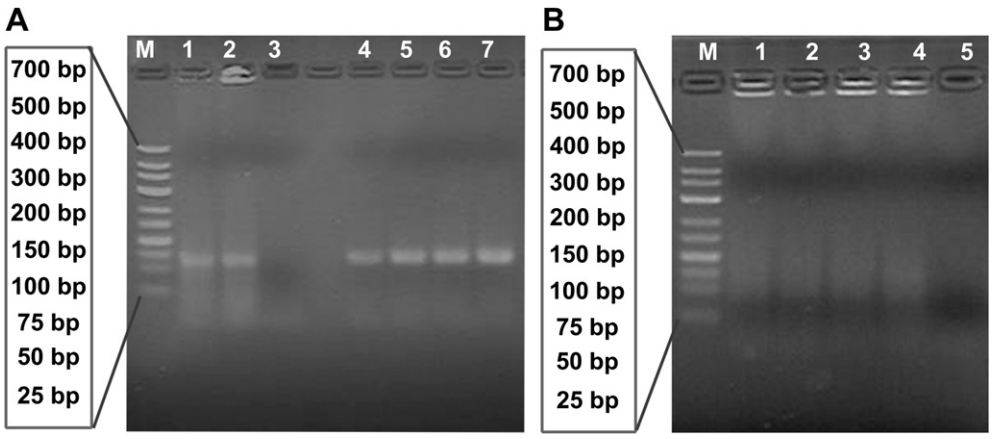

Fig. 1. Enrichment of HCC metastasis-related aptamers using subtractive cell-SELEX strategy. (A) After 9 rounds of enrichment, the single strand DNA bound to HCCLM9 was eluted and amplified by PCR to produce double strand DNA for further analysis. (A1), a 76 bp double strand DNA lane was obtained after 8 cycles of PCR; (A2), the same product as A1 after 10 cycles of PCR; (A3), negative control of PCR; (A4), using the double strand DNA from A1 as template, double strand DNA was obtained after 4 cycles of PCR; (A5–A7), the same as A4 after 6, 8 and 10 cycles of PCR, respectively. (B) After 10 rounds of enrichment, the single strand DNA bound to HCCLM9 was eluted and amplified by PCR, but did not produce the 76 bp DNA lane, even after 10, 12, 14 and 16 cycles of PCR (B1-B4). (B5) negative control of PCR. Molecular marker (from top to bottom) was 700 bp to 25 bp.

were derived from the same patient, so they share the same genetic background but have dramatically different metastasis potentials. Therefore, these cell lines could help search for cell surface specific molecules on HCCLM9, for both prediction and treatment of metastasis and recurrence.

SELEX stands for systematic evolution of ligands by exponential enrichment [13], which is an efficient strategy for high throughput in vitro selection of bio-library pool. The technology usually takes repetitive in vitro selection and PCR amplification, to obtain aptamers which are short single-stranded nucleic acid oligomers with a specific three-dimensional configuration, enabling them well-fittingly and specifically bind to target molecules. Aptamers have a wide application in clinical diagnosis, basic researches and drug developments because of their unique features including specific recognition, no immunogenicity, easy chemosynthesis and modification [14]. In recent years, a modified SELEX process using whole living cells as target was developed and designated as cell-SELEX [15], which could select aptamers from unknown complex targets.

Presently there have been no researches on applying subtractive cell-SELEX method to select DNA-aptamers specifically binding to human metastatic HCC cells surface molecules. This study was designed to identify single-strand DNA-aptamers (cell-molecular probe) that specifically recognize surface molecules of HCCLM9 but not MHCC97-L. Subtractive cell-SELEX strategy was used to generate aptamers, using MHCC97-L as subtractive cells and HCCLM9 as target cells. Flow cytometry assay was applied to determine enrichment of aptamers library pool, the binding affinity and specificity of the selected aptamers. Then the candidate DNA-aptamers were conjugated with biotin to form specific bio-probes to recognize HCC cells in cultured cell lines, animal models of HCC and human HCC tissues. Furthermore, the biotin-conjugated aptamer was coupled with magnetic particles to capture HCC cells in experimental conditions mimic human peripheral blood.

2. Materials and methods

2.1. Cell lines

MHCC97-L cell (low metastatic potential, used as subtractive control cell in this study) and HCCLM9 cell (high metastatic potential, used as target cell in this study) were established from the same parent cell line MHCC97 [12]. Other HCC cell lines HepG2 and Huh-7, human breast cancer cell line MDA-MB-231, human lung cancer cell line H1299, Human colon adenocarcinoma cell line SW48, human gastric cancer cell line MGC803, and human cervical cancer cell line HeLa were maintained at our laboratory.

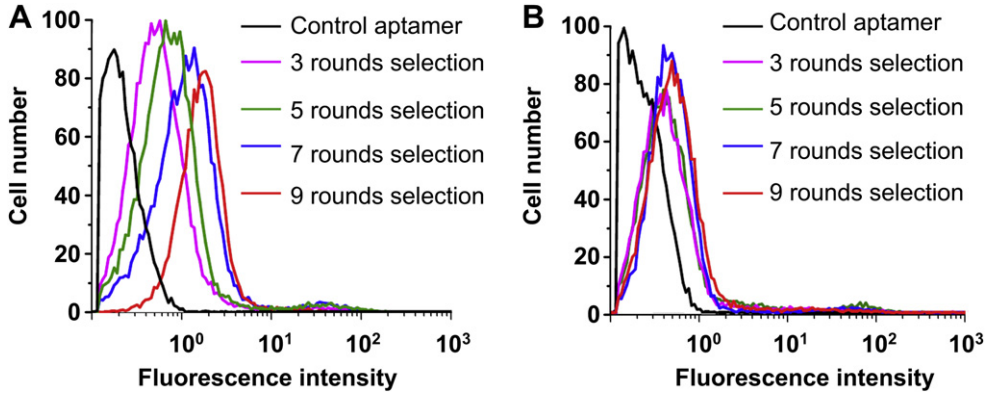

Fig. 2. Flow cytometry assay to monitor the binding of selected library with HCCLM9 (target cells) and MHCC97-L (subtractive cells). With increasing rounds of enrichment, significant increases in fluorescence intensity were detected on HCCLM9 cells (panel A) but not on MHCC97-L (panel B), suggesting the enrichment of HCCLM9 specific aptamers. (A) Increasing fluorescence intensity bound to HCCLM9 with the 3, 5, 7 and 9 rounds of selected library; (B) Fluorescence intensity bound on MHCC97-L with the 3, 5, 7 and 9 rounds selected library by flow cytomtry.

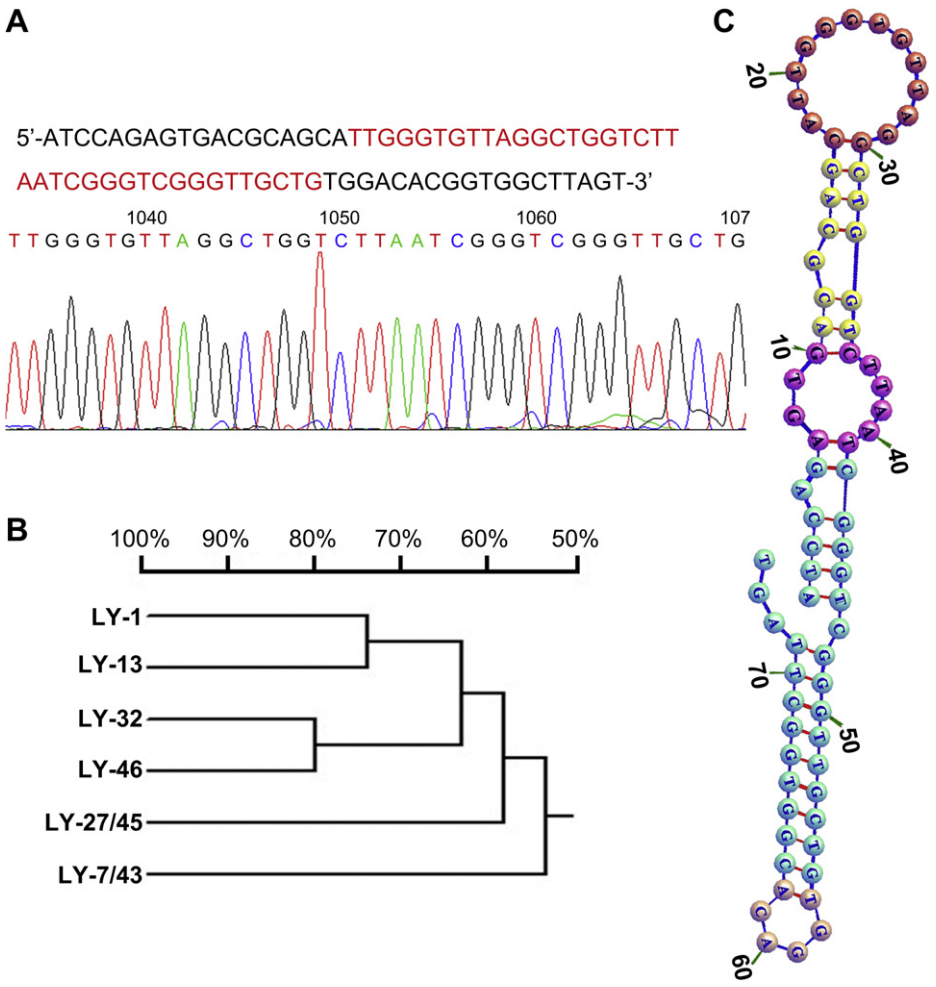

Fig. 3. Multiple sequence alignment analysis, sequencing and structure prediction of aptamer LY-1. (A)The sequence analysis of LY-1 aptamer generated from HCCLM9 cells, with a 38 bp core sequence marked in red font. (B) The homologous analysis of aptamers generated from HCCLM9 cells. (C) The structure prediction of aptamer LY-1 generated from HCCLM9 cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Antibodies and reagents

Rabbit anti-human monoclonal antibody cytokeratin (CK) 19 and mouse anti-human AFP monoclonal antibody were purchased from R&D Systems. PE-CD45 (mouse anti-human) monoclonal antibody was purchased from BD Biosciences. Yeast tRNA and BSA and Salmon sperm DNA were purchased from Fisher Scientific (Thermo Fisher Scientific Inc., USA). Streptavidin coated magnetic particles (Dynabeads, M-280 Streptavidin) used for separating the single strand DNA and capturing cells and QD605-streptiavidine were purchased from Invitrogen. The AmpliTaq Gold 360 PCR Master Mix was purchased from ABI (Applied Biosystems Inc., Foster City, CA). The FITC-labeled control aptamer NK8 (bound to Mycobacterium tuberculosis) [37] was synthesized by SBS Genetech Co., Ltd (Shanghai, China) and purified by reverse phase high performance liquid chromatography (HPLC) (Agilent Technologies, USA).

2.3. Random DNA library and primers

The cell-SELEX DNA library contains a 40-base central random sequence flanked by primer sites on either side (5′-ATC CAG AGT GAC GCA GCA-N40-TGG ACA CGG TGG CTT AGT-3′). The FITC-labeled forward primer (5′-FITC-ATC CAG AGT GAC GCA GCA-3′) and biotin-labeled reverse primer (5′- Bio-ACT AAG CCA CCG TGT CCA-3′) were used in PCR to obtain the double-labeled DNA and to separate the single-stranded DNA by streptavidin-coated magnetic particles. The FITC-labeled sequences were used to monitor progress of selection by flow cytometry (Beckman Coulter, USA). All sequences were synthesized by SBS Genetech Co., Ltd and purified by reverse phase HPLC.

2.4. Cell culture

HCC cell lines MHCC97-L, HCCLM9, HepG2 and Huh-7, human breast cancer cell line MDA-MB-231, human lung cancer cell line H1299, human colon adenocarcinoma cell line SW48, human gastric cancer cell line MGC803, and human cervical cancer cell line HeLa were cultured in high glucose DMEM with 10% FBS and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.5. Subtractive cell-SELEX procedure [15]

In this study, HCCLM9 was used as target cells and MHCC97-L as subtractive cells. Both cell lines were counted and tested for viability before experiments. The

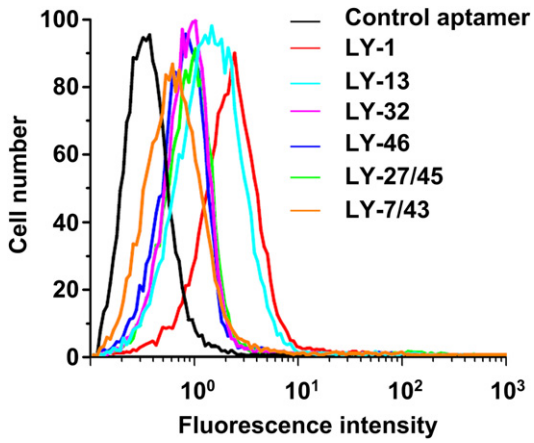

Fig. 4. The fluorescence intensity of the selected aptamers bound to HCCLM9 cells by flow cytometry analysis. Compared with control, all the selected aptamers had increases fluorescence intensity, and the LY-1 the highest.

ssDNA library (8 nmol) was first denatured at 95 °C for 5 min and kept on ice for 10 min, then dissolved in 1 mL pre-cooled binding buffer (PBS- 1 M $MgCl_2$ -0.1 mg/mL yeast tRNA-1 mg/mL BSA-0.1 mg/mL Salmon sperm DNA). Target cells (1×10^7) were washed, dissociated (PBS -0.02% EDTA), and then incubated with the ssDNA library on ice in an orbital shaker for 60 min. After incubation, the cells were washed 3 times to remove unbound DNA sequences. DNase-free water (500 μl) was added to the adhesive cells, which were scraped off, re-suspended and transferred into a 1.5 mL microfuge tube. The cell mixture was heated at 100 °C for 5 min, centrifuged at 13,100 g for 5 min and the supernatant containing eluted ssDNA was collected. The bound sequences were amplified by PCR using FITC- and biotin-labeled primers, using ABI 9600 Cycler (Applied Biosystems Inc., Foster City, CA, USA) at 95 °C for 40 s, 56 °C for 40 s, and 72 °C for 40 s, followed by the final extension for 7 min at 72 °C. The selected sense ssDNA strands were separated from the biotinylated antisense ssDNA by streptavidin-coated magnetic particles (Dynabeads, M-280 Streptavidin, Invitrogen) and alkaline denaturation. From the fourth round of selection, the selected DNA pool was used to perform subtractive selection (the selected DNA pool firstly incubated with subtractive cells, then the supernatant containing unbound ssDNA sequence incubated with target cells) to filter out sequences that may bind to the molecules on both the target and subtractive cell lines. To enrich the aptamers with high affinity and specificity, the washing stringency was enhanced gradually by extending the washing time with increasing volume of washing buffer and increasing the number of washes. Furthermore, the target cell number and the concentration of ssDNA pool and the incubation time were gradually reduced. The entire selection process was repeated according to the extent of enrichment, as monitored by flow cytometry. The detailed procedures were listed in Table 1 (see supplementary data).

2.6. Flow cytometry to monitor the binding of enriched ssDNA pool

To monitor the binding of enriched ssDNA pool during cell-SELEX, FITC-labeled ssDNA pools were incubated with target cells HCCLM9 or subtractive cells MHCC97-L (1×10^6 for each) in 500 μL binding buffer on ice for 30 min. Cells were washed

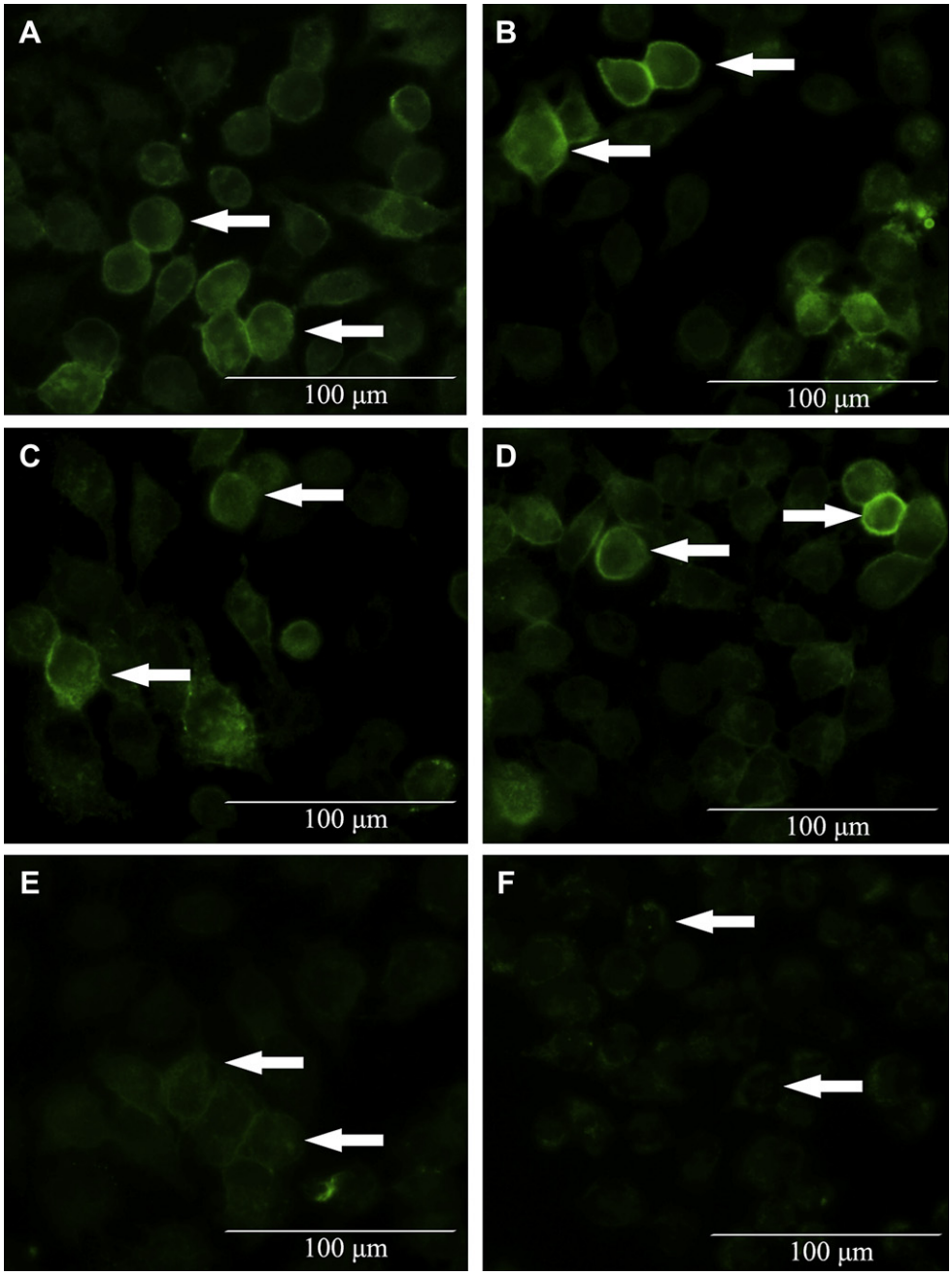

Fig. 5. The fluorescence imaging of selected aptamers bound to HCCLM9 cells by fluorescent microcopy analysis. All the aptamers had obvious cell membrane binding property. (A) The fluorescence imaging of aptamer LY-13 bound to HCCLM9 cells. (B) The fluorescence imaging of aptamer LY-1 bound to HCCLM9 cells. (C) The fluorescence imaging of aptamer LY-32 bound to HCCLM9 cells. (D) The fluorescence imaging of aptamer LY-27/45 bound to HCCLM9 cells. (E) The fluorescence imaging of aptamer LY-46 bound to HCCLM9 cells. (F) The fluorescence imaging of aptamer LY-7/43 bound to HCCLM9 cells.

twice after incubation and the fluorescence intensity was determined by flow cytometry. The FITC-labeled control aptamer NK8 (bound to Mycobacterium tuberculosis) [37] was used as a negative control. The 3, 5, 7, and 9 rounds of enriched ssDNA pool were analyzed by flow cytometry (Beckman Coulter, USA).

2.7. Cloning and sequencing of enriched ssDNA pool and multiple sequence alignment analysis

In this study, 10 rounds of selection were performed. On the basis the flow cytometry results, the enriched ssDNA pool of 9 rounds of selection was PCR-amplified using unmodified primers and cloned into Escherichia coli using the TA cloning kit (Invitrogen). The candidate aptamer sequences were determined by Invitrogen Co., Ltd (Shanghai, China). The sequencing results were subjected to multiple sequence alignment analysis with the DNAMAN (version7 Lynnon Corporation, Quebec, Canada) to identify highly conserved motifs in the selected DNA sequences. The conserved sequences with high repeats among selected pools were then synthesized and tested for specificity and affinity.

2.8. Flow cytometry to study the binding affinity and specificity

The selected aptamers were synthesized and labeled with FITC. The screening of potential aptamers and the binding affinity assay were performed using flow cytometry. The aptamers with the best binding affinity (high fluorescence intensity) were chosen to further identify the binding affinity constant and specificity. To determine the binding affinity of the aptamers, target cells (1×10^6) were incubated with varying concentrations of FITC-labeled aptamer in 500 μL binding buffer on ice for 30 min. Cells were washed twice after incubation and the fluorescence intensity was determined by flow cytometry. The FITC-labeled control aptamer NK8 was used as negative control. All binding assays were in triplicate. The mean fluorescence intensity of the control aptamer NK8 was subtracted from that of the aptamer with the target cells to determine the specific binding of the labeled aptamer. The equilibrium dissociation constant (Kd) 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 $Y = B\ max\ X/(kd + X)$, using Prism software (V5.0 trial, GraphPad Software, Inc, CA, USA). To determine the cell specificity of the selected aptamer, human cancer cell lines including HCC cell lines MHCC97-L, HepG2 and Huh-7, breast cancer cell line MDA-MB-231, lung cancer cell line H1299, colon adenocarcinoma cell line SW48, gastric cancer cell line MGC803, cervical cancer cell line HeLa, and peripheral blood white cells (WBC) were used in binding assays by flow cytometry.

2.9. Verification of the selected aptamer possible binding sites on target cell by flow cytometry

To verify the binding of aptamers to target cell-surface markers, cells were examined by enzymatic treatment. The cultured target cells were washed twice with PBS, then incubated with 1 mL Trypsin-free cell dissociation solution (PBS-0.02% EDTA) or 1 mL Trypsin-free cell dissociation solution (PBS-0.02% EDTA) containing 0.1 mg/mL proteinase K in PBS at room temperature for 10 min. To quench the proteinase digestion, the target cells were quickly mixed with 1 mL pre-cooled PBS (containing 10% FBS). Then the treated cells (1×10^6) were used for the aptamer binding assay by flow cytometry described as above.

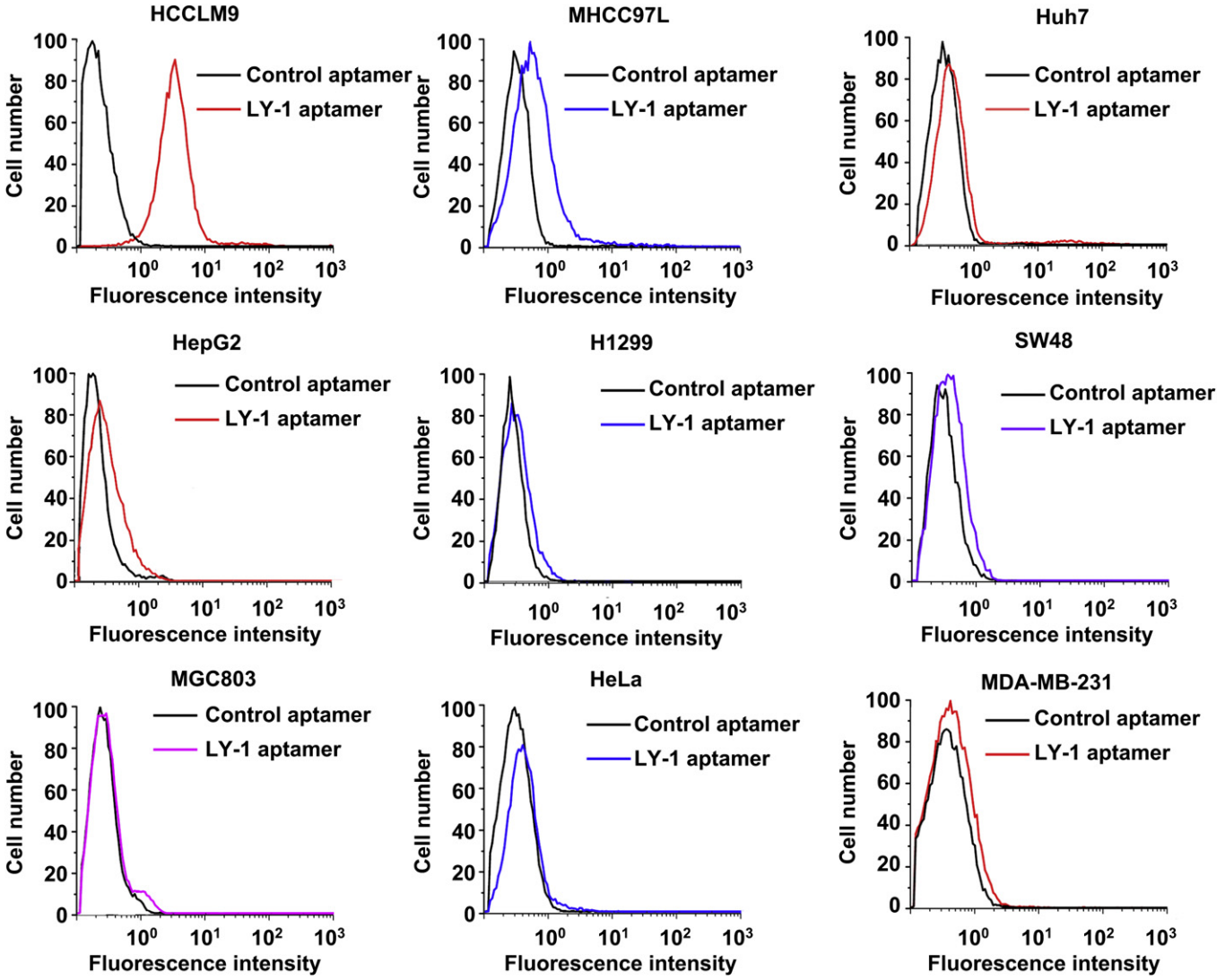

Fig. 6. The binding specificity study of aptamer LY-1 to different cancer cell lines as determined by flow cytometry. There was a significant increase in fluorescence intensity in HCCLM9, but no changes in other cancer cell lines, indicating that aptamer LY-1 was specific to HCCLM9.

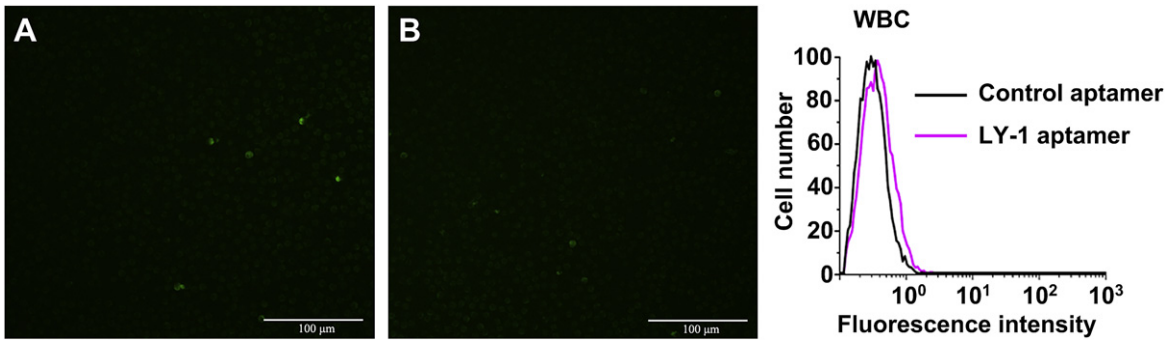

Fig. 7. The fluorescence imaging and fluorescence intensity of selected aptamer LY-1 bound to WBC by fluorescent microcopy and flow cytometry analysis. Both results indicated that LY-1 aptamer was not specific to WBC. (A) The fluorescence imaging of control aptamer unbound to WBC. (B) The fluorescence imaging of aptamer LY-1 unbound to WBC.

2.10. Fluorescence imaging of live cells bound to the selected aptamer

The FITC or biotin 5′-tagged aptamer was synthesized by SBS Genetech Co., Ltd (Shanghai, China). Cells were cultured in chamber slides, grown overnight, and rinsed with PBS. For fluorescence imaging, the selected individual aptamer labeled with FITC or biotin was incubated with cell monolayer in chamber slides in binding buffer on ice for 30 min. After washing, the cells incubated with the biotinylated aptamer were then continued to incubate with QD605-streptiavidine (Q10101MP, invitrogen) at room temperature for 30 min. Cells bound to FITC- or QD605-labled aptamer were imaged with Olympus BX51 fluorescence microscope equipped with an Olympus DP72 camera (Olympus Optical Co., Ltd., Tokyo, Japan).

2.11. Nude mice model of HCC spontaneous pulmonary metastasis

Male athymic BALB/c nu/nu mice, 4-wk old, were obtained from Beijing HFK Bio-Technology Co., Ltd [animal quality certificate No. SCXK(Jing) 2009-0004] and maintained in an Animal Biosafety Level 3 Laboratory at the Animal Experimental Center of Wuhan University. After 3 days of adaptation, the animals were used for in vivo study, and the protocols were approved by the Animal Care Committee of Wuhan University. HCCLM9- nude mice were produced as described previously [12]. All mice were sacrificed under deep anesthesia by peritoneal injection of 3% pentobarbital sodium in approximately 6 wks after model construction. Liver and lung samples were collected and stored at −80 °C refrigerator. Some lung samples were fixed in 10% neutral formalin solution and embedded in paraffin. Four micrometer thick sections were cut from each paraffin block and stained with hematoxylin and eosin for microscopic examination to validate lung metastasis.

2.12. Recognition of the HCC cells in mice model tumor tissue and clinical specimens by the selected aptamer LY-1 labeled with QD605

Preparation for the formalin-fixed paraffin-embedded mice model liver tumor, lung metastasis tumor tissues, and clinical specimens were briefed as the following. Tissue sections (4 μm thick) were preheated at 60 °C for 2 h and then deparaffinized in xylene 3 times each for 5 min. Tissue hydration was carried out by a series of immersion steps at decreasing ethanol concentrations (100%, 95%, 95% and 85% ethanol for 5, 2, 2, and 2 min, respectively), followed by rinsing in water for 5 min. The slides were pretreated in 0.01 M citrate buffer (pH 6.0) and heated in a microwave oven at 98 °C for 20 min for antigen retrieval. After cooling down in the citrate buffer for another 30 min at room temperature, the tissue slides were washed in water before use. For fluorescence aptamer staining, tissue sections were blocked with pre-cooled binding buffer (PBS- 1 M $MgCl_2$ -0.1 mg/mL yeast tRNA-1 mg/mLBSA-0.1 mg/mL Salmon sperm DNA) at 4 °C for 60 min and then incubated with biotinlated aptamer in binding buffer. After incubation at 4 °C for 60 min, these tissue sections were washed 3 times with PBS. After washing, these tissue sections were then continued to incubate with QD605-streptiavidine at room temperature for 30 min. Finally, the stained sections were imaged by an Olympus BX51 fluorescence microscope equipped with an Olympus DP72 camera (Olympus Optical Co., Ltd., Tokyo, Japan).

2.13. Blood and tumor tissue clinical samples collection

To prepare for artificial blood samples environment, peripheral blood samples were collected in EDTA-K2 anticoagulant tube from healthy male volunteers. HCC specimens from 66 patients were obtained from the Department of Pathology at Wuhan University Zhongnan Hospital and Hubei Cancer Hospital. All tumors were fixed with formalin and embedded with paraffin. Pathological diagnosis was made according to the histology of tumor specimens and examined by experienced pathologists. Major clinic-pathological characteristics of these patients were listed in Table 2 (see supplementary data).

2.14. Recognition of the HCC cells in real peripheral blood samples using the selected aptamer LY-1 labeled with FITC

To evaluate the recognition capacity of the selected aptamer in a complex biological environment, HCCLM9 cells (1×10^5) were prepared as described above and mixed with the whole blood. After lysis of red blood cells in NH_4Cl solution, the remaining cells (HCCLM9 cells and WBC) were incubated with FITC-labeled aptamer and PE-CD45 (BD Biosciences) on ice for 30 min. After incubation and washing, the samples were analyzed by flow cytometry. In order to test the recognition sensitivity of the FITC-labeled aptamer, different amounts HCCLM9 cells (10^4, 10^3 and 10^2 HCC cells) were respectively mixed with the whole blood, the other treatment steps were the same as described above.

2.15. Capture the HCC cells in real peripheral blood samples by the aptamer-conjugated magnetic particles

To evaluate the targeting capture capacity of the selected aptamer LY-1 in a complex biological environment, HCCLM9 cells (1×10^5) were prepared as described above and mixed with the whole blood. After lysis of red blood cells in NH_4Cl solution, the remaining cells (HCCLM9 cells and WBC) were incubated with bio-labeled aptamer on ice for 30 min. After washing, the cells were then incubated with streptavidin-coated magnetic particles (Dynabeads, M-280 Streptavidin) at room temperature for 30 min. After washing, the suspension of the cells-aptamer-magnetic particles was dropped on the glass slides pre-treated with poly-lysine. After being dried in room air, the slides were gently washed once in PBS and immediately fixed for 5 min in methanol solution which was pre-cooled at −20 °C. The cells were first incubated with rabbit anti-human keratin 19 and mouse anti-human AFP primary anti-bodies (R&D Systems) overnight at 4 °C, then washed and incubated with FITC-conjugated anti-rabbit or anti-mouse IgG secondary antibody (dilution 1:200, Santa Cruz Biotechnology Inc) for 60 min at room temperature. In order to evaluate the capture efficiency of the aptamer-conjugated magnetic particles, different amounts HCCLM9 cells (10^4, 10^3 and 10^2 HCC cells) were respectively mixed with the whole blood. The other treatment steps were the same as described above.

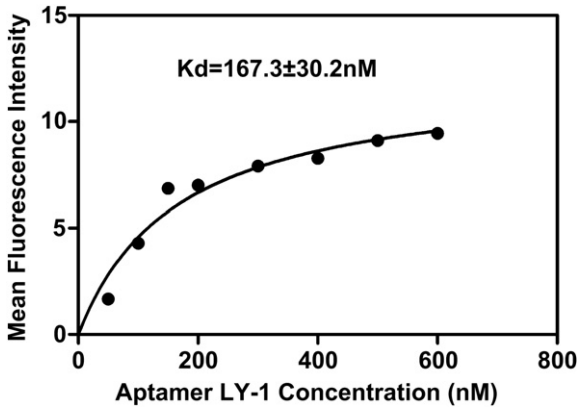

Fig. 8. Binding curve of the aptamer LY-1 with target cells HCCLM9. Cells were incubated with varying concentrations of FITC-labeled aptamer in triplicate. The mean fluorescence intensity of the control aptamer (background binding) at each concentration was subtracted from the mean fluorescence intensity of the corresponding aptamer.

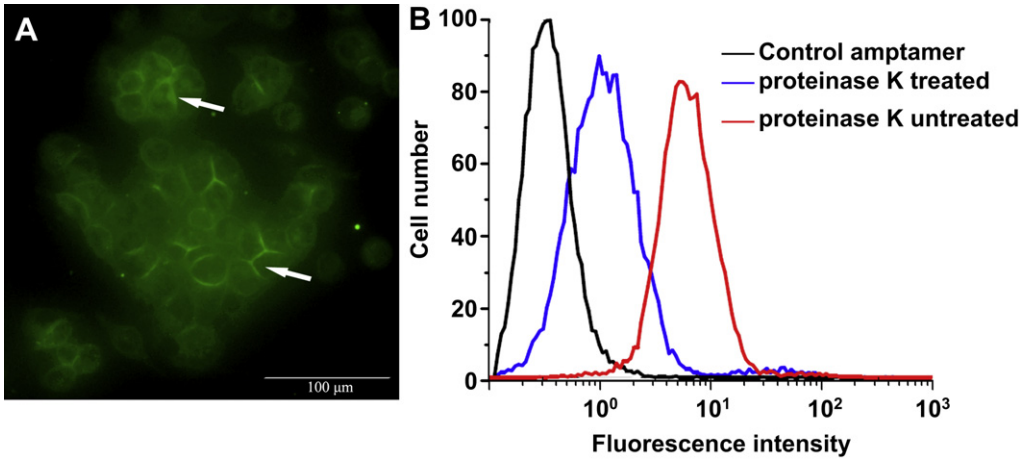

Fig. 9. Possible binding sites of aptamer LY-1 to target cell as determined by cytometry and fluorescence microcopy. Without proteinase K treatment, the HCCLM9 cells had significant cell membrane binding to LY-1 (panel A and red curve of panel B). After proteinase K treatment, there was remarkable decrease in cell membrane fluorescence intensity (the blue curve of panel B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Selection and identification of HCC metastasis-related aptamers

Using subtracted cell-SELEX strategy with MHCC97-L as subtractive cells and HCCLM9 as target cells, we screened one DNA lane after 9 rounds of enrichment (Fig. 1A). On further enrichment, this DNA lane disappeared (Fig. 1B). Therefore it was concluded that 9 rounds of enrichment was optimal. We further evaluated the selected DNA strands using flow cytometry (Fig. 2). With increasing number of enrichment up to 9 rounds of selection, the fluorescence intensity bound on HCCLM9 cells increased significantly (Fig. 2A), while there was no change in fluorescence intensity on MHCC97-L cells (Fig. 2B). Therefore, it was evident that the selected single strand DNA had preferential and specific binding to HCCLM9 cells.

3.2. Selection, sequencing and structure prediction of HCCLM9 specific aptamers

After 9 rounds of the enrichment, the selected ssDNA library was PCR-amplified and cloned into *Escherichia coli*. Fifty clones were subjected to sequence study and definite results were obtained from 38 clones, including 23 clones yielding the same sequence named as LY-1, 9 clones yielding the same sequence named as LY-13, 2 clones yielding the same sequence named as LY-7/43, another 2 clones yielding the same sequence named as LY-27/45, and the remaining 2 clones each yielding one sequence named as LY-32 and LY-46. After sequence analysis (Fig. 3A), homologous analysis (Fig. 3B) and structure prediction (Fig. 3C), these 6 aptamers were labeled with FITC at the 5′end, and their binding affinity to HCCLM9 cells was evaluated. Both flow cytometry and fluorescent microcopy assays (Fig. 4 and Fig. 5) revealed that LY-1 had the highest binding capacity. Therefore, LY-1 was selected for detailed study.

3.3. Binding study of LY-1

Different HCC cells (HCCLM9, MHCC97-L, Huh-7 and HepG2), human lung cancer cell line H1299, human colon adenocarcinoma cell line SW48, human gastric cancer cell line MGC803, human cervical cancer cell line HeLa, human breast cancer cell line MDA-MB-231, and human peripheral WBC were used to test the binding specificity of LY-1. As shown in Fig. 6 and Fig. 7, LY-1 had highly specific binding capacity to HCCLM9 and low specific binding capacity to MHCC97-L, but no or little binding to other cancer cells and WBC. The apparent dissociation constants (Kd) of the LY-1

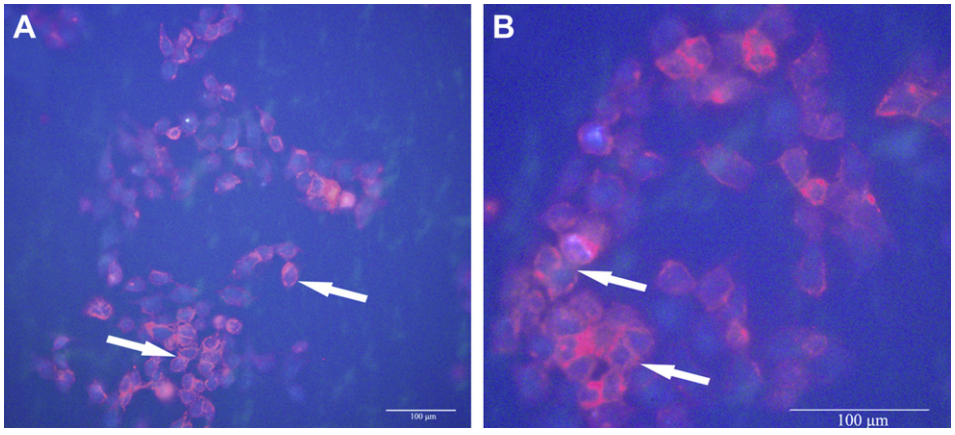

Fig. 10. The QD605 fluorescence imaging of the selected aptamer LY-1 bound to HCCLM9 cells by fluorescent microcopy analysis.

aptamer–cell interaction was Kd = 167.3 ± 30.2 nM (Fig. 8). In order to investigate the possible binding site of LY-1, fluorescence binding assays were conducted before and after HCCLM9 cells were treated with proteinase K. As shown in Fig. 9, there was evident cell membrane fluorescence before proteinase K treatment (Fig. 9A and Fig. 9B red line), and significantly fluorescence decrease after proteinase K treatment (Fig. 9B blue line). These results suggest that the binding site of the aptamer LY-1 is most likely on the cell membrane of HCCLM9.

3.4. QD605 labeled LY-1 aptamer as specific molecular probe to recognize HCC cells in cell culture, animal models tissue and clinical HCC specimens

We then explored the possibility whether the LY-1 aptamer could be a potential probe to recognize HCC cells in vitro. Biotinlated-LY-1 aptamer was first added to cultured cells, followed by QD605-streptiavidine. As shown in Fig. 10, there was high QD605 fluorescence on the cell membrane in cultured HCCLM9

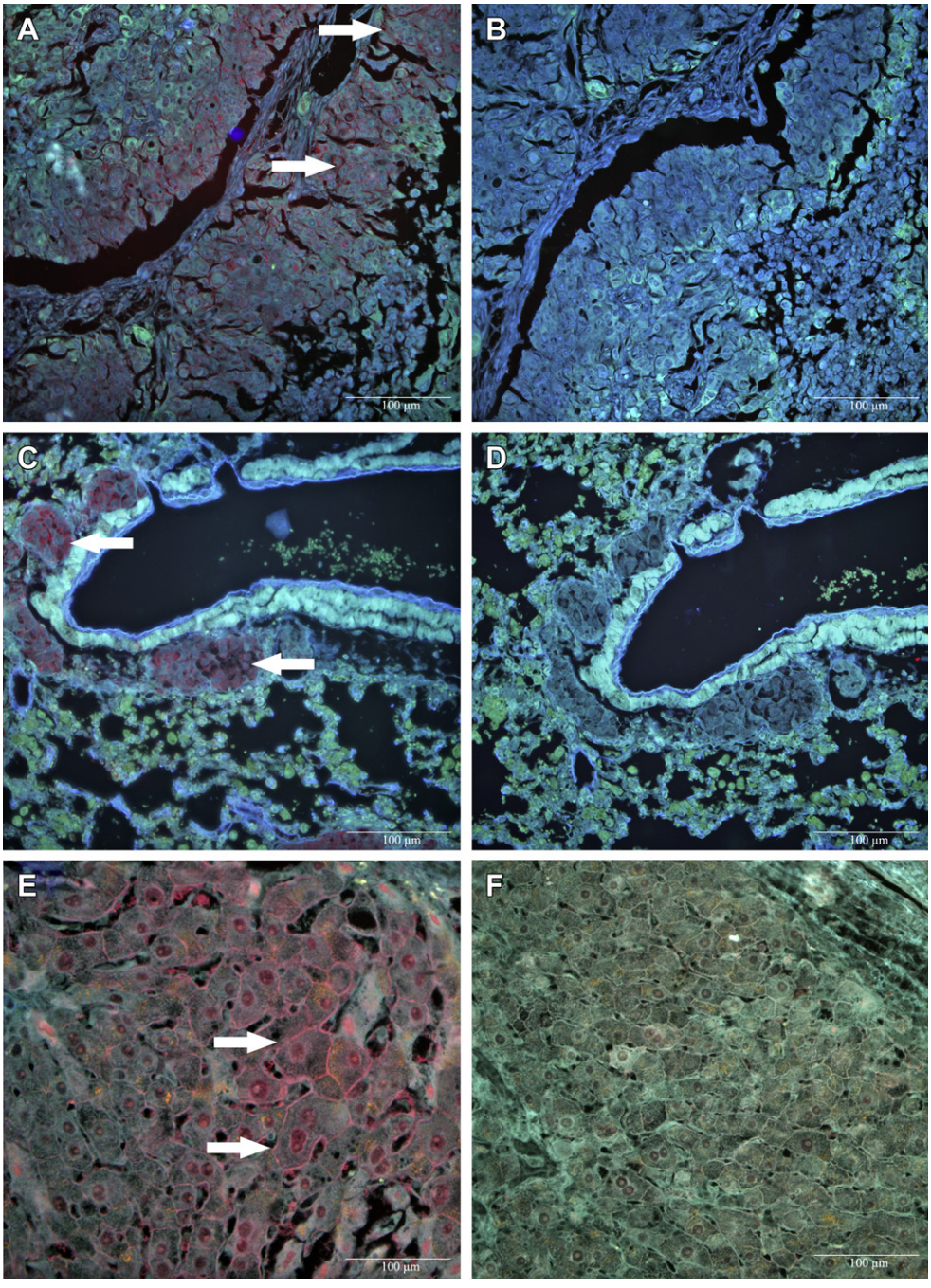

Fig. 11. The QD605 fluorescence imaging of the aptamer LY-1 bound to HCCLM9 cells in the formalin-fixed paraffin-embedded mice liver tumor and lung metastasis tumor tissues and HCC cells in the formalin-fixed paraffin-embedded human HCC tissues from patients. (A) aptamer LY-1 bound to the HCC cells in mice model liver tumor tissue; (B) The control aptamer unbound to the HCC cells in mice model liver tumor tissue; (C) aptamer LY-1 bound to the HCC cells in mice model lung metastasis tumor tissue; (D) The control aptamer unbound to the HCC cells in mice model lung metastasis tumor tissue; (E) aptamer LY-1 bound to the HCC cells in human HCC tissue; (F) The control aptamer unbound to the HCC cells in human HCC tissue.

cells. Furthermore, in the xenograft model of HCCLM9, the HCC cells in both the liver tumor (Fig. 11A and B) and lung metastasis (Fig. 11C and D) were also clearly stained by this probe.

Moreover, among the 66 HCC specimens, there were 6 specimens with clear and specific QD605-LY-1 labeling (Fig. 11E and F). The 6 HCC patients were all positive for serum hepatitis B surface antigen (HBsAg), elevated serum AFP and distant metastasis. Detailed information on these 6 patients was listed in Table 3 (see supplementary data).

3.5. FITC labeled LY-1 to recognize HCC cells in peripheral blood

To test whether aptamer LY-1 could identify HCC cells in a simulated peripheral blood environment, we mixed 10^5, 10^4, 10^3 and 10^2 HCCLM9 cells into 1 mL of peripheral blood from a healthy volunteer, respectively. After lysis of erythrocytes with NH_4Cl solution, the suspension containing HCCLM9 cells was incubated with FITC-labeled LY-1 aptamer on ice. Flow cytometric study (Fig. 12) demonstrated that as few as 10^2 HCC cells in 1 mL of peripheral blood could be identified by the FITC-labeled LY-1 aptamer.

3.6. Capture of circulating HCC cells based on aptamer LY-1 conjugated magnetic particles

We used aptamer LY-1 as recognition molecule and magnetic particles as separation media to capture circulating HCC cells. We mixed 10^5, 10^4, 10^3 and 10^2 HCC cells into 1 mL of peripheral blood from a healthy volunteer, respectively. After lysis of erythrocytes with NH_4Cl solution, the suspension containing HCCLM9 cells was incubated with biotinlated-labeled aptamer LY-1 on ice, and then circulating HCC cells were captured by streptavidin-coated magnetic particles. The captured cells were further identified by immunofluorescence cytochemistry to recognize AFP and CK19, and DAPI to stain the nuclei. As shown in Fig. 13, the circulating HCC cells were indeed surrounded by magnetic particles, suggesting specific capture of these cells. It was also found that 2 HCC cells could be captured from 1 mL peripheral blood containing 10^2 HCC cells by the magnetic particles-aptamer conjugates.

4. Discussion

The most important biological behaviors of malignant tumor are invasion and metastasis, which seriously affect the tumor prognosis [16]. These behaviors are closely associated with the cancer cell invasiveness, and tumor cell membrane molecules are directly involved in many aggressive behaviors such as adhesion, invasion and movement [9–11,16]. The composition and characteristics of cell membrane proteins are modified during malignant transformation and make them likely candidates for cancer biomarkers [9,17]. The identification of tumor cell membrane protein targets is important in understanding tumor progression, developing diagnostic tools, and identifying new therapeutic targets [17,18]. Correct understanding of tumor cell surface protein requires efficient molecular probes to recognize them. To be effectively used for tumor diagnosis, classification and therapy, such probes should have specific binding to target tumor cells [19,20]. Aptamers, as DNA/RNA probes, could help address such challenges by recognizing proteins, peptides and other small molecules with high affinity and specificity [19,21].

Recently, aptamer selection against complex targets, especially whole live cells, has attracted increasing attention. Using the cell-SELEX approach, several promising aptamers have been

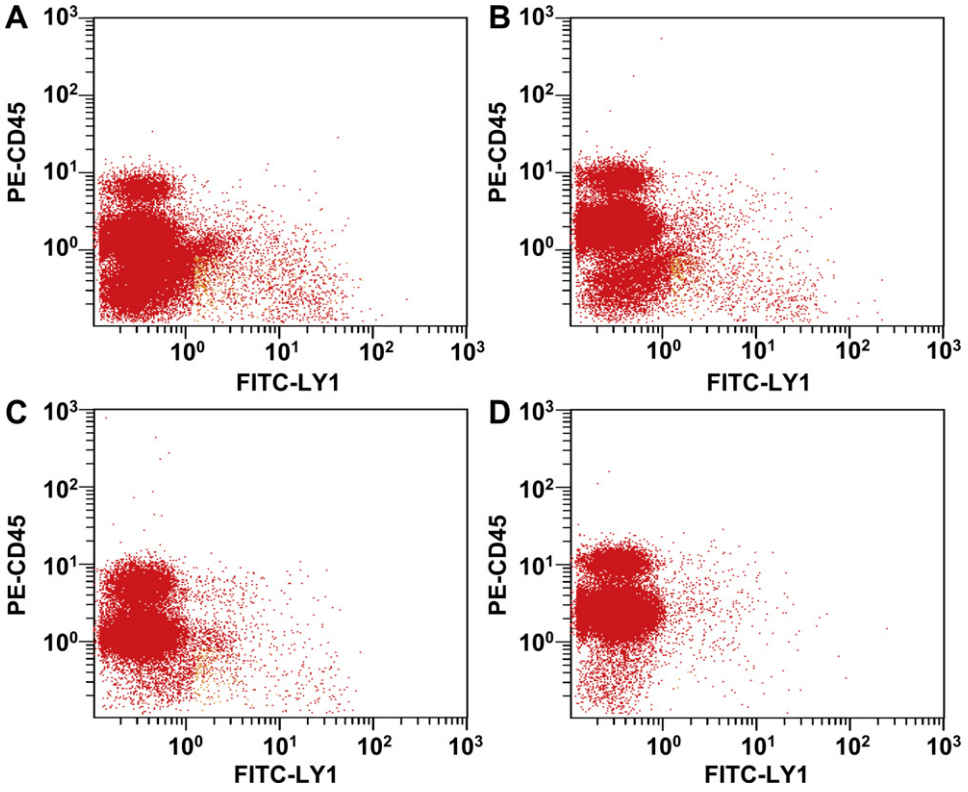

Fig. 12. Recognition of the HCCLM9 cells by the molecular probe aptamer LY-1 in the complex mixture with whole blood from healthy volunteer. FITC-labeled aptamer LY-1 and monoclonal antibody PE-CD45 were used to detect the HCC cells mixed with WBC. The aptamer LY-1 only recognized the HCC cells and did not bind to CD45-positive WBC and other blood cells. The FITC-labeled aptamer LY-1 could recognize HCC cells when (A) 10^5, (B) 10^4, (C) 10^3 and (D) 10^2 HCCLM9 cells mixed into the 1 mL peripheral whole blood.

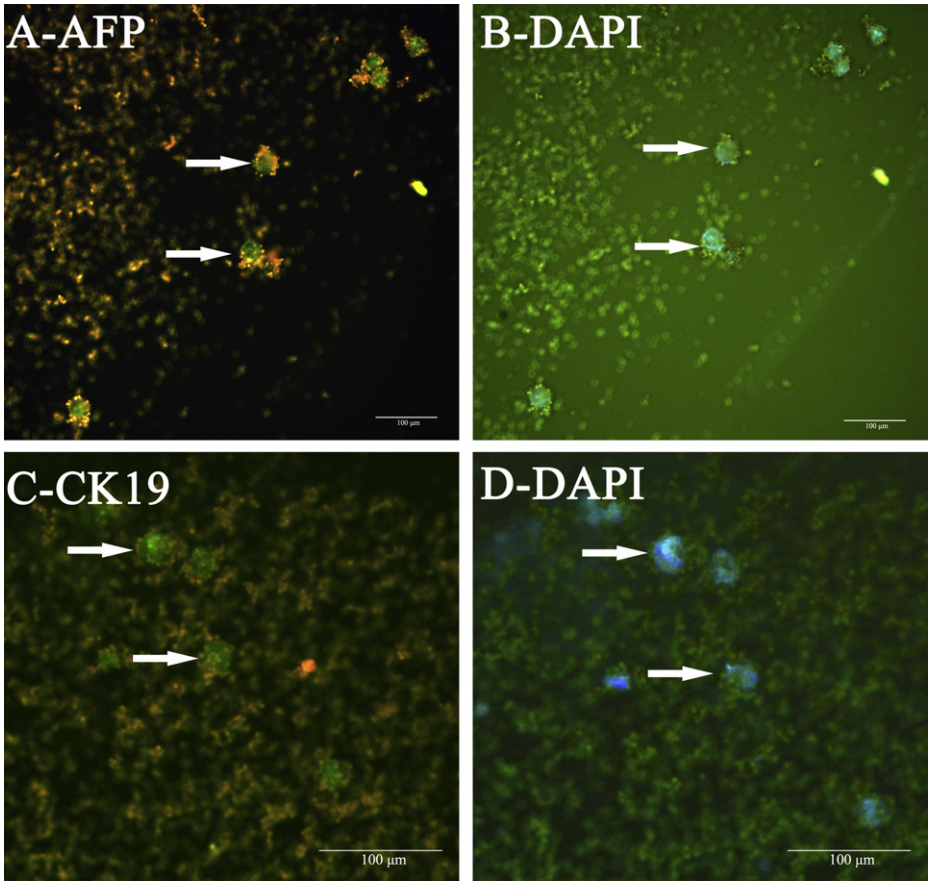

Fig. 13. Capture of the HCCLM9 cells by biotinated apatamer LY-1 as recognition molecular probe and streptavidin-coated magnetic particles as separation medium in the complex mixture with whole blood from healthy volunteer. The captured cells were stained with monoclonal anti-bodies against AFP and CK19. The nucleus of the captured cells was also stained by the nucleic acid organic dye DAPI. When 1×10^4 HCCLM9 cells mixed into the 1 mL of peripheral whole blood, the aptamer LY-1 -magnetic particles conjugates could specifically recognize and capture HCC cells.

selected for cancer cells, including small cell and non-small cell lung cancer [22], liver cancer (mouse) [23], and lymphocytic and myeloid leukemia [19]. To our knowledge, there has been no report on using aptamer strategy to select metastasis-related cell surface molecules from highly metastatic human HCC cell lines. In our study, we modified the subtractive cell-SELEX strategy, using the highly metastatic HCCLM9 as target cells and the low metastatic MHCC97-L as subtractive cells, through 9 rounds of selection and identification by both flow cytometry and fluorescence imaging, obtained a specific aptamer LY-1, binding the highly metastatic human HCC cell membrane surface with high affinity. To test whether aptamer LY-1 could be used for HCC cells specific recognition, other cancer cells from lung cancer, breast cancer, colorectal cancer, gastric cancer and cervical cancer and WBC were used as control, the aptamer LY-1 showed specific targeting to HCCLM9 cells and little or no response to other cancer cells. To our knowledge, this is the first report on aptamers against highly metastatic human HCC cells. The specific molecular probe aptamer LY-1 generated from cell-SELEX provide basis for clinical application.

The specific recognition of targets in complex clinical samples remains a major challenge for many molecular probes. To determine the capability of aptamer LY-1 as a molecular probe for specific receptor recognition, we used aptamer LY-1 to identify HCC cells in complex clinical samples (tissue and whole blood). Quantum dots (QDs) have been developed as labels for the sensitive detection [20]. Compared with organic fluorescent dyes, QDs are relatively photostable and have a very broad excitation range, making them better for imaging detection [24,25]. In this study, QDs-labeled aptamer LY-1 was used to recognize HCC cells cultured in vitro, HCC cells in the liver and lung tissue sections from the animal model of HCC with lung metastasis, and HCC cells from clinical liver cancer tissues. These studies demonstrated that the functionalized aptamer LY-1 labeled with QDs could recognize not only the highly metastatic HCC cells cultured in vitro, but also HCC cells in liver and lung tissue section in animal model of lung metastasis, and more importantly HCC cells in clinical liver cancer tissues. Based on the specific binding of aptamer LY-1 to highly metastatic HCC cells, we hypothesized that the HCC cells identified by aptamer LY-1 in clinical liver cancer tissues have high metastatic potential, if this hypothesis proved to be correct by additional tests and related clinical information, the molecular probe aptamer LY-1 conjugated QDs would be used in target molecular imaging of HCC and predict early metastasis of HCC. The capacity of the selected aptamer LY-1 to identify the highly metastatic HCC cells in complex tissue specimens makes it a potentially effective molecular probe to recognize HCC for both diagnostic and therapeutic applications.

To confirm whether the aptamer LY-1 could identify HCC cells in a simulated peripheral blood environment, FITC-labeled aptamer LY-1 and monoclonal antibody CD45 were used to detect HCC cells mixed with normal human whole blood. The aptamer LY-1 only recognized the HCC cells and did not bind to CD45-positive WBC and other blood cells. We also found that HCC cells could be identified from 1 mL peripheral blood containing 10^2 HCC cells by the FITC-labeled aptamer LY-1. These studies indicate that this

aptamer could serve as a targeting molecule to capture circulating HCC cells.

Circulating tumor cells (CTCs) have a feature of "self-seeding" [26], which means they can return to the primary tumor to develop and enhance tumor growth. CTCs are important in prognostic evaluation, early detection of metastasis and individualized treatment. Due to lack of specific target molecules, the clinical detection of circulating HCC cells is not possible. So far, technologies of CTCs enrichment and separation are mostly based on density gradient centrifugation, cell size-based membrane filtration and immunomagnetic particles capture [27]. The standard method of CTCs separation is magnetic activated cell sorting technology based on the tumor cell surface epithelial antigen. Epithelial cell adhesion molecule (EpCAM) is a specific adhesion molecule widely expressed on surfaces of epithelial cells and tumor cells of epithelial origin. Cellsearch system based on EpCAM antibody beads has been applied to detect CTCs of breast cancer [28], colon cancer [29] and prostate cancer [30]. Though HCC cells are epithelial cells, EpCAM is only expressed in about 35% of HCC tissue samples [31]. Moreover, epithelial CTCs may lose epithelial markers during dissemination through the process called epithelial-to-mesenchymal transition (EMT). Cellsearch system is not suitable for the separation, detection of circulating hepatocellular carcinoma cells [32]. Tan et al. [33] developed a method to rapidly collect and detect leukemia cells from whole blood samples using a two-nanoparticle assay with aptamers as the molecular recognition probe, in which the aptamer-integrated magnetic nanoparticles were used for target cell extraction, and aptamer-integrated fluorescent nanoparticles were used for sensitive cell detection. In our study, the molecular probe aptamer LY-1 conjugated magnetic particles was used for target cell recognition and extraction, the human antibody AFP and CK19 and the nucleic acid organic dye DAPI were used for cell identification. Our findings suggest that aptamer LY-1 conjugated magnetic particles could be specific molecular probe to recognize and capture circulating HCC cells from peripheral blood, using strategies such as the nucleic acid aptamer-based microfluidic chip. Indeed, there are some groups that have developed a highly sensitive method for rapid capture of target cells based on aptamers combined with microfludic chip [21,34–36].

5. Conclusion

In summary, using whole living cell-SELEX system, we screened out a molecular probe aptamer LY-1 that can distinguish the high metastatic potential human HCC cell from other cells, and validated its specificity and sensitivity as potential molecular probes. When combined with fluorescent nanoparticles QDs, the aptamer LY-1-QDs conjugates could specifically recognize not only highly metastatic HCC cells in liver and lung tissue section in animal model but also in clinical specimens. Furthermore, the aptamer LY-1- magnetic particles conjugates could effectively detect and extract metastatic HCC cells from complex mixture whole blood. These results suggest that aptamer LY-1 could be developed into a promising molecular probe for early prediction HCC metastasis.

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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.02.018.

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