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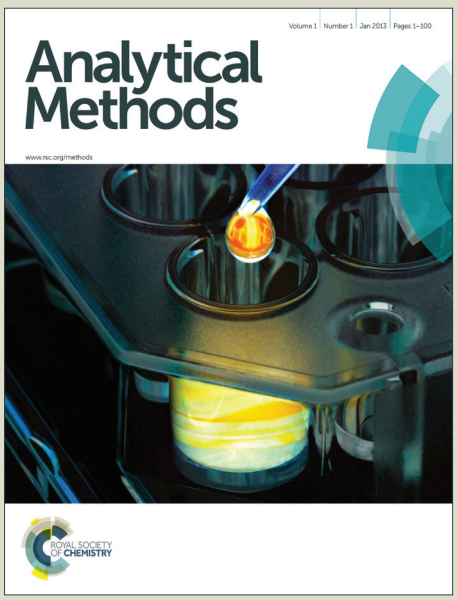

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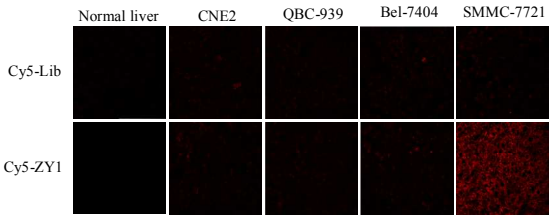

A newly developed hepatocellular carcinoma-specific aptamer for highly specific identifying SMMC-7721 carcinoma in real biological samples

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TECHNICAL NOTE

Whole cell-SELEX aptamers for fluorescence staining of frozen hepatocellular carcinoma tissues

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Hepatocellular carcinoma is one of the most common and deadly cancers in the world, the development of specific molecular probes for hepatocellular carcinoma diagnosis is of great significance. Recently, aptamer ZY1, which showed excellent specificity against SMMC-7721 human hepatocellular carcinoma cells, was selected by our group through cell-SELEX. In this study, aptamer ZY1 was labelled with Cy5 to serve as fluorescence probes, its efficacy in fluorescence staining of frozen SMMC-7721 xenograft tissues was tested. The results indicated that the aptamer not only showed high specificity in identifying SMMC-7721 carcinoma, but also exhibited high efficiency in differentiating liver carcinomas of different subtypes. Moreover, the time required for tissue staining was as short as 10 min. These results strongly support the potential of the aptamer for future application in cancer diagnosis.

Introduction

Hepatocellular carcinoma is the six most prevalent cancer and the third most frequent cause of cancer-related death, it causes approximately 700,000 deaths worldwide in 2008.[1] As with all forms of cancer, an accurate and specific diagnosis is an inevitable task for cancer researchers. During the past several decades, antibodies have been utilized as clinically validated molecular probes for hepatocellular carcinoma diagnosis. Despite the universality and versatility of antibodies, there are some disadvantages like the time-consuming production process and the variation between different batches.[2] Thus, alternative molecules that circumvent these limitations are highly desirable.

Aptamers, generated by SELEX,[3, 4] are small single-stranded DNA or RNA oligonucleotides that display high binding affinity and specificity for target molecules.[5-8] As novel ligands, aptamers possess several advantages over antibodies, including excellent binding affinity and specificity, economical and reproducible synthesis, convenient and flexible modification, high consistency between different batches, lack of immunogenicity, and fast tissue penetration.[9, 10] These favourable characteristics not only make aptamers successfully used in various applications,[11-20] but also make aptamers a highly suitable probe for cancer recognition and diagnosis. Particularly, the recently developed whole cell-SELEX technology, a process to select aptamers against whole intact cancer cells,[21] has further strengthened the function of aptamers in differentiating cancer cells even in the same type.

By applying the whole cell-SELEX technology, we have developed panels of aptamers against SMMC-7721 human hepatocellular carcinoma cells.[22] Among the selected aptamers, one particular aptamer, termed sequence ZY1, showed especially strong binding affinity for SMMC-7721 cells. In this study, aptamer ZY1 was chosen as the molecular probe for tissue staining to test its capability in differentiating different cancer types in real biological samples.

Experimental

Materials

Cy5-labeled aptamer ZY1 (Cy5-ZY1: 5'-Cy5-TTG ACT TGC CAC TGA CTA CCA ATA GTC GAA GAC TGA TGG TTG AGC TGA TGA TCC TAC GGT GAA GTC AGT CGG TCG TCA TC-3') and Cy5-labeled 80mer sequence-randomized DNA library (Cy5-Lib: 5'-Cy5- N_{80} -3') were synthesized by Sangon Biotech. Co., Ltd (Shanghai, China). The Cy5-Lib or Cy5-ZY1 aptamers were dissolved in ultrapure water (>18 MΩ, Milli-Q, Millipore) to give a stock concentration of 10 μM, without further denaturation and left on ice for further experiments. Bull Serum Albumin (BSA) and yeast tRNA were purchased from Dingguo Changsheng Biotechnology Co., Ltd (Beijing, China). All other reagents were of analytical grade. pH 7.4, phosphate buffered saline (PBS) was prepared using ultrapure water. Binding buffer was prepared by adding 1

mg/mL BSA and 0.1 mg/mL yeast tRNA into the PBS containing 4.5 g/L glucose and 5 mM $MgCl_2$.

Cells

Five *in vitro* cultured cell lines were used in this study: SMMC-7721 (human hepatocellular carcinoma), L02 (normal human hepatocytes), Bel-7404 (human hepatocellular carcinoma), QBC-939 (human cholangiocarcinoma) and CNE2 (human nasopharyngeal carcinoma). SMMC-7721, L02, Bel-7404 were purchased from the Shanghai Institute of Cell Biology of the Chinese Academy of Science. QBC-939 was obtained from XiangYa Hospital, Central South University and CNE2 was provided by the Cell Center of our lab. All cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (heat inactivated), 100 μg/mL streptomycin and 100 IU/mL penicillin at 37 ℃ in an incubator containing 5% CO_2. The cell density was determined using a hemocytometer, and this was performed prior to any experiments.

Animals

Male BALB/c nude mice were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. They were 5-6 weeks old at the start of experiment. All animal operations were in accord with institutional animal use and care regulations, according to protocol No. SYXK(Xiang)2008-0001, approved by Laboratory Animal Centre of Hunan.

Flow cytometry assays

Anchorage-dependent cells were first washed twice with cooled PBS, detached with 0.02% EDTA and 0.5% trypsin, and then washed again with cooled PBS. Then, Cy5-labeled probes were incubated with 2×10^5 cells in 200 μL binding buffer at 4 ^oC for 30 min in the dark. Next, cells were washed twice with 0.5 mL of binding buffer and resuspended in 0.2 mL of binding buffer. The fluorescence was determined with a FACScan cytometer (BD Biosciences, Mountain View, CA, USA) by counting 10, 000 events.

Tumour growth

Five- to six-week-old male BALB/c nude mice received a subcutaneous injection of 4×10^6 *in vitro* propagated human cancer cells into the right back leg. Tumours were then allowed to grow to 1-1.5 cm in diameter for 20-30 days.

Tissue section preparation for fluorescence staining

Immediately after sacrifice of the mice by vertebral dislocation, normal liver, SMMC-7721, Bel-7404, QBC-939 and CNE2 xenograft tissues were taken out, frozen in a cryostat (CM 1850, Leica, Germany), and embedded in optimal cutting temperature (OCT) compound. Then, frozen tissue sections of 10 μm were cut in the cryostat and immediately placed onto charged glass slides. Next, tissue sections were immersed in PBS for 3 minutes to remove OCT medium and used for fluorescence staining. For fluorescence staining, tissue sections were incubated with 250 nM Cy5-ZY1 probe or the control Cy5-Lib probe at 4 ^oC for 10 min. After three washes with cooled PBS, fluorescence images of stained sections were taken by confocal laser scanning microscopy (FV500-IX70, Olympus, Japan). The Cy5 was excited with a 633 nm laser and fluorescence signals were collected with a 60× objective.

Results and discussion

Specific detection of SMMC-7721 cells *in vitro*

As mentioned above, we decided to use Cy5-labeled ZY1 (Cy5-ZY1) as the probe for fluorescence staining of SMMC-7721 xenograft tissues. First, the capability of Cy5-ZY1 in specifically detecting target cells *in vitro* was demonstrated. Flow cytometry assays were conducted by incubating 250 nM Cy5-ZY1 with normal hepatocyte L02 cells, hepatocellular carcinoma SMMC-7721 cells, hepatocellular carcinoma Bel-7404 cells, cholangiocarcinoma QBC-939 cells and nasopharyngeal carcinoma CNE2 cells in binding buffer. Cy5-Lib (250 nM) was used as the negative control probe. As shown in Fig. 1a, compared with nonspecific signals from Cy5-Lib, Cy5-ZY1 exhibited much higher fluorescence labelling *in vitro* cultured SMMC-7721 cells. In contrast, little signal differences between Cy5-ZY1 and Cy5-Lib stained cells were detected in other four cell lines (Fig. 1b-e). These results revealed that the Cy5-ZY1 probe exhibited high specificity for the identification of SMMC-7721 cells from other cell types and even some subtypes.

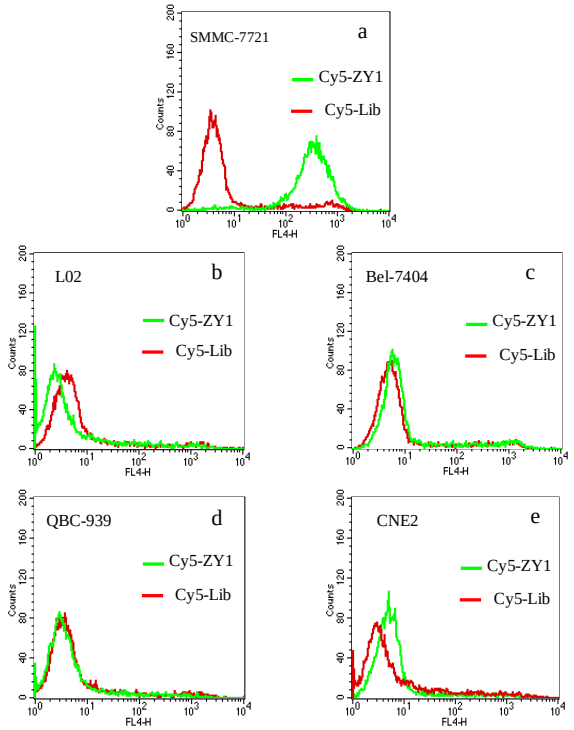

Fig. 1 Investigating the recognition specificity of Cy5-ZY1 *in vitro*. Flow cytometry analysis of different *in vitro* cultured cells in binding buffer using Cy5-ZY1, including (a) SMMC-7721, (b) L02, (c) Bel-7404, (d) QBC-939, (e) CNE2 cells. Cy5-Lib was used as the negative control probe.

Fluorescence staining of SMMC-7721 Xenograft Tissues

Since the Cy5-ZY1 probe specifically detected target cells *in vitro*, we further tested the feasibility of Cy5-ZY1 probe for fluorescence staining of SMMC-7721 xenograft tissues. To maximally preserve the antigens at their original state, SMMC-7721 tissue was removed immediately after sacrifice of the mice and used for frozen section. After immersing in PBS for 3 min, the frozen section was used for staining with 250 nM Cy5-ZY1 and Cy5-Lib control at 4 °C for 10 min. The results of fluorescence images were illustrated in Fig. 2A. Tissue sections gave bright red fluorescence after incubation with the Cy5-ZY1 probe, whereas very limited background fluorescence signal was observed from the Cy5-Lib control probe. This indicated that the SMMC-7721 hepatocellular carcinoma tissues could be effectively stained by aptamer ZY1. In addition, since the selection of aptamer ZY1 was performed at 4 °C, we performed tissue staining at 25 °C to test whether Cy5-ZY1 probe could be used for tissue staining at room temperature. Notably, under this condition, the SMMC-7721 hepatocellular carcinoma tissues still could be stained by Cy5-ZY1 probe, despite the fluorescence signal was weaker than that at 4 °C (Fig. 2B). These results were similar to those of flow cytometry assays (Fig. 3). To ensure tissue sections could give bright fluorescence signals after incubation with Cy5-ZY1 probe, 4 °C of operation temperature was chosen for further investigation.

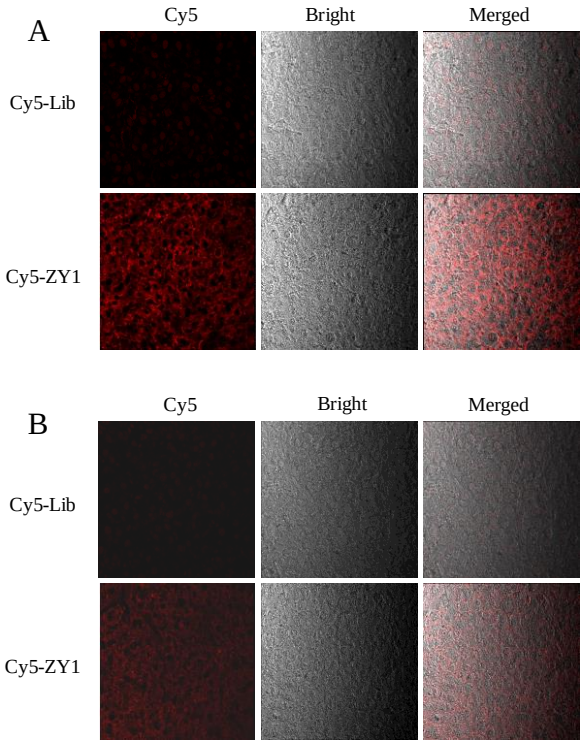

Fig. 2 Fluorescence images of SMMC-7721 xenograft tissues stained by Cy5-Lib control probe (top) and Cy5-ZY1 probe (bottom). (A) Tissue staining was performed at 4 °C. (B) Tissue staining was performed at 25 °C.

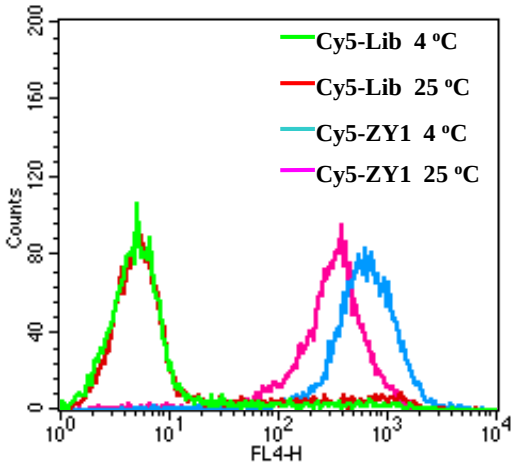

Fig. 3 The binding of Cy5-ZY1 (250 nM in binding buffer) to SMMC-7721 cells at 4 °C and 25 °C. The Cy5-Lib was used as negative control.

Furthermore, to achieve the best staining performance, the effect of incubation time and the concentration of ZY1 probe were also investigated. Initially, the incubation time with tissues was studied. As shown in Fig. 4A, the fluorescence signals enhanced significantly with the incubation time increased from 5 to 10 min, while, as the incubation time was increased to 15 min, the background fluorescence signal enhanced obviously. Thus, 10 min incubation time with tissues was chosen for further experiment. Subsequently, the concentration of Cy5-ZY1 probe was investigated. As shown in Fig. 4B, bright red fluorescence could be observed at 250 nM, 300 nM, or 500 nM, however, the background fluorescence signal increased with increased probe concentration. Thus, 250 nM probe concentration was used in the following experiment.

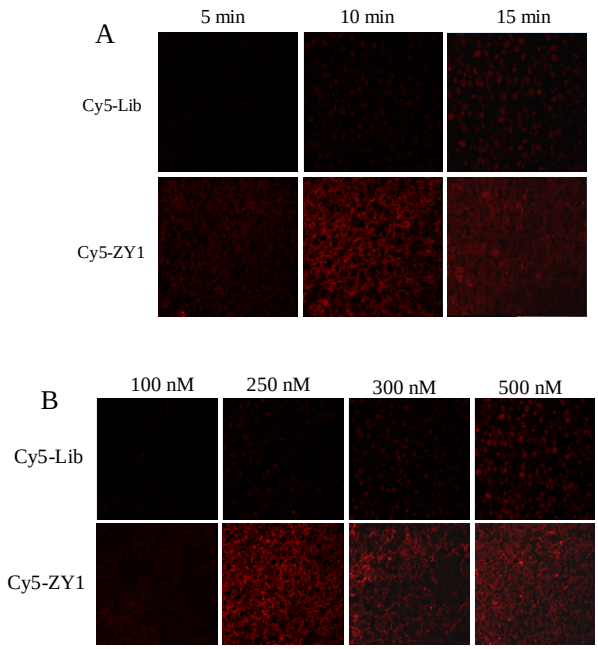

Fig. 4 The effect of different conditions for fluorescence staining of SMMC-7721 xenograft tissues. (A) Different incubation time with tissues. Concentration of Cy5-ZY1 probe: 250 nM; operation temperature: 4 °C. (B)

Different concentrations of Cy5-ZY1 probe. Operation temperature: 4 °C; incubation time with tissues: 10 min.

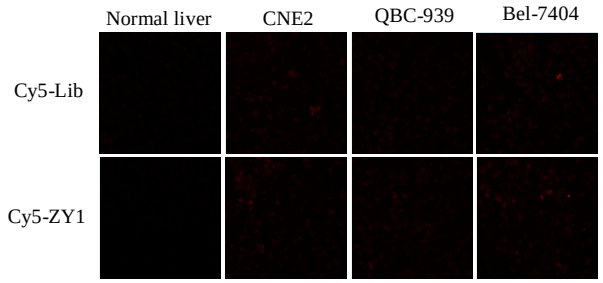

Fig. 5 Investigating the recognition specificity of Cy5-ZY1 in tissues. Fluorescence images of different tissues, including normal liver, CNE2, QBC-939 and Bel-7404 xenograft tissues, stained by Cy5-ZY1. The Cy5-Lib probe (top) was used as the negative control.

Specificity of Cy5-ZY1 for staining SMMC-7721 xenograft tissues

Under the optimized conditions, a systematic comparison investigation was performed to validate the capability of Cy5-ZY1 in specific fluorescence staining of SMMC-7721 hepatocellular carcinoma tissues. Sections of different tissues, including normal liver, CNE2, QBC-939 and Bel-7404 carcinoma tissues were prepared and incubated with Cy5-ZY1 probe as described above. The Cy5-Lib probe was used as a negative control in parallel to rule out nonspecific tissue staining. As shown in Fig. 5, no cross-reaction or background staining by the Cy5-ZY1 probe was observed in any of these tissues, which positively demonstrated that Cy5-ZY1 retained its high affinity and specificity for target cells in tissues.

Conclusions

In conclusion, the aptamer ZY1 have been successfully applied as useful probes for fluorescence staining of frozen carcinoma tissues. First, the aptamer ZY1 specifically detected target cells both *in vitro* and in tissues. In addition, the aptamer probe provided tissue staining with facile operation and quick response. Moreover, its high specificity for tissue staining was repetitiously confirmed, which greatly supported its capability in classifying different cancer types and even subtypes. This work might make a great contribution to introduce the aptamer into liver carcinoma diagnosis in pathology laboratory.

Acknowledgements

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