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ORIGINAL ARTICLE

Immunocytochemistry Based on a Cell-Type-Specific Aptamer for Rapid Immunostaining of Adenocarcinoma Cells in Clinical Serosal Fluids

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Abstract

All too often, conventional immunocytochemistry (ICC) via an antibody on cytological samples is limited to a few smears due to scant cellularity. To circumvent these limitations, this study employed a cell-type-specific aptamer as the core tool in ICC protocols for a timely and highly specific ICC diagnosis. S6, an aptamer against A549 lung carcinoma cells, was adopted instead of antibodies in this study for differentiating cancer cells in serosal fluids. Here, we developed three different strategies for discriminating the adenocarcinoma cells in effusion cytology specimens using the S6 aptamer in ICC. These strategies included a biotin-labeled S6 aptamer, an FAM-labeled S6 aptamer, and an activatable S6 aptamer. A total of 112 serosal fluid specimens with known diagnoses were evaluated by all three modes of use of the S6 aptamer. ICC procedures based on biotin-labeled or FAM-labeled S6 aptamers required time-consuming washing to avoid interference from nonspecific adsorption. ICC procedures based on an activatable S6 aptamer probe showed a weak fluorescence signal in the absence of target cells, but the procedures showed a strong fluorescence signal due to alteration of the conformation without any complicated washing steps, in the presence of targets. The specificity and sensitivity are higher in all three different ICC protocols based on the S6 aptamer than those for antibody protocols for differentiating adenocarcinoma cells in clinical effusion cytology. ICC based on cell-type-specific aptamers, instead of on a panel of a set of antibodies, is promising as an auxiliary method for the diagnosis of cancer.

Keywords Aptamer · Immunocytochemistry · Adenocarcinoma cells · Serosal fluid

Introduction

In recent years, cytology has played an increasingly important role in the diagnosis of various disease processes, particularly when cytological specimens are the only specimen types available from patients with cancer. Immunocytochemistry (ICC) has already proven to be important to most cytology samples in order to assist in the differentiation of cancer from other cells [1]. Currently, conventional immunohistochemistry (IHC) or ICC is used in the diagnosis of specific types of cancers. This technique requires multiple antibodies that can target specific molecular markers on a tumor cell in order to properly interpret results. However, ICC is largely not very useful for cytological specimens because of the limited number of specimens in most cases. ICC stands in contrast to IHC, in which a sufficient number of consecutive sections are usually available for each tissue specimen. This may hamper the performance or preclude the use of ICC based on antibodies for some cytological specimens.

Aptamers are single-stranded DNA or RNA (mainly DNA) sequences selected by genetic enrichment techniques. They have emerged as a novel class of antibody-like molecules, which are used as diagnostic/therapeutic agents [2–4]. Aptamers can be readily modified by different reporter molecules to meet specific applications. For example, aptamers can be conjugated to fluorescent molecules for visualization with fluorescence microscopy, or to biotin for a standard immunohistochemical program. In light of the possibility of aptamer functionalization (by fluorophore or biotin conjugation), they are found to possess several advantages over antibodies in terms of cost, ease of production, and absence of batch-to-batch variation. Based on the characteristics of aptamers, aptamers have been proposed as a potential substitute for conventional

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antibodies in enzyme-linked immunosorbent assays (ELISA) and IHC to detect various chemical molecules or cells [5].

In the area of IHC staining, the application of aptamers as versatile tools for immunohistochemical tissue analysis has just begun. To date, the application of aptamers in IHC is a new field, and only a limited number of studies have investigated the suitability of aptamers in IHC with chromogenic or immunofluorescent staining of formalin-fixed or frozen tissues. Bakhitiar et al. scrutinized the technical feasibility of diagnosis using IHC employing aptamers as the core tool. They concluded that IHC diagnosis based on aptamers was feasible for an average diagnostic pathology laboratory [6]. In summary, aptamers possess a diagnostic potential, which is timely and more sensitive than the antibodies in IHC.

In particular, cell-type-specific aptamers have been developed using whole-cell-SELEX technology with whole living cells as a target based on different cancer cell lines, including lung cancer [7, 8], leukocytosis [9], lymphoma [10], ovarian cancer [11], and colon cancer [12]. The selection of cell-type aptamers adopts a counterselection strategy to avoid selecting aptamers nonspecifically recognizing the cell surface of non-target cells and to help select cell-specific aptamer probes. Thus, cell-specific aptamers can distinguish target tumor cells from normal cells or other cells. Cell-type-specific aptamers are promising for use in ICC for an auxiliary diagnosis of cancer instead of a panel of a set of antibodies. However, few papers have reported the use of aptamers for clinical cytology immunohistochemistry in complex biological environments, such as body fluid samples, so far.

S6 aptamer probes have been developed for A549 adenocarcinoma (the most common subtype of non-small cell lung cancer) by Zhao's group [8]. They confirmed that aptamer probes showed selective binding to cells of NSCLC adenocarcinoma and NSCLC large cell carcinoma, but minimal binding properties with normal cells. This is important for the detection of cancer cells. In the present study, we used the S6 aptamer with three different ICC strategies against the lung adenocarcinoma A549 cell line for the identification of cancer cells in effusions. The three ICC strategies are the following: (1) The ICC involved a biotin-labeled S6 aptamer (an "always-on" probe). This is similar to conventional IHC, which uses a streptavidin-peroxidase method based the action of 3,3′-diaminobenzidine (DAB). (2) The ICC involved a direct fluorescent-labeled S6 aptamer (another "always-on" probe). This detects the target cells through a fluorescent signal. (3) The ICC involved an activatable probe (normally in the "off" state). In this protocol, the S6 aptamer detects the tumor cells through activated fluorescence. Following the method mentioned in Zhao's paper [13], the activatable probe in this study was designed with six complementary bases at both ends of the aptamer sequence to form a hairpin structure, which is labeled with a quencher (Eclipse) at the 3′-terminus and with a fluorophore (FAM) at the 5′-terminus. This arrangement enables close proximity of the quencher to the fluorophore. Before interaction with cells, the fluorescence of the probe is quenched due to fluorescent resonance energy transfer (FRET) between the fluorophore and the quencher. As a result, in the absence of target cells, the activatable probe showed minimal fluorescence. When specific binding with the target occurred, however, the activatable probe showed a strong fluorescent signal because the target caused a conformational change in the aptamer, forcing the separation of the fluorophore from the quencher.

This study confirms that cell-type-specific aptamers can be successfully applied to ICC for effusion cytology for the rapid diagnosis of adenocarcinoma cells. In particular, the activatable probe method is useful in the diagnosis of cancer as it causes minimal background signal, and because it is easy to handle. It provides rapid diagnosis with higher specificity and sensitivity than the other methods. Cell-type-specific aptamers provide a new perspective for differentiating cancerous cells in ICC procedures.

Materials and Methods

Reagents

An activatable hairpin aptamer probe based S6 aptamer (Simply "A-S6 apt") against A549 cancer cells was designed with an additional six complementary bases, and was labeled with a quencher (Eclipse) at the 3′-terminus and a fluorophore (FAM) at the 5′-terminus. A secondary structure prediction (shown in Fig. 1) from the sequence was generated using RNAstructure 5.8 software. Also, biotin-labeled and FAM fluorescence-labeled S6 aptamers ("Bio-S6 apt" and "FAM-S6 apt", respectively) were constructed in the present study. The nucleic acid sequences used in this study are all listed in Table 1 and were synthesized as oligonucleotides by Shanghai Sangon Biotechnology (Shanghai, China). All DNA oligonucleotides were dissolved in sterile distilled water as stock solutions and stored at −20 °C until use. Washing buffer was prepared by adding 5 mmol $MgCl_2$ and 4.5 g glucose into 1 L of Dulbecco's PBS without calcium and magnesium (Beyotime Institute of Biotechnology, Shanghai, China). The binding buffer was prepared by adding 1 mg/mL bovine serum albumin (Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd., China) and 500 μg/mL heparin (Shanghai Sangon Biotechnology, China) to the washing buffer.

A549 Cell Culture and Preparation

The A549 adenocarcinoma cell line was cultured in RPMI 1640 culture medium containing 10% fetal bovine serum, 100 units/mL penicillin (Sigma), and 100 units/mL

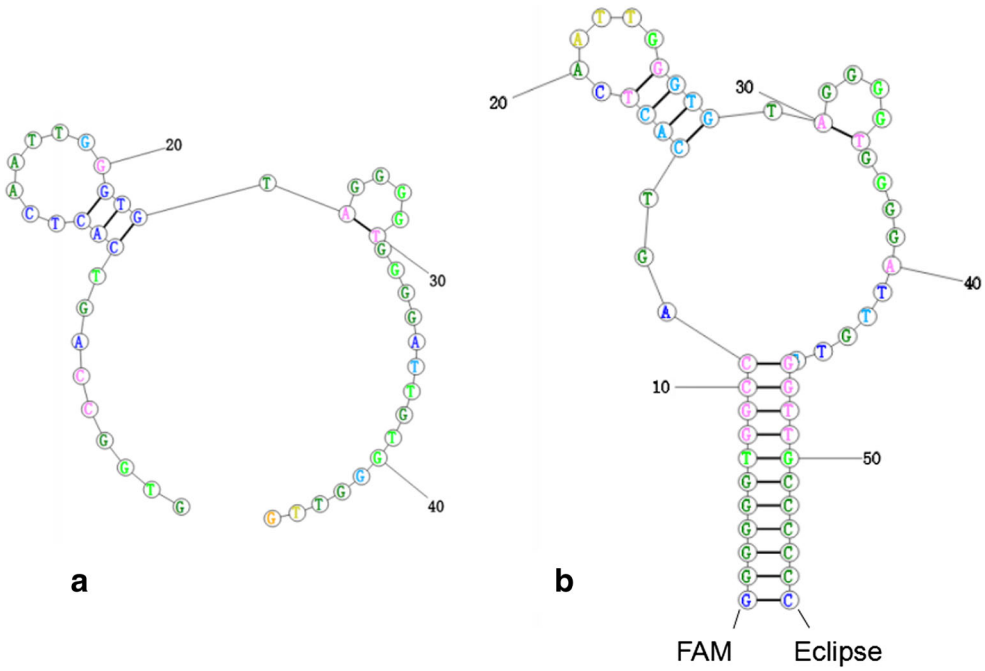

Fig. 1 Highly probable second structure of S6 aptamer predicted by RNAstructure 5.8 software. **a** original sequence of S6 aptamer; **b** the activatable probe sequence, which presented a hairpin structure

streptomycin (Sigma). The cultured cells were washed twice with cooled PBS and were then detached by treatment with 0.02% EDTA/0.25% trypsin. Then, cell suspensions were prepared in PBS at a density of 5.0×10^5 cells/mL. Six liquid-based cytology (LBC) slides were prepared using a sedimentation method from an aliquot of the cell suspension to verify if aptamer probes can bind selectively to tumor cells through an ICC procedure.

Collection and Preparation of Clinical Serosal Fluids

A total of 112 clinical cytological specimens with known diagnoses were collected for study at the Department of Cellular Pathology of the First Affiliated Hospital of Chongqing Medical University. The specimens included 82 pleural effusions, 6 pericardial effusions, and 24 peritoneal effusions (see Table 2). All effusion cell specimens were centrifuged, and then the resulting cell pellet of each specimen was resuspended in PreserveCyt solution (Guangzhou Anbiping Pharmaceutical Technology Co., Ltd). Four liquid-based cytology (LBC) slides for each specimen were prepared using a sedimentary method with an aliquot of the appropriate cell suspension. One LBC slide was used for Papanicolaou staining and was processed for routine cytomorphologic evaluation. Each of other 3 LBC slides was washed three times with PBS after antigen retrieval with citrate buffer for ICC

Table 1 The Sequences of oligonucleotides used in this work

Type	Description	Sequence(5′—3′)
A-S6 apt	Activatable-S6 probe (there are six extending spacers bases underlined at each terminus with the fluorescent group and the quench group,respectively.)	5’-FAM-GGG GGG GTG GCC AGT CAC TCA ATT GGG TGT AGG GGT GGG GAT TGT GGG TTG CCC CCC- Eclipse −3’
A-random sequence	The sequence was used as control (there are six extending spacers bases underlined at each terminus with the fluorescent group and the quench group,respectively.)	5’-FAM-GGG GGG GTG NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN TTG CCC CCC- Eclipse −3’
Bio-S6 apt	Biotin-modified S6 aptamer at 5′ end.	5’-Bio-GTG GCC AGT CAC TCA ATT GGG TGT AGG GGT GGG GAT TGT GGG TTG-3’
Bio-random sequence	The sequence was modified with Biotin at 5’end and used as control.	5’-Bio-GTG NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN TTG-3’
FAM-S6 apt	Fluorescence-modified S6 aptamer at 5’end	5’-FAM-GTG GCC AGT CAC TCA ATT GGG TGT AGG GGT GGG GAT TGT GGG TTG-3’
FAM-random sequence	The sequence was modified with fluorescence at 5’end and used as control.	5’-FAM-GTG NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN TTG-3’

Table 2 Origin and diagnosis of body fluids included in study*

Primary Site	Histological type	Pleural effusion	Pericardial effusion	Peritoneal effusion	No. of specimens
lung tumors	Aden.CA	51	6	0	57
	NSCC	2	0	0	2
	SC	2	0	0	2
	SCC	3	0	0	3
non-lung tumors	Gastric CA	1	0	7	8
	Breast CA	3	0	0	3
	Ovarian CA	0	0	13	13
	Lymphoma	2	0	0	2
	Mesothelioma	1	0	0	1
	Colon CA	1	0	1	2
benign	Reactive effusion	16	0	3	19
total		82	6	24	112

* *Aden* Adenocarcinoma, *NSCC* Non-small cell carcinoma, *SC* Squamous carcinoma, *SCC* small cell carcinoma, *CA* cancer

experiments using the S6 aptamer with one of the three detection modes mentioned above.

Bio-S6 Apt Labels Cancer Cells

The protocol for the ICC procedure based on Bio-S6 apt is shown in Fig. 2a. Endogenous biotin-blocking liquid (Beijing Leagene Biotech. Co., Ltd.) and 3% H_2O_2 were added onto LBC slides of serous effusions to block endogenous peroxidase and biotin in cells. The LBC slide was washed four times with binding buffer with each wash being 5 min. Then, 300-nM Bio-S6 apt diluted in a binding buffer was incubated with cells for 20 min at room temperature. The slides were washed three times with washing buffer with each wash being 3 min. Subsequently, the specimens were treated with 1:1000 diluted streptavidin-horseradish peroxidase conjugate (Shanghai Sangon Biotechnology, China) for 30 min at room temperature and washed five times with washing buffer with each wash being 3 min. Then they were visualized by DAB chromogen (Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd., China) and counterstained with hematoxylin. Finally, the slides were mounted, followed by microscopic examination. Similarly, two LBC slides of cultured A549 lung carcinoma cells were labeled with Bio-S6 apt and Bio-random sequence following the above procedure.

Fluorescence ICC Based on FAM-S6 Apt

A schematic of the ICC procedure based on FAM-S6 apt is shown in Fig. 2b. LBC slides of patients' serous effusions were incubated with binding buffer to diminish the problem of background binding for 20 min at room temperature. Subsequently, 300-nM FAM-S6 apt diluted in binding buffer was incubated with cells for 20 min at room temperature in darkness and washed five times with washing buffer with each wash being 3 min. Finally, the slide was counterstained for nuclei with DAPI (Sigma) and mounted on glass slides. The specimen was observed with a fluorescence microscope (Olympus BX53). Similarly, fluorescence ICC was performed on two LBC slides of cultured A549 lung carcinoma cells with FAM-S6 apt and FAM-random sequence following the above procedure.

Fluorescence ICC Based on A-S6 Apt (the Activated Fluorescence Signal)

The activatable aptamer probe itself (with its usual "signal off" characteristic) showed a negligible fluorescence signal (low background). The fluorescence signal is activated by specifically recognizing the target cells. At first, we verified whether A-S6 apt can fluoresce when A-S6 apt binds to the target cells. The suspended A549 cells were transferred to a centrifuge tube, washed with binding buffer three times, and resuspended in binding buffer at a density of 5.0×10^5 cells/mL. Then, A-S6 apt (final concentration 300 nM) was mixed with the cell suspension. After incubation at room temperature in the dark for 20 min, the fluorescence emission spectra of FAM were recorded with an excitation of 492 nm with a Cary Eclipse fluorescence spectrophotometer (Varian, U.S.). Next, an ICC experiment based on A-S6 apt was carried out on LBC slides of patients' serous effusions. A schematic of this procedure is shown in Fig. 2c. LBC slides were incubated with binding buffer for 20 min at room temperature. Subsequently, 300 nM A-S6 apt was incubated with cells for 20 min at room temperature in the dark. Then the cells were washed three times with washing buffer. Finally, the slide was counterstained for nuclei with DAPI and mounted on glass slides. The specimen was observed with a fluorescence

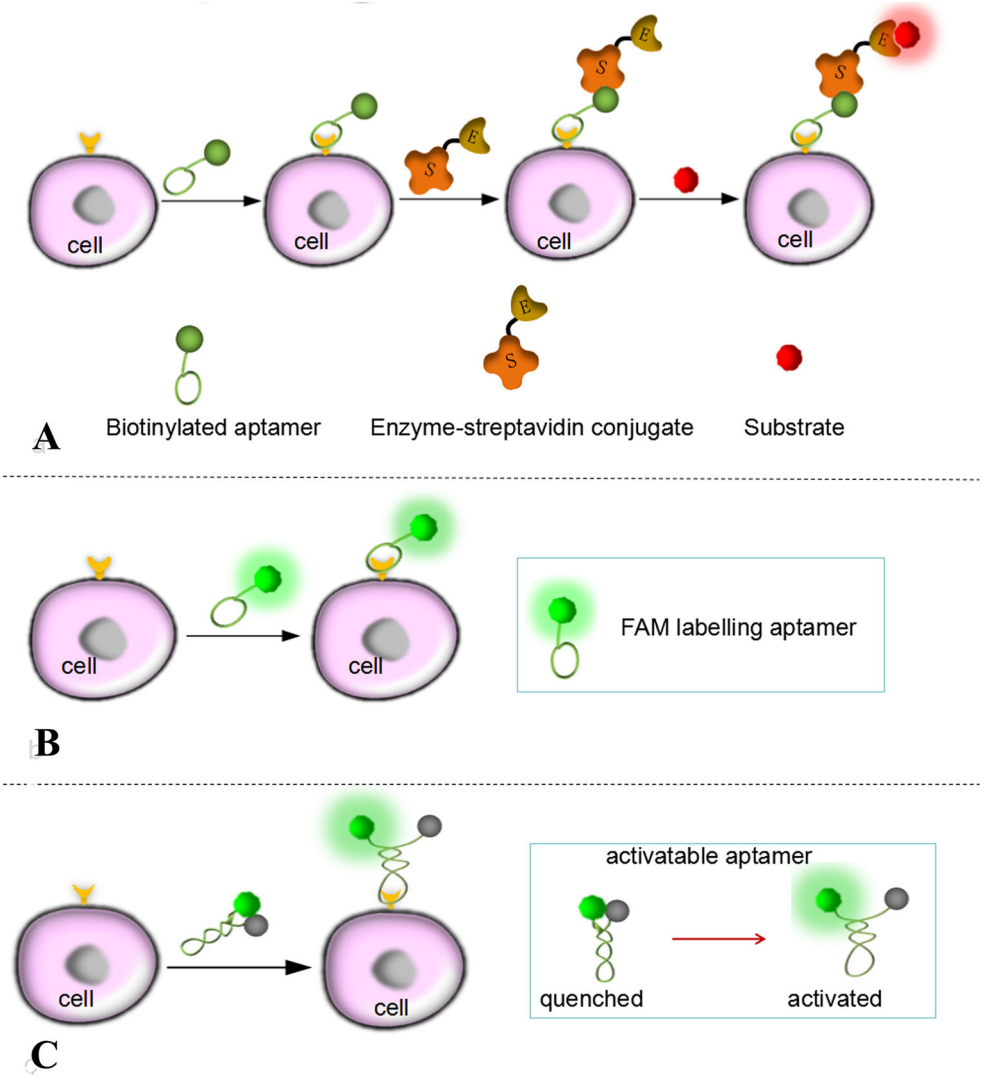

Fig. 2 The schematic diagram of ICC procedure based on three modes of aptamer. ICC which Bio-S6 apt labeled cancer cells (**a**);ICC based on FAM-S6-apt labeling cancer cells(**b**); ICC based on the activated fluorescence signal(**c**)

microscope (Olympus BX53). Similarly, an ICC experiment was carried out on two LBC slides of cultured A549 lung carcinoma cells with FAM-S6 apt and FAM-random sequence following the above procedure.

ICC Control and Assessment

Immunostaining necessitates the use of positive as well as negative controls, which is usually an easy procedure in IHC, but a challenging task to ICC. In general, clinical serous specimens harbor different types of cells, such as mesothelial cells, histiocytes, neutrophils, lymphocytes, and other cell types. These non-epidermal derived cells in specimens can act as negative controls. The target tumor cells should be positive in ICC; thus, they are their own positive control. Therefore, no additional negative controls or positive controls were used in the clinical samples.

ICC immunoreaction results from all samples were confirmed by two qualified cytopathologists (Dairong, Li; Tao, Wan) blinded to the clinical outcome. Results were recorded according to the staining intensity or fluorescence intensity of the cytoplasm. Results were considered positive when the staining intensity of malignant tumor cells was stronger than that of non-epidermal-derived cells in specimens. Otherwise, results were considered negative. In cases of disagreement, the results were determined by negotiation.

Results

Cultured A549 Cell Labeling by the Three Different ICC Modes

We employed fluorescence spectra analysis to validate the ability of A-S6 apt to bind specifically to A549 cells. The activatable S6 apt probe itself (300 nM) showed a negligible fluorescence signal (Fig. 3), implying an complete quenching of the fluorophore. When the A-S6 apt was incubated with

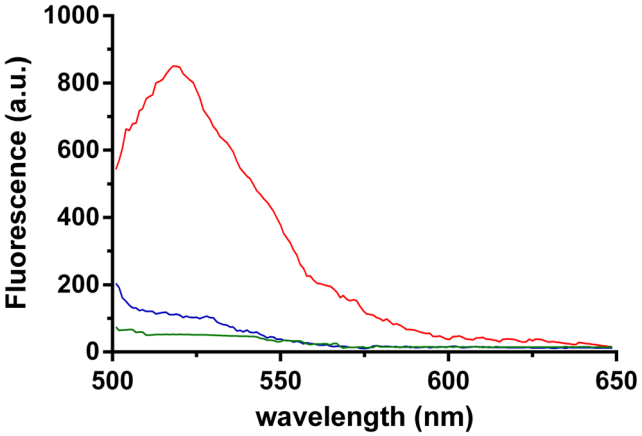

Fig. 3 Fluorescence spectra analysis. The designed activatable-S6 probe itself(300 nM) in binding buffer (without incubation with A549 cells, green line), A549 cells suspension alone (1×10^5 cells /ml, blue line), and the activatable-S6 probe (300 nM) after incubated with A549 cells (1×10^5 cells /ml) for 20 min (red line)

A549 cells for 20 min, a higher fluorescent signal was recorded by the fluorescence spectrophotometer, as shown in Fig. 3. The results of the fluorescence spectra analysis demonstrated that the activated S6 probe may bind specifically to the target cells (A549 lines) and therefore emit fluorescent signals.

The cytoplasm of A549 cells after ICC staining using the Bio-S6 aptamer showed brownish (positive reaction) staining, but no staining (negative reaction) based on the Bio-random sequence, as depicted in Fig. 4a. The cytoplasm of A549 cells was stained with green fluorescence using the FAM-S6 apt, whereas no fluorescence signal was displayed by the FAM-random sequence (negative control), as depicted in Fig. 4b. Similarly, fluorescent ICC of A549 cells gave a positive reaction for A-S6 apt but a negative reaction for A-random sequences (see Fig. 4c).

Evaluation of Papanicolaou Staining Results in Clinical Samples

Clinical cytological diagnosis based on Papanicolaou staining of one LBC slide was performed routinely and was evaluated before each ICC experiment to avoid the possibility that ICC results might be affected by an insufficient number of cells. Finally, all specimens in the study were classified as having adequate cellularity for diagnosis.

ICC Based on Bio-S6 Apt Staining for Clinical Samples

After ICC staining based on the Bio-S6 aptamer, the nucleus was blue due to counterstaining with hematoxylin. The coloring strength in the cytoplasm after staining the cells was recorded as follows: 0, no staining; 1, pale yellow; 2, brownish-yellow; 3, tan. The cytoplasm of common neutrophils, lymphocytes, mesothelial cells, and red cells in clinical specimens showed no staining, which was considered to be a negative reaction. If the cytoplasm of the tumor cells showed different degrees of yellow, they would be considered to show positive reactions for S6-aptamer-biotin; otherwise, they showed negative reactions. Representative staining results of clinical samples are also shown in Fig. 5.

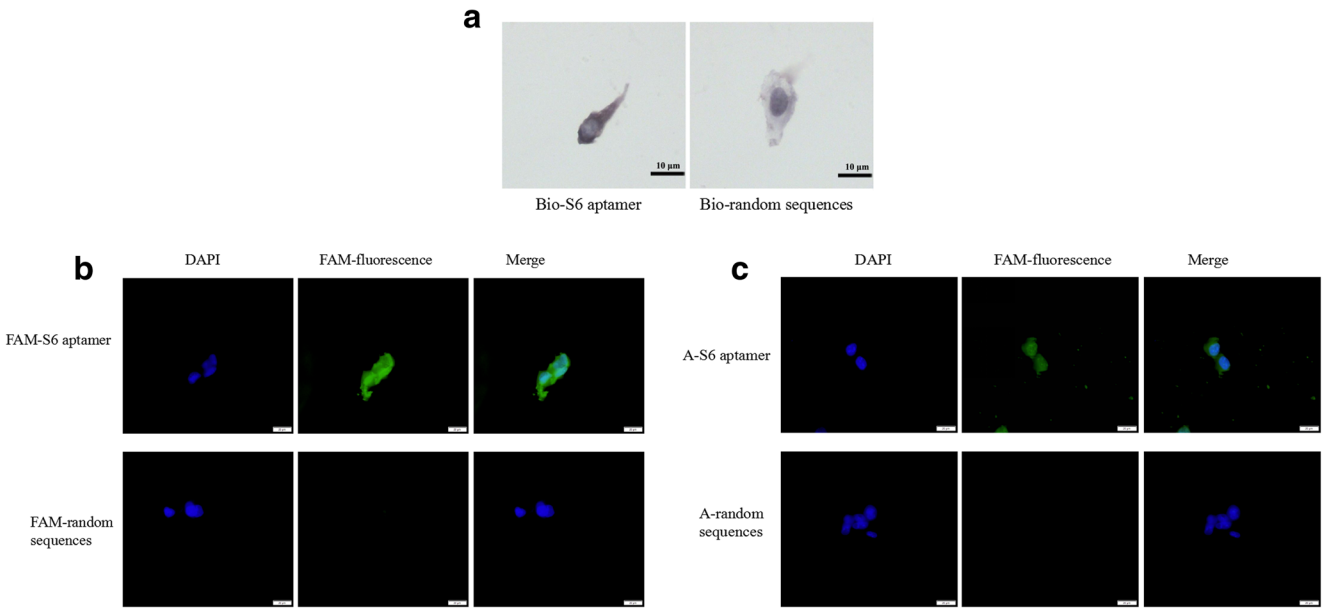

Fig. 4 A549 culture cells (×40) labelled by S6 aptamer with three different ICC manners. A549 cells were labelled by Bio-S6 aptamer and Bio-random sequence(**a**); A549 cells were labelled by fluorescent probes based on FAM-S6 apt and FAM-random sequence (**b**);A549 cells were labelled by activatable probes based on A-S6 apt, A- random sequences(**c**)

Fluorescence ICC Using the Direct Fluorescent-Labeled S6 Aptamer for Clinical Samples

In this study, adenocarcinoma cells on an LBC slide are very easily distinguished through fluorescent ICC using FAM-S6 apt. The cytoplasm of positive cells was stained with green fluorescence by FAM-S6 apt, whereas no fluorescence signal was displayed for normal cells (noncancerous cells, as control). Representative images of clinical samples are shown in Fig. 6a.

Fluorescence ICC by the Activatable Probe for Clinical Samples

Fluorescent ICC by the activatable probe was performed on LBC slides of patients' samples. Figure 6b shows that activatable S6 apt (A-S6 apt) binds specifically to adenocarcinoma cells in the specimens, and that fluorescence accumulates mainly on the cell surface with cell nuclei being counterstained blue by DAPI (a nucleus marker).

Evaluation of the Application of the Three ICC Protocols in Clinical Effusions

Bio-S6 apt and FAM-S6 apt probes can not only bind specifically to most lung adenocarcinoma cells with strong positive reactions, but can also bind to adenocarcinoma cells of non-lung primary sites, such as breast cancer, ovarian adenocarcinoma, and gastric adenocarcinoma with positive reactions to varying degrees. A-S6 apt probes can bind specifically to most lung adenocarcinoma cells with strong positive reactions, however, these probes showed lower positive rates in adenocarcinoma cells from non-lung origins. None of the aptamer probes reacted with mesothelioma, lymphoma, lung small cell carcinoma, or common benign cells in effusion samples. The detection rates with Bio-S6 apt, FAM-S6 apt, and A-S6 apt staining cells in the present study are listed in Table 3. The sensitivity and specificity in diagnosing adenocarcinoma cells with Bio-S6 apt, FAM-S6 apt, and A-S6 apt in the present study are listed in Table 4. Obviously, A-S6 apt ICC showed the highest specificity for diagnosing adenocarcinoma cells in specimens.

Discussion

Cell-type-specific aptamers have been developed by cell-SELEX without prior knowledge of molecular signatures on the cell surface. Currently, it is difficult for us to produce their corresponding antibodies based on cell surface molecules. These aptamers can recognize protein markers overexpressed

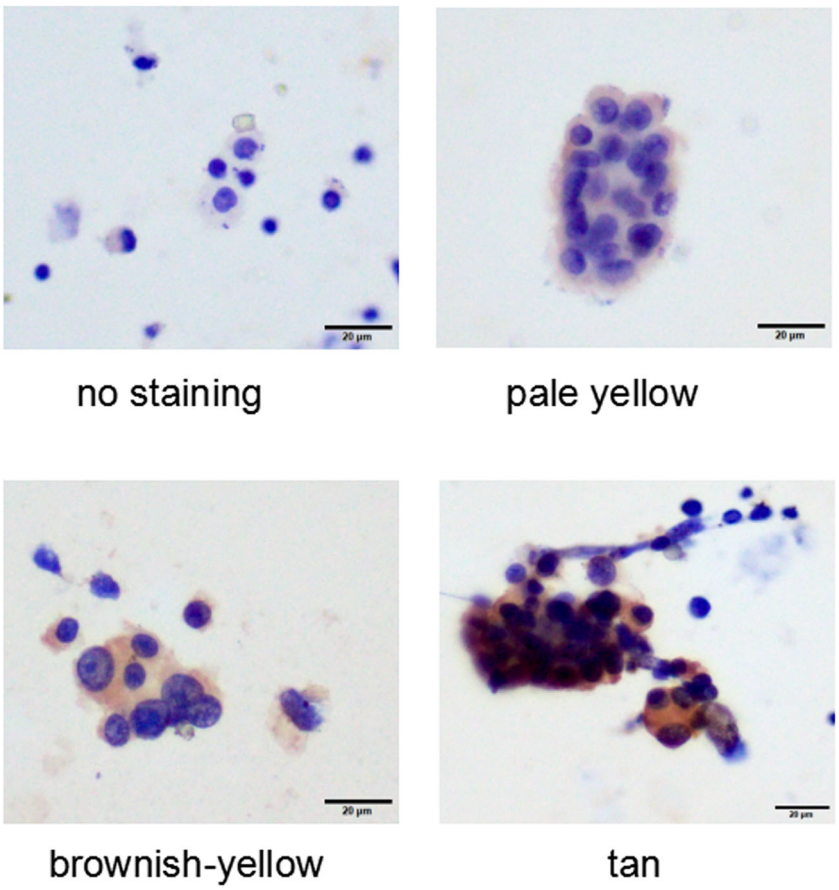

Fig. 5 Representative images (×40) from the cases based on Bio-S6 apt staining with DAB chromogen visualization in cytoplasm [the nucleus was blue due to counterstaining with hematoxylin]

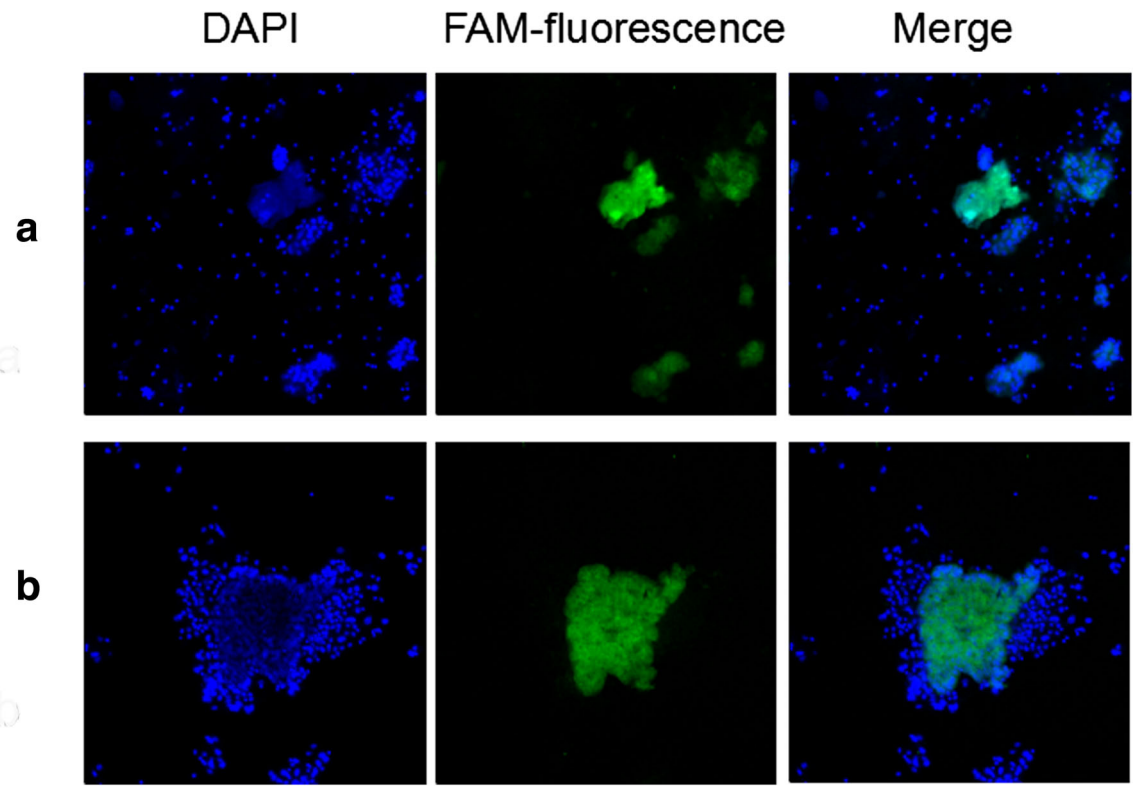

Fig. 6 The fluorescence images (×10) from representative cases with both Fam-S6 apt staining(**a**) and A-S6 apt staining(**b**). The target cell cluster showed green fluorescence

on the cancer cell membrane. Thus, the aptamers preferentially bind to diseased cells, especially cancer cells and aid in early diagnosis and targeted intervention [14]. The S6 aptamer against A549 cells was adopted in this study for differentiating cancer cells in clinical serosal fluids without the corresponding antibodies. That is, no corresponding antibodies were used as controls. The Bio-S6 apt, FAM-S6 apt, and A-S6 apt probes that were designed in this study could not only distinguish the cancerous cells quickly from large quantities of noncancerous cells, but could also identify the type of the cancerous cells in body fluid (a complex biological sample).

Aptamer-based tumor cell detection technology is currently mainly dependent on the "always on" analysis model [8, 15]. Particularly, direct fluorophore-tagged aptamer probes in the "always on" form are usually used for cancer cell detection [8, 16, 17]. As the probe signal is always present with a high background signal, its application in ICC needs many washing steps to avoid interference from nonspecific adsorption. Therefore, any ICC method based on an "always on" analysis model is relatively complex.

Cancer diagnosis during an ICC procedure with a minimal background signal originating from nontarget cells is crucial. As an alternative, "signal off" activatable aptamer probes, in which normally quenched signals are activated only after targeting cancer cells, have been developed for direct cancer detection [13, 18–20]. The A-S6 aptamer (an activatable probe) used in this study was constructed with the addition of six complementary bases at both ends of the sequence. The secondary structure of the sequence was predicted by RNAstructure 5.8 software and presented a hairpin structure, where the fluorescent group was close to the quenching group so that the fluorescence was quenched effectively. This aptamer emits fluorescence when binding with the target to cause it to undergo a conformational change. In this study, the

Table 3 Comparison of detection rates (%) of ICC based on S6 aptamers with three modes

	Bio-S6 apt(%)	FAM-S6 apt(%)	A-S6 apt(%)
Lung adenocarcinoma($n = 57$)	94.7	94.7	91.2
Non-lung adenocarcinoma($n = 26$)	84.6	88.5	73.1
Other malignancies($n = 10$)	20.0	20.0	0.0
Reactive effusion($n = 19$)	0.0	0.0	0.0

Table 4 The Sensitivity and specificity in diagnosing adenocarcinoma cells with S6 aptamer ICC

	Bio-S6 apt	FAM-S6 apt	A-S6 apt
Sensitivity	91.60%	92.8%	85.5%
Specificity	93.1.0%	93.1%	100%

A-S6 aptamer itself showed a weak fluorescence signal, indicating complete fluorescence quenching in the absence of A549 cells. However, in the presence of A549 cells, we observed an apparent fluorescence signal peak at 518 nm (excited by 492 nm light), which is the characteristic fluorescence of FAM, as seen in Fig. 3. Furthermore, we used the A-S6 aptamer for the ICC procedure in clinical effusion specimens. After incubation for 20 min, we observed a very strong fluorescence signal on adenocarcinoma cells. The A-S6 aptamer for ICC can avoid high background signal caused by nonspecific adsorption without any complicated washing steps.

Of course, both "always-on" fluorescent probes and activatable probes substantially reduce operating time in comparison to conventional ICC methods. All can be used for rapid diagnostics, but this is especially true for the activatable probe, because when it is mixed with the specimens, we can observe the emergence of fluorescence within minutes. The ICC method based on activatable aptamer probes can be applied to cytological specimens for quickly distinguishing malignant cells from nontarget cells. The technique will be useful for rapid on-site evaluation and can provide an on-site preliminary diagnosis. It may be beneficial to patients who will undergo minimally invasive sampling, such as medical thoracoscopy, transbronchial needle aspiration, and similar procedures [21, 22]. Additionally, we can further observe cell morphology with hematoxylin stain on slides that have already been stained by the activatable aptamer probe.

Overall, among the three ICC procedures, the easiest to handle and the one with the highest specificity of ICC were observed with the A-S6 apt probe. Nevertheless, activatable aptamer probes still have other problems including the fact that their organic synthesis can be inconvenient due to the coexistence of both the fluorophore and the quencher group and that undesired stimuli such as nuclease degradation may give false positive results and background elevation.

Conclusion

ICC examinations based on aptamers constitute a very promising detection method that quickly differentiates target cells from a large number of nontarget cells. ICC based on a cell-type-specific aptamer with shorter diagnostic time than that currently obtainable with antibodies will be a major breakthrough. In addition, clinical specimens containing only a few cells can be used in cancer diagnosis by ICC based on a cell-type-specific aptamer, replacing multiple antibodies. Our study is only the first step toward finding an acceptable method for ICC based on aptamers. More work in this field is needed.

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Compliance with Ethical Standards

Medical Ethics All specimens were obtained from the First Affiliated Hospital of Chongqing Medical University (Chongqing, China) in compliance with a protocol approved by the Institutional Review Board of the First Affiliated Hospital of Chongqing Medical University (Chongqing, China).

Conflict of Interest The authors declare that there is no conflict of interest regarding the publication of this paper.

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