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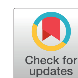
Colorimetric assay of rare disseminated tumor cells in real sample by aptamer-induced rolling circle amplification on cell surface



Lulu Xu [a,b,1], Zheng Jiang [c,1], Yudong Mu [d], Yuhong Zhang [a], Qian Zhan [a], Jin Cui [a], Wei Cheng [a,*], Shijia Ding [b]

[a] The Center for Clinical Molecular Medical Detection, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, PR China
[b] Key Laboratory of Clinical Laboratory Diagnostics (Ministry of Education), College of Laboratory Medicine, Chongqing Medical University, Chongqing 400016, PR China
[c] College of Medical Informatics, Chongqing Medical University, Chongqing 400016, PR China
[d] Department of Clinical Laboratory, Tumor Hospital of Shaanxi Province, Xi'an, 710061, PR China

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ABSTRACT

The identification of tumor cells disseminating in body fluids plays a critical role in the accurate diagnosis of cancer. Herein, a simple and pragmatic colorimetric strategy has been developed for ultrasensitive detection of tumor cells based on aptamer-induced rolling circle amplification (RCA) on cell surface. An Epithelial Cell Adhesion Molecule (EpCAM)-targeted aptamer was selected as recognition agent to specifically recognize and bind with tumor cells. Following, the aptamer-induced RCA performed in situ, presenting numerous G-quadruplexes sequences on the cell surface. G-quadruplex/hemin DNAzyme then formed and catalyzed colorimetric reaction for tumor cells detection. The proposed aptamer-induced RCA strategy displayed remarkable signal amplification performance and high specificity. The designed colorimetric assay could distinguish as low as 10 cancer cells in 10000 times of benign cells mixture, showing very high sensitivity and selectivity. Moreover, it has been successfully verified that the established strategy could directly identify tumor cells disseminated from various primary sites in multiple types of clinical body fluid samples. Thus this proposed strategy provided a simple, handy and powerful platform for ultrasensitive detection of tumor cells and had great potential for clinical application in convenient assay of rare disseminated tumor cells in body fluid samples for early cancer screening.
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1. Introduction

The tumor cells disseminate from the primary tumor site into body fluids like blood, urine, ascites and pleural fluid, is an early event in cancer progression [1,2]. Moreover, malignant body fluids may be the initial presenting manifestation in the late stage of certain types of cancers. For example, malignant pleural fluids present in nearly 50% of advanced non-small cell lung cancer patients [3]. An invasive procedure may only limit to those patients who can tolerate surgery and biopsy examination [4]. Thus, malignant body fluids are vitally important category of clinical specimen for cancer screening and diagnosis, which provide a higher yield of tumor cells and are more available and less invasive than surgery or biopsy. Regrettably, disseminated tumor cells in body fluids are rare events and usually mixed in with a large number of benign cells, such as blood cells and exfoliated cells [5,6]. So, the characterization of rare tumor cells in body fluids has proven to be difficult.

Some technologies have been applied to detect tumor cells in clinical laboratory such as histochemical staining, immunohistochemistry and flow cytometry. Histochemical staining exists a variety of interfering effects, and its sensitivity may not be sufficient to accurately identify tumor cells with low abundance in body fluid specimens [7]. Immunohistochemistry as a direct detection method is the most commonly employed to pathological diagnosis of cancer by using formalin-fixed and paraffin-embedded tissue sample, especially, with the development of tyramine amplification method and multiplexed immunohistochemistry [8,9]. But, those immunohistochemistry based techniques are more complex to analyze the body fluid specimens because of the time-consuming preparation of cytopathological slides, fixation, antigen retrieval, and so on [10,11]. Compared with immunohistochemistry, flow cytometry technology can overcome the difficulty of assessment

* Corresponding author at: The First Affiliated Hospital of Chongqing Medical University, The Center for Clinical Molecular Medical detection, 1 youyi Road, Yuzhong District, Chongqing, 400016, PR China.
E-mail address: chengwei@hospital.cqmu.edu.cn (W. Cheng).
[1] These authors contributed equally to this work.

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and achieve the sensitive analysis of multiple cell surface and intracellular markers. Nevertheless, many problems have been noted including its inactivation of antibody, and costly reagent and instrumentation [12]. Furthermore, variable affinity and specificity of different batches of antibodies inevitably influence on the precision of the immunohistochemistry and flow cytometry. Thus, a simple, low-cost and pragmatic tool for the detection of rare tumor cells disseminating in body fluids with high analytical sensitivity and specificity is still highly desirable.

Aptamers, known as chemical antibodies, are synthetic DNA/RNA nucleic acid single strands selected in vitro by exponential enrichment with high affinity and specificity [13,14]. Aptamers offer superior advantages over protein antibodies, such as small size, stability, high binding affinities, low cost, ease of modification and lack of immunogenicity [15,16]. The presence of many high-affinity aptamers that specifically bind to different biomarkers on tumor cell surface created a great opportunity to develop aptamer-based biosensors for the identification of tumor cells. Many new aptasensors have been developed successfully for tumor cells detection by using single fluorescence-labeled aptamer or hairpin-structured aptamer probe [17–20]. These means have proven to be simple, fast and low-cost. However, the single aptamer-binding triggered conformational alteration may only have a 1:1 ratio of binding event to signal output without signal amplification [21], resulting in a low analytical sensitivity. These means could not meet the clinical requirement for the detection of rare disseminated cancer cells in body fluids. On the other hand, aptamers were modified on the surface of biosensor [22], or labeled with nanoparticles [23,24] to capture and detect tumor cells. These strategies have greatly improved the sensitivity of designed aptasensors. Unfortunately, the variability of the preparation and modification of nanoparticles often affected the reproducibility and quantification of the methods [25,26]. These defects maybe show little impact on the analysis of mimetic samples of pure tumor cell, but inevitably compromise the sensitivity and reproducibility of the biosensing methods for the detection of low abundance of tumor cells in complicated real sample.

Herein, aiming at further improving the efficiency and practicality of aptamer-based methodology for ultrasensitive and specific analysis of rare tumor cells in complicated real sample, a simple and pragmatic biosensing strategy was developed by in situ accomplishing aptamer-induced rolling circle amplification (RCA) on cell surface and G-quadruplex/hemin DNAzyme-catalyzed colorimetric assay. Epithelial cell adhesion molecule (EpCAM) is a transmembrane glycoprotein [27] and overexpressed in most solid cancers such as colon, lung, head and neck, and breast [28,20]. Therefore, EpCAM is an ideal biomarker for distinguishing tumor cell and anti-EpCAM antibodies have been used to identify circulating tumor cells in the peripheral blood for individualized treatment of cancer [6]. So, optimized EpCAM aptamer [29] was employed to specifically bind to EpCAM on the surface of the target cells and induced subsequent RCA to synthesize a lot of repetitive G-quadruplex sequence in situ. Horseradish peroxidase-mimicking G-quadruplex/hemin DNAzyme then catalyzed colorimetric assay for tumor cells detection without additional separation or capture of tumor cells. Based on the specific recognition of EpCAM aptamer, powerful signal amplification of RCA and simplicity of DNAzyme-catalyzed colorimetric assay, the proposed strategy exhibited very high sensitivity and selectivity, and could directly identify tumor cells disseminated from various primary sites in multiple types of clinical body fluid samples. Though RCA- generated DNAzyme have been used as signal amplification strategy for the detection of the protein and pathogen [30,31]. These strategies still needed aptamer-functionalized microbeads for the protein separation or antibody-immobilized biosensor surface for the pathogen capture. Thus, our designed colorimetric assay provides a novel, convenient and pragmatic strategy for tumor cell biosensing, and a potential tool for detection of rare disseminated tumor cells in clinical body fluid specimens.

2. Experimental

2.1. Materials and reagents

T4 DNA ligase, Phi29 DNA polymerase, Exonuclease I (Exo I), and Exonuclease III (Exo III) were purchased from Thermo (Waltham Mass, USA). Hemin and 2,2′-azino-bis (3-ethylben-zothiazoline-6-sulfonic acid) ($ABTS^{2-}$) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 30% hydrogen peroxide solution (H_2O_2), bovine serum albumin (BSA), diethylpyrocarbonate (DEPC), deoxynucleotide solution mixture (dNTPs), phosphate buffer saline (PBS), Triton X-100, dimethyl sulfoxide (DMSO) and DNA oligonucleotides were obtained from Sangon Biotechnology Co. Ltd. (Shanghai, China). Salmon sperm DNA was purchased from Solarbio (Beijing, China). DAPI (Beyotime Institute of Biotechnology, Shanghai, CN). All DNA oligonucleotides were purified using high-performance liquid chromatography and listed in Table S1. Body fluids sample and cell lines used in this study were obtained from the First Affiliated Hospital of Chongqing Medical University.

All buffer solutions were prepared using Millipore-Q water (≥18 MΩ, Milli-Q, Millipore). Hemin stocking solution (10 mM) was prepared in dimethyl DMSO and stored in the dark at −20 °C and was diluted to the required concentrations with 25 mM 4-(2-hydroxyethyl)-1-piperazineëthanesulfonic acid (HEPES) (pH 7.4) containing 100 mM KCl, 200 mM NaCl, 0.05% Triton X-100 and 1% DMSO prior to use.

2.2. Apparatus

The absorbance signal was detected by the UV–vis spectrophotometer (UV-2550, Shimadzu, Kyoto, Japan). The gel electrophoresis was performed on the DYY-6C electrophoresis analyzer (Liuyi Instrument Company, China) and imaged on a Bio-rad ChemDoc XRS (Bio-Rad, USA). NanoDrop 1000 ultraviolet spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used to measure the concentrations of DNA suspensions. Fluorescence labeled cells were visualized under an Olympus BX53 fluorescence microscope (Olympus, Tokyo, Japan) and examined by FC-500 Flow Cytometer (Beckman Coulter, Inc., Fullerton, CA).

2.3. Cell culture

Involved cell lines were cultured in DMEM Medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin and streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. After being digested with 0.25% trypsin/EDTA solution for 2–3 min, cells were collected, centrifuged at 800 rpm for 3 min and then washed twice with PBS. The cells were quantified with Cedex XS cell counter (Roche) and diluted to a certain concentration by binding buffer (5 mM $MgCl_2$ and 0.1 mg/mL salmon sperm DNA in PBS pH = 7.4) [29]. In order to reduce adhesion of cells to plastic tips, all tips should be treated with 30% BSA before use [32].

2.4. Preparation of a circular template for RCA

4 μM padlock probe and 4 μM primer were mixed in T4 DNA ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM ATP, pH 7.4). In order to ensure that padlock probe can fully hybridize with primer, the mixture was heated to 95 °C and slowly cooled down to room temperature (RT). Then, after 30 Weiss U of T4 DNA ligase was added, ligation process was performed for 16 h at 16 °C to

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