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Label-free colorimetric detection of cancer related gene based on two-step amplification of molecular machine

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ABSTRACT

Highly sensitive detection of K-ras gene is of great significance in biomedical research and clinical diagnosis. Here, we developed a colorimetric biosensing system for the detection of proto-oncogene K-ras based on enhanced amplification effect of DNA molecular machine, where dual isothermal circular strand-displacement amplification (D-SDA) occurs on two arms in one-to-one correspondence. Specifically, we designed a primer-locked hairpin probe (HP) and a primer-contained linear polymerization template (PPT). In the presence of target gene, HP can hybridize with PPT, forming a DNA molecular machine with dual functional arms (called DFA-machine). Each of the two probes in this machine is able to be extended by polymerase on its counterpart species. Moreover, with the help of nicking endonuclease, the dual isothermal polymerization is converted into dual circular strand-displacement amplification, generating a large amount of anti-hemin aptamer-contained products. After binding to hemins, the aptamer/hemin duplex, horseradish peroxidase (HRP)-mimicking DNAzyme, was formed and catalyzed the oxidation of colorless ABTS by H_2O_2, producing a visible green color. The proposed colorimetric assay exhibits a wide linear range from 0.01 to 150 nM with a low detection limit of 10 pM. More interestingly, the mutations existing in target gene are easily observed by the naked eye. It should be noted that this colorimetric system was proved by the analysis of K-ras gene of SW620 cell lines. The simple and powerful DFA-machine is expected to provide promising potential in the sensitive detection of biomarkers for cancer diagnosis, prognosis and therapy.

1. Introduction

Biomolecules or genes related to the biological processes in nature are attracting increasing attention in biomedicine and health because their appearance or change is often the early molecular events of carcinogenesis. The point mutations in cancer related genes not only indicate tumor occurrence but also are important therapeutic targets (Vanhattum and Waldmann, 2014). It is generally known that point mutations of proto-oncogenes might cause malignant transformations of the cells. For example, in human bladder carcinoma, a G to T transversion in codon 12 of the Ha-ras and Ki-ras proto-oncogene converts them into oncogenes (Patolsky et al., 2001). Although K-ras gene is a kind of cancer related gene, which is a proto-oncogene. The detection of K-ras mutations in either primary or metastatic tumors from patients with metastatic colorectal cancer could be used to predict response to targeted therapies such as cetuximab and panitumumab (Santini et al., 2008). Therefore, the development of detection methods to accurately screen the point mutations in cancer related genes is extremely urgent.

In recent years, various signal amplification strategies were used to develop sensing systems for the detection of target genes of interest, including hybridization chain reaction (HCR) (Kahn et al., 2015; Trifonov et al., 2016; Wu et al., 2015), exonuclease-aided signal amplification (Guichi et al., 2014; Zuo et al., 2010), exponential isothermal polymerization amplification (Qiu et al., 2015; Song et al., 2014; Wei et al., 2009; Xie et al., 2013), rolling cycle amplification (RCA) (Pei et al., 2015; Wen et al., 2012; Zhuang et al., 2014), nicking endonuclease signal amplification (NESA) (Qiu et al., 2013; Weizmann et al., 2008), isothermal circular strand-displacement amplification (SDA) (Du et al., 2015; Huang et al., 2015; Lu et al., 2015; Wang et al., 2013) and other protocols (Kato and Oishi, 2014; Wu et al., 2009). Each of these amplification strategies has its own advantages and disadvantages, depending on the target DNA species and detection environments. For example, although PCR, as a powerful amplification

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method, can detect even a single molecule of target gene and has a very wide response range, it requires a thermal cycler and precise temperature control and is not suitable for the detection of short RNAs and point-of-care diagnostics. Therefore, there is an urgent need to develop new sensing systems for the detection of nucleic acids in order to meet at different requirements at various circumstances.

Because qualitative and semiquantitative assessment can be accomplished without any advanced or complicated instrumentation, visual detection is an increasingly attractive tool in many research fields. It is a promising sensing method for rapid diagnostics especially in emergency circumstances and in resource-limited rural areas, where cost-effective, rapid, and convenient detection are essential. One type of G-rich sequence, called anti-hemin aptamer (H-aptamer), is capable of self-assembling into unusual G-quadruplex structures by stacking several four-hydrogen-bonded G-tetrads (Zhou et al., 2013) and then binds to hemin, forming horseradish peroxidase (HRP)-mimicking DNAzyme. This complex exhibit superior peroxidase-like activity and is able to catalyze the oxidation of ABTS by H_2O_2 to generate green-colored oxidized products, indicating a colorimetric signaling strategy for the detection of biomarkers. Due to the perfect visibility, low cost, easy to operate, and compatibility with different amplification techniques, G-quadruplex/hemin DNAzyme-based colorimetric assay is often adopted in biosensing applications since the technique was reported by Travascio' in 1998 (Travascio et al., 1998). For instance, G-quadruplex/hemin DNAzyme-based sensitive sensing systems were successfully developed for quantitative detection of metal ions (Kong et al., 2010), specific detection of DNA sequences (Deng et al., 2008; Shimron et al., 2012; Wang et al., 2011; Weizmann et al., 2006), the activity analysis of enzymes (Xiao et al., 2004) and drug screening (Kong et al., 2008).

On the basis of the above considerations, we proposed a colorimetric sensing system for K-ras gene detection based on the amplification effect of DNA molecular machine with dual functional arms (DFA) each of which is able to perform the circular isothermal strand-displacement amplification (SDA). The DNA nanodevice is called DFA-machine because of its dual functional arms. In the presence of target gene, a continuous stream of H-aptamer-contained product could flow out from the two functional arms of DNA machine, and a dramatically enhanced colorimetric signal is achieved due to the formation of a large number of DNAzymes after addition of hemins. The point mutation in target gene may impede the operation of DFA-machine, and the colorimetric signal is inevitably compromised. Therefore, the mutant target DNA can be recognized by naked eye. Compared with one functional arm-designed DNA machine, DFA-machine is able to implement the ultrasensitive and highly selective detection of K-ras. The real sample analyses performed also display the potential of proposed colorimetric assay in the field of gene mutation detection.

2. Materials and methods

2.1. Materials and reagents

Oligonucleotides designed in this study were synthesized and purified with PAGE by Invitrogen Bio Inc. (Shanghai, China), and their sequences are listed in Table S1. All lyophilized oligonucleotides were dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH=8.0) and stored at 4 °C before usage. The Klenow fragment (3′–5′ exo$^-$) polymerase (KF polymerase) and Nt. BbvCI endonuclease were purchased from New England Biolabs Ltd. The deoxynucleotide triphosphates (dNTPs) were obtained from Dingguo Changsheng Biotechnology Co., Ltd. Hemin and 2,2′-azino-bis(3- ethylbenzothiozoline)–6-sulfonic acid (ABTS) were ordered from Sigma-Aldrich Co. LLC. Ultrapure water obtained from a Kerton lab MINI water purification system (resistivity≥18.2 MΩ cm) was used throughout the experiments. All other chemicals were of analytical grade and were used without further purification.

2.2. Apparatus

Absorbance spectrum of DFA-machine was collected by a UV-2700 spectrophotometer (Shimadzu, Japan) in the wavelength range of 370–500 nm. The absorbance peak at 417 nm was recorded and used to evaluate the sensing performance of colorimetric assay system. The SDA reaction temperature was controlled by a TU-200 Block Heater (Yiheng Co. Ltd., Shanghai, China).

2.3. Target gene assay

HP was annealed at 90 °C for 5 min and then allowed to gradually cool down to room temperature to fold into a hairpin structure. The resulting HP solution was then stored at 4 °C for further use. For the target gene detection, a 0.5 μL aliquot of target K-ras gene at given concentration was added into a 0.5 mL centrifuge tube containing the mixture of 2.5 μL of 10×NE buffer 2 (10 mM pH=7.9 Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT), 1 μL of 10 μM HP and 1 μL of 10 μM PPT2, and then diluted by 18.2 μL of water. After 1 μL of 10 mM dNTPs, 0.5 μL of 5 U/μL KF polymerase, and 0.25 μL of 10 U/μL Nt. BbvCI endonuclease were added, the resulting solution was kept at the temperature of 37 °C for 120 min. Subsequently, the mixture was incubated at 80 °C for 20 min to terminate SDA reaction. The final volume of colorimetric sensing system was 25 μL, from which the concentrations of HP, PPT and K-ras were calculated.

2.4. Colorimetric measurement

A 2 μL droplet of freshly-prepared hemin was added into above-mentioned reaction solution, and the obtained mixture (27 μL) was incubated at 37 °C for 60 min to form DNAzymes. Then, 85.5 μL of 2×HEPES buffer (50 mM HEPES, 20 mM KCl, 0.4 M NaCl, 2% DMSO, 0.1% Triton X-100, pH =5.2), 5 μL of 36 mM ABTS, and 2.5 μL of 4 mM H_2O_2 were added, and the resulting solution (120 μL) was allowed to react for 30 min. The hemin concentration mentioned in subsequent experiments indicates that in 27 μL of mixture, while the concentration of ABTS and H_2O_2 was calculated from 120 μL of final reaction solution.

2.5. Cell lysis and gene extraction

Because the colon cell line expresses mutant K-ras (Ebi et al., 2011; Singh et al., 2012), the colon tumor cell line SW620 was used as a model cell line to demonstrate the DFA-machine-based colorimetric assay for the visual detection of K-ras gene. SW620 cell lines obtained from Cell Bank of Chinese Academy were cultured in RPMI 1640 media in tissue culture flasks. The media were supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The resulting cells were suspended in 200 μL of PBS, while the culture medium was discarded. Subsequently, the cells were spun down at 5000 rpm for 5 min in PBS. Total DNAs were extracted from cells using the Universal Genomic DNA Extraction Kit (Takara Biotechnology Co. Ltd.) according to the manufacturer's procedures. The newly-extracted DNAs were dried by vacuum rotary evaporation, followed by dissolved in 100 μL of ultrapure H_2O. The DNA concentration was determined by UV on Q5000 UV/Vis spectrometer (Quawell Technology, Inc., USA).

2.6. Asymmetric polymerase chain reaction (PCR) amplification

Two groups of primers were involved for the asymmetric PCR amplification. The first group of primers were used for control PCR amplification: Sense-primer 1: 5′-TCTACTGGGACGGAACAGCTT-3′;