Analytical Biochemistry 538 (2017) 20–25

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Ultrasensitive microRNA-21 detection based on DNA hybridization chain reaction and SYBR Green dye

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ARTICLE INFO

Article history:
Received 20 June 2017
Received in revised form
6 September 2017
Accepted 10 September 2017
Available online 15 September 2017

Keywords:
Label-free fluorescence method
MicroRNA-21
Ultrasensitive
Hybrid chain reaction
SYBR Green I
Gastric cancer

ABSTRACT

It is extremely important for quantifying trace microRNAs in the biomedical applications. In this study, an ultrasensitive, rapid and efficient label-free fluorescence method was proposed and applied for detecting microRNA-21 in serum of gastric cancer patients based on DNA hybridization chain reaction (HCR). DNA H1 and DNA H2 were designed and used as hairpin probes, the HCR was proceeded in the presence of target microRNAs. Amounts of SYBR Green I dyes were used as signal molecules to intercalate long DNA concatemers from HCR, which guaranteed the model of label-free fluorescence and strong fluorescence density. The detection method showed a wide linear region from 1 fM to 10^5 fM, and the limit of detection was 0.2554 fM (at S/N = 3) for microRNAs. The results showed that this method had an excellent specificity and reproducibility. Furthermore, the label-free fluorescence strategy exhibited a sensitive response to microRNA-21 in real serum samples of gastric cancer patients and the results obtained were in accordance with reference method ($R^2 = 0.994$). Overall, the proposed strategy could be satisfactory for rapid, ultrasensitive and efficient detection of microRNA-21, and held great potentials in clinic diagnosis of gastric cancer.

Introduction

MicroRNAs are small non-coding RNA molecules that have crucial roles in the regulation of gene expression and post-transcriptional regulators of the immune response, which were involved in the whole cellular processes including cell proliferation, differentiation and apoptosis [1–3]. Previous researches have indicated that there is a closely relationship between the abnormal expression of microRNAs and several human diseases [4–7]. To minimize health risks, the quantitative detections of microRNAs has gradually become a research hotspot and shown great application potential for clinical diagnostics.

Among the large number of microRNAs [8], the excessive expression of microRNA-21 has been found in the serum of cancer patients, including medulloblastoma [9], glioblastoma [10], colon cancer [11], hepatocellular cancer [12], and breast cancer [13], when compared to the corresponding normal tissues. Recently, the increasing of microRNA-21 expression had been verified in the early head and neck squamous cell carinoma tissue samples [14]. Thus, sensitive and accurate detection for microRNA-21 will make contribution to clinic diagnosis and prognosis in cancer curing [15]. And it could help clarifying the relationship between microRNA-21 and the cancer, and even the pathways of microRNA-21 participating in malignancies.

Various techniques and methods have been developed for detecting the expression level of microRNAs. Quantitative real-time polymerase chain reaction (qRT-PCR) was one of the most commonly used methods and had been widely applied for microRNAs analysis [16–18]. Additionally, Chan et al. reported a northern blotting method for quantitative analysis microRNA-21 in glioblastoma multiforme tumor tissues [19]. Thum et al. developed an in situ hybridization method for microRNA-21 detection in myocardial fibroblasts [20]. Wang et al. examined the expression of microRNA-21 in human serum by surface plasmon resonance strategy [21]. Cissell et al. presented a method for sensitive and specific detection of microRNA-21 using bioluminescence-based techniques [22]. These strategies have shown high sensitivity and specificity, and provided multiple selections. Nevertheless, expensive instruments, environment contamination, complicated and time-consuming operations, were still the existence deficiencies.

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http://dx.doi.org/10.1016/j.ab.2017.09.007
0003-2697/

The development of sensitive, simple, rapid, and economical methods to detect microRNAs would be more required and significant.

The sequence amplification-base strategies were ideal ways to improve the sensitivity of microRNAs detection significantly, such as exponential isothermal amplification [23], loop-mediated isothermal amplification [24], rolling circle amplification [25], nicking endonuclease signal amplification [26], exonuclease-aided signal amplification [27], hybridization chain reaction amplifying signal [28] and strand displacement amplification [29]. These researches have made remarkable progress and could be important reference for developing higher sensitive detection methods for microRNA-21. Among these sequence amplification-base strategies, hybridization chain reaction attracted greatly interests due to its enzyme-free nucleic acid isothermal amplification technology, where a the initiator sequence and two stable hairpin probes were self-assembled to extend the double-stranded DNA concatemers [30]. The hybridization chain reaction amplifying signal strategy is a good choice for ultrasensitive microRNA detection. Up to now, the HCR has been developed for analyzing microRNAs. In 2014, a novel technique called allosteric hairpin DNA switch-HCR detection technology was applied for quantitative measurements of microRNA let-7a [31]. However, it need streptavidin aptamer and special chemiluminescence reagent (3,4,5-trimethoxylphenyl-glyoxal). Recently, the electrochemical biosensing technology has widely applied in the field of microRNA [32,33], but it requires complicated multistep procedures due to the assembly of the biosensor.

The present work aims to develop an ultrasensitive, simple, rapid and low cost HCR method for microRNA-21 detection in serum samples of gastric cancer patients using label-free fluorescence strategy. For this purpose, the hairpin probes DNA H1 and DNA H2 were designed and synthesized according to the sequence of microRNA-21. In the detection process, the microRNA-21 could specifically trigger the HCR between DNA H1 and DNA H2, yielding long double-stranded DNA. Subsequently, the SYBR Green I dyes were added to the reaction solutions, generating a strong fluorescence signal. Finally the expression level of microRNA-21 was quantified according to the fluorescence intensity. Furthermore, the developed method was evaluated and applied to analysis of microRNA-21 in the serum of gastric cancer patients. What is more, the method has universality for any variants of the miRNA. It should be noted that different miRNAs need to resynthesis of the label-free specific DNA probes. but it is not necessary to reoptimization of the assay conditions for the each new target miRNA. And the organic fluorescence dye-labeled DNA probe has been widely applied in nucleic assay using fluorescence technique because the easily commercialized synthesis.

Experimental section

Reagents and solutions

Diethypyrocarbonate (DEPC) was supplied by Solarbio (Beijing, China). Tris (hy-droxymethyl) aminomethane (Tris), dithiothreitol (DTT), EDTA and tris (2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Aladdin (Shanghai, China). DNase I was provided by New England Biolabs (Ipswich, MA). ATP (adenosine 5′-triphosphate disodium salt hydrate) was synthesized by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). SYBR Green I (10000 × stock solution in dimethyl sulfoxide) was purchased from Xiamen Bio-Vision Biotechnology Co. Ltd. (Xiamen, China). All other chemicals used were of analytical reagent grade, and purchased from Sigma Aldrich (Shanghai, China).

The buffer solutions employed in this work were as follows. TE buffer: 10 mmol/L Tris-HCl, 1 mmol/L EDTA (pH 8.0). 5 × reaction buffer: 3.75 mmol/L NaCl, 0.5 mmol/L EDTA, 250 mmol/L Tris-HCl, 50 mmol/L $MgCl_2$, 5 mmol/L ATP, 50 mmol/L DTT (pH 7.5). DNase I reaction buffer: 10 mmol/L Tris-HCl, 2.5 mmol/L $MgCl_2$, 0.5 mmol/L $CaCl_2$ (pH 7.6). 4 × SPSC reaction buffer: 1 mol/L NaCl, 200 mmol/L Na_2HPO_4 (pH = 7.5). All the solutions and redistilled deionized water were treated with DEPC and autoclaved to protect from RNase degradation.

The HPLC-purified DNA oligonucleotides and microRNAs were supplied by Sangon Biotech Co., Ltd. (Shanghai, China) and TaKaRa Biotechnology Co., Ltd. (Dalian, China), respectively, which have been list in Table 1.

The synthesized microRNAs and DNA were dissolved in TE buffer (containing 10 mmol/L Tris-HCl and 1 mmol/L EDTA, pH 8.0) and stored at −20 °C.

Sample preparation

The serum samples were collected from the Tumor Center of Taian City Central Hospital. A volume of 20 μL serum from non-cancer human or gastric cancer patients was uniformly mixed with 1 μL DNase I reaction buffer, 1 μL EDTA and 10 μL DNase, and incubated at 37 °C for 30 min. Then, the mixture solution was incubated at 65 °C for 10 min to inactive DNase. Finally, the reaction product was stored at −80 °C until analyzed.

Detection procedure

HCR step

The DNA H1 and DNA H2 were heated at 95 °C for 5 min, and then gradually cooled down to room temperature in about 2 h. A volume of 20 μL for DNA H1 (10^{-7} mol L^{-1}), 20 μL of DNA H2 (10^{-7} mol L^{-1}) and 20 μL various concentrations of microRNA-21 were added into 20 μL 4 × SPSC reaction buffer, the mixture was reacted at 37 °C for 2 h in a constant temperature incubator.

Fluorochrome intercalation step

SYBR Green I working solution was prepared through 100 times dilution of 10000 × SYBR Green I using 5 × reaction buffer. Then, the HCR product was then incubated with 20 μL SYBR Green I working solution for 15 min.

Fluorescence measurement step

Fluorescence intensity was recorded on a F-4600 spectrophotometer (Hitachi, Japan). The excitation wavelength was set at 493 nm, and the emission spectra were collected from 510 to 600 nm with both excitation and emission slits of 10 nm. the measurement was conducted in the total volume of 100 μL sample system.

Evaluation of the developed method

The concentration of probes (DNA H1 and DNA H2) and time of HCR reaction were investigated to optimize the developed method. A series of microRNA-21 concentration were detected using this detection strategy, the standard curve was constructed and the sensitivity of method was evaluated. Additionally, the specificity of this detection strategy was evaluated by testing the fluorescence intensity for four different RNAs.

Detection of real samples

After collection and preparation of real serum samples, the expression level of microRNA-21 for samples were analyzed by the developed strategy as above procedure. Afterwards, the samples were further confirmed by qRT-PCR on a Bio-Rad CFX96 ™ Real-