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A target-triggered dual amplification strategy for sensitive detection of microRNA

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ABSTRACT

The accurate and quantitative analysis of microRNA (miRNA) expression is critical for biomedical research and clinical theranostics. In this study, we report a novel sensor for the sensitive detection of miRNA based on a duplex-specific nuclease (DSN)-assisted dual signal amplification strategy. A chimeric probe (DNA/2-OMe-RNA) that consists of a miRNA recognition DNA sequence and a Taqman probe hybridization RNA sequence (2′-O-methyl RNA) was designed and synthesized. One molecule of target miRNA can trigger cyclical cleavage of the chimeric probes to produce 2′-O-methyl RNA by DSN in the first round of amplification. The 2′-O-methyl RNA molecules can subsequently hybridize with Taqman probes and initiate the second round of cyclical amplification to generate detectable fluorescence by DSN. The proposed strategy exhibits high specificity in discriminating cognate miRNAs, and the dual signal transduction process enables the detection of miRNA concentrations as low as 7.3 fM. We further applied this assay to miRNA quantification in cancer cells to confirm its applicability. The present study provides a sensitive, specific and simple method for miRNA detection and holds great potential for further application in biomedical research and in the clinical laboratory.

1. Introduction

MicroRNAs (miRNAs) belong to a class of endogenous small molecules that are involved in the regulation of gene expression in a variety of life processes (Baker, 2010; Inui et al., 2010). The deregulation of miRNA expression in specific tissues has been reported in various pathological processes such as viral diseases, cardiovascular diseases, immune disorders and cancers (Arenz, 2006; Jopling et al., 2005). Recent studies have also confirmed that miRNAs are also present in the extracellular matrix of various tissues, including peripheral blood and other biofluids (Grasedieck et al., 2013; Weber et al., 2010). Moreover, most of these extracellular miRNAs (ex-miRNAs) carry specific information and have been shown to be associated with various diseases. Since (ex-miRNAs) are easily accessible and relatively non-invasive, they are regarded as a promising new group of biomarkers.

In comparison with other nucleic acids including DNA and messenger RNA, the unique characteristics of miRNAs, such as small size, sequence homology among family members (even single-base mismatch), and low abundance in biological samples, make accurate analysis a great challenge (Baker, 2010). In the past decade, a variety of effective methods have been developed for miRNA detection, among which northern blotting is regarded as the gold standard (Valoczi et al., 2004), although it has limitations such as poor sensitivity, large sample consumption and a tedious experimental protocol. The microarray-based technique is another conventional assay which has the advantage of high throughput and multiplexing capacity (Thomson et al., 2004), but poor sensitivity and a lengthy hybridization time restrict its wide application. In order to improve the sensitivity and efficiency of miRNA detection, a variety of methods which rely on signal amplification strategies have been proposed, such as real-time PCR (RT-PCR) (Chen et al., 2005; Shi and Chiang, 2005), exponential amplification reaction (EXPAR) (Jia et al., 2010) and rolling circle amplification (RCA) (Cheng et al., 2009; Zhou et al., 2010), among others.

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Among these approaches, RT-PCR methods, including stem-loop RT-PCR and polyA-tailed RT-PCR, are the most widely used miRNA analysis techniques, both of which have the advantage of high sensitivity and specificity. Unfortunately, these techniques require precise temperature control and the essential step of reverse transcription, which increases the experimental time and risk for RNase contamination. EXPAR, which was first devised by the Galas group for the efficient amplification of short oligonucleotides (8–16 nucleotides) (Van Ness et al., 2003), has been successfully applied to miRNA real-time detection by Li and coworkers, with a detection limit of about 15 aM (Jia et al., 2010). However, this method is mainly limited by strong nonspecific background amplification, which is mainly caused by interactions between the template and DNA polymerase. RAC-based methods are also widely used in miRNA analysis owing to their high sensitivity (at the fM level) and specificity (able to discriminate single-base pair mismatches); however, these methods are very time-consuming. In a paper by Cheng et al. (2009) a detection limit of 10 fM was achieved by allowing the experiment to proceed as long as 8 h. Therefore, the development of a rapid, convenient and sensitive miRNA detection method is still in great demand.

Recently, a novel and stable enzyme called duplex-specific nuclease (DSN) was isolated from the hepatopancreas of the Kamchatka crab (*Paralithodes camtschaticus*) by Shagin et al. (2002). This nuclease exhibits a strong cleavage preference for double-stranded (ds) DNA and DNA in DNA-RNA hybrid duplexes, while it shows little activity toward single-stranded (ss) DNA or single- or double-stranded RNA. More interestingly, this enzyme has a good ability to discriminate between perfectly matched and slightly mismatched (up to one mismatch) short duplexes. Due to its unique enzymatic characteristics, DSN has attracted increasing interest and shows great potential for application in the biological and biomedical sciences. Using DSN, several fluorescence signal amplification methods for miRNA detection have been developed (Degliangeli et al., 2014; Yin et al., 2012). Despite their remarkable advantages, such as simple methodology, fast experimental protocols and high specificity, the detection limit of these methods remains in need of further improvement for more extensive application. A further improvement in sensitivity could be achieved by introducing a dual signal amplification process. Herein, on the basis of DNA/2-OMe-RNA chimeric probes (DR-CPs) and a DSN-assisted cascade signal amplification strategy, we report a novel and more sensitive fluorescence method for the quantitative detection of miRNA. In this study, due to the ultrahigh selectivity of DSN, the proposed strategy can distinguish between varied homologous sequences that contain as little as a single base mismatch. Meanwhile, by the prominent signal amplification efficiency of DR-CPs assisted dual signal amplification strategy, the proposed sensitive method allows for the detection of target miRNA in a wide dynamic range of 10 fM to 10 pM and has a detection limit as low as 7.3 fM, making this method advantageous for analyzing biological samples. Moreover, we successfully applied this method for analyzing total RNA samples from human cancer cell clines, demonstrating its potential application in practical sample analysis.

2. Experimental methods

2.1. Materials and reagents

The HPLC-purified microRNAs, DNA/2-OMe-RNA chimeric probes, Taqman probes and other oligonucleotide probes were synthesized by Takara Biotechnology Co. Ltd. (Dalian, China). All oligonucleotide sequences are listed in Table S1 (Supporting information). RNase inhibitor and DEPC-treated water were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Streptavidin-coated magnetic microspheres (MMS, ~0.8 μm in diameter, 10 mg/mL) were purchased from Bangs laboratories, Inc. (Indiana, USA). DSN was obtained from Newborn Co. Ltd. (Shenzhen, China). All chemicals were purchased from Sigma-Aldrich, Inc. (Saint Louis, MO, USA) and used without further purification.

2.2. Procedure for miRNA detection

First, the detection of miRNA was performed in a 30 μL amplification reaction mixture containing 1 × DSN buffer (50 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$ and 1 mM DTT), 0.2 U DSN (dissolved in 25 mM Tris–HCl, pH 8.0; 50% glycerol), 20 U RNase inhibitor, 3 μL prepared functional MMs and different concentrations of target miRNA at 60 °C for 30 min After that, the MMs were separated from the reaction solution using a magnetic separation rack, and the solution (27 μL) was transferred to a new centrifuge tube. This was followed by the addition of Taqman probes (3 μL, 3 μM) to a final concentration of 300 nM. Subsequently, the reaction mixture was incubated at 60 °C for 30 min prior to the fluorescence measurement.

2.3. Fluorescence measurement

The amplification products (30 μL) were diluted to a final volume of 60 μL with 30 μL 10 mM EDTA. Fluorescence measurements were carried out on a Perkin-Elmer LS-55 fluorometer equipped with a xenon lamp excitation source. The excitation wavelength was 495 nm, and the spectra were recorded between 500 and 650 nm. The fluorescence intensity at 517 nm was chosen as the optimal experimental condition for data analysis.

2.4. Gel electrophoresis

Products of the DSN enzymatic amplification reaction were analyzed by 3% agarose gel electrophoresis in 0.5 × TBE buffer (45 mM Tris-boric acid, 1 mM EDTA, pH 8.0) at a 110 V constant voltage at room temperature for 40 min The gels were stained by 4 S Red Plus and analyzed using a UVItec Platinum Gel Imaging system (Cambridge, UK).

3. Results and discussion

3.1. Principle of the miRNA assay

The principle behind the miRNA assay based on DR-CPs and DSN-assisted dual signal amplification is shown in detail in Scheme 1. DR-CP with a biotin group at its 3′ terminus was rationally designed, consisting of two main regions: a target miRNA recognition DNA sequence at its 3′ end and a Taqman probe recognition 2′-O-methyl RNA sequence at its 5′ end (Scheme 1(B)). This probe attached to the surface of streptavidin-coated MMs through a biotin/streptavidin interaction. When the target miRNA is added to the reaction solution, it hybridizes with the DR-CP to form a DR-CP/miRNA duplex. Once the DNA/RNA heteroduplex is formed, the target recognition sequence of the DR-CP is selectively hydrolyzed by DSN. As a result, the 2-OMe-RNA fragments are released and the target miRNA strands are recycled, thus forming a target-recycling amplification through which thousands of 2-OMe-RNA fragments are cleaved off the MMs by one target miRNA strand during hybridization/DSN incubation. Afterwards, the MMs together with the unreacted DR-CPs are separated from the reaction solution using a magnetic separation rack. This is followed by the addition of Taqman probes (as a signal output). Similarly, the 2-OMe-RNA fragments and Taqman probes also form