Ultrasensitive Detection of Nasopharyngeal Carcinoma-Related MiRNA through Garland Rolling Circle Amplification Integrated Catalytic Hairpin Assembly

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ABSTRACT: MiRNA is reported to be closely related to nasopharyngeal carcinoma and has the potential to be a biomarker for the early diagnosis of nasopharyngeal carcinoma. However, the detection of miRNAs remains to be improved, given their complexity and low sensitivity. Herein, we propose here a novel miRNA detection method through the integration of garland RCA and CHA. In detail, the method is composed of two important signal amplification processes. For the first signal amplification process, the target miRNA could initiate garland RCA and then generate a nicking site on the products with the assistance of Nb.BbvCI enzymes. Afterward, a CHA process is induced with a designed H probe through the two signal amplification processes; the method exhibited a much-improved sensitivity. At last, we believe that this method is a promising approach capable of being applied in screening, diagnosing, and prognosticating multiple diseases.

INTRODUCTION

Nasopharyngeal carcinoma is a malignant tumor derived from the epithelium of the nasopharyngeal region through a complex process of multifactor regulation and occurs on the posterior and sidewalls of the nasopharyngeal cavity.1−3 Nasopharyngeal carcinoma is closely related to the Epstein–Barr virus infection, genetic susceptibility genes, and environmental carcinogens. The emerging increased incidence rate, high mortality misdiagnoses, or miss of atypical early symptoms make it a global health issue. Therefore, a potential noninvasive, convenient, and low-cost serological detection method is highly desired and will greatly contribute to the early diagnosis of nasopharyngeal carcinoma.4,5 MicroRNAs are a group of noncoding RNA sequences composed of 20−24 nucleotides, which mainly regulate the expression of downstream genes by combining with the 3′ noncoding region (3′ UTR) of the target gene.6,7 Particularly, the circulating miRNAs, which could avoid degradation of RNA enzymes via bind with Argonaute protein, are ideal biomarkers for cancer diagnosis.

Currently, the most classic methods for sensitive detection and quantification of miRNAs are qRT-PCR, Northern blot analysis, and microarray.6,9 Although the above methods have been widely applied for experimental or clinical miRNA quantification, some shortcomings could not be neglected due to the short length, low expression level, and high homologous sequence similarity of miRNAs.10 For example, the requirements of complicated primer design, a cumbersome detection device, and relatively high costs of qRT-PCR and Northern blot analysis made them remain improvable (especially in biofluid samples like a serum, where the miRNA concentration ranges from fM level to pM level). In recent years, a variety of miRNA detection methods have been developed,11−14 mainly including bioluminescence, fluorescence, electrochemical, and nanopore sensors. These methods made great progress to the former miRNA detection strategies and avoid some of the drawbacks. Zhang et al. proposed a sensitive approach for miR-21 detection by integrating rolling circle amplification (RCA) and trans-cleavage activity of CRISPR-Cas12a.15 Zhao et al. developed a novel DNA nanosensor for controllable miRNA detection and successfully applied it for in situ miRNA imaging.16 However, the efficacy of these techniques, as well as some other new approaches need further improvements for better sensitivity, acceptable costs, and even simple detection components. Herein, there is a high demand to develop a sensitive and easy-to-operate miRNA detection method for better detection performance. In recent years, isothermal signal amplification techniques have attracted abundant attention due to their advantages in amplification efficiency and constant temperature requirements and have been utilized for both in vitro and in situ miRNA detection. Among them, RCA has

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been demonstrated to be able to amplify miRNA in vitro and has also been applied to amplify RNA for in situ imaging or logic gate construction recently. Through the RCA-based amplification, the single-strand nucleic acids (miRNA, short DNA) could be transferred into long ssDNA products. However, RCA could only provide limited amplification, which could not meet the detection demand of miRNA with extremely low concentration.

We propose here a completely novel miRNA detection methods through the integration of both RCA and strand displacement amplification (SDA). In the method, target miRNA could especially recognize and hybridize with the two terminals of dumbbell structure padlock and thus cyclize it with the assistance of T4 DNA ligase. With the cyclized padlock as a template and miRNA as a primer, a wreath structure RCA product is obtained. Afterward, the hybridization section of the stem-loop structure in the wreath structure RCA product could be recognized by Nb.BbvCI enzymes and form a nicking site. Therefore, the RCA products are divided into large amounts of hairpin structure probes that could be recognized by the H probes. After the chain replacement by H probe from the nicking site, the fluorescence moiety and the corresponding quenching moiety differ and thus the fluorescence appears. Eventually, we have demonstrated the method for the low-cost and convenient detection of miRNAs with an fM-level sensitivity and single-base specificity.

## RESULTS AND DISCUSSION

### Principle of the Proposed Garland RCA-Integrated CHA System

The working mechanism of the method for miRNA detection is illustrated in Scheme 1. There are two steps included in the whole sensing system: the garland RCA process and H probe-based CHA. In the garland RCA process (Scheme 1a), target miRNA could recognize the two terminals of the designed dumbbell structure padlock and gradually open the hybridization section of the dumbbell structure padlock. As a result, the dumbbell structure padlock cyclized under the simultaneous assistance of target miRNA and T4 DNA ligase. With the target miRNA as a primer and the cyclized padlock as a template, rolling circle amplification initiated and gradually produce the ssDNA garland products. In detail, the obtained garland RCA products are composed by repeated hairpin structure probes that contain a Nb.BbvCI enzyme recognition site in the stem section. Therefore, when Nb.BbvCI enzymes existed, it could specifically recognize and cut a single chain in the stem section and thus generate a nicking site. In the CHA process (Scheme 1b), we carefully designed a hairpin structure probe (H probe), which could specially hybridize with the nicking site in the stem section and gradually open the Nb.BbvCI enzyme-treated garland RCA products. During the chain replacement process, the fluorescence of fluorescence moiety, which is quenched by the corresponding quenching moiety, recovered. Meanwhile, the target miRNA could subsequently hybridize with RCA products from the other terminal to release the H probe and thus from the CHA process. As the result, the obtained fluorescence intensity shows a positive correlation with the amount of target miRNA in the whole sensing system.

### Investigation of RCA-Integrated CHA for miRNA Detection

It is widely reported that the upexpression of let-7b-5p is closely related to the pathological process of nasopharyngeal carcinoma, and accurate and sensitive detection of let-7b-5p would contribute to the early diagnosis and prognosis of nasopharyngeal carcinoma. With the nasopharyngeal carcinoma-related miRNA (let-7b-5p) as a target, we first investigated whether it could specially recognize the designed padlock and initiate garland RCA, especially in a complicated clinical sample through the PAGE gel electrophoresis. As shown in Figure 1a, the target miRNA is lower than the 25 bp ladder and the dumbbell structure probe is about 50 bp. When the target miRNA mixed with the dumbbell structure probe, a new band appeared at 70 bp, indicating the successful hybridization of them. In addition, the products of miRNA and padlock complex treated with T4 DNA ligase and phi29 enzymes were stocked in the well, suggesting the generation of an extremely long DNA product, which even could not flow down. Furthermore, we optimized four designed padlocks, which were different in the secondary structure and hybridization section with the target miRNA, by characterizing the RCA products with SYBR I. In detail, the synthesized padlock is ligase by the T4 DNA ligase and phi29 enzymes were stocked in the well, indicating the successful hybridization of them. Eventually, the four designed padlocks could all initiate the following RCA process with the target miRNA (100 nM) and padlock 3 provided the most ideal efficiency (Figure 1b).

Therefore, padlock 3 was chosen for the following amplification process. To demonstrate the feasibility of the method for miRNA detection even from clinical samples, we put the gradient amount of synthesized miRNA into human serum (7%) to mimic the clinical detection conditions and detect the obtained fluorescence. As shown in Figure 1c, both miRNA from PBS buffer and serum could provide a comparable detection performance, demonstrating the potential feasibility of the method for clinical application. We then performed a fluorescence assay to investigate the importance of each component in the whole sensing system, such as the Nb.BbvCI enzymes and H probes. From the result in Figure 1d, we observed a significantly enhanced fluorescence intensity compared with the control group, while the obtained fluorescence intensity showed no obvious increments compared with the control group when the Nb.BbvCI enzymes

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**Scheme 1. Working Principle of the Proposed Strategy for Ultrasensitive miRNA Detection**

(a) The first step of the method for target miRNA-initiated garland RCA and Nb.BbvCI enzyme-based nicking site generation. (b) CHA process of the attached signal amplification and signal output.
or the H probes absent, indicating that both of them play a pivotal role in the miRNA detection system.

**Optimization of the Experimental Conditions.** We first investigated the incubation time for the Nb.BbvCI enzyme-based nicking site generation process by monitoring the fluorescence intensity at different times. From the result in Figure 2a, we observed a gradually increased fluorescence intensity before 1 h and the obtained fluorescence showed no significant increments after 1 h, indicating that incubating the Nb.BbvCI enzymes with garland RCA products for about 1 h could provide ideal performance. Afterward, we verified the concentration of Nb.BbvCI enzyme. As shown in Figure 2b, 2U of Nb.BbvCI enzyme induced the most enhanced fluorescence intensity, suggesting that the concentration could provide the best cut efficiency. Therefore, 2U was chosen as the best concentration of Nb.BbvCI for nicking site generation. Eventually, we studied the optimized concentrations of H probe for miRNA detection in the SDA process. From the result, the obtained fluorescence intensity of the system increased with the concentration of the H probe varying from 1 pM to 100 nM and no more fluorescence increments were observed with the concentration above 10 nM (Figure 2c). Therefore, 10 nM was chosen as the optimized concentration of the SDA process.

**Sensitivity and Specificity of the Proposed Method for miRNA Detection.** Under optimized experimental conditions, we then studied the sensitivity of the proposed method by detecting synthesized miRNA with different concentration gradients (10 fM–100 pM). From the result in Figure 3a, the obtained fluorescence intensity at 570 nm gradually increased with the concentration verifying between 10 fM and 100 pM. Furthermore, a fluorescent–concentration curve was obtained by fitting the data with the exponential curves as $Y = 290.9 \times \lg C + 1582$ (Figure 3b, $R^2 = 0.983$, C refers to the concentration of target miRNA). These results indicated the ability to amplify miRNA signal with the
relatively high detection sensitivity of the RCA reaction. Meanwhile, the specificity of the method both in 1 × PBS buffer and in clinical serum samples was assessed by the comparison of the obtained fluorescence intensity for 10 pM of target miRNA detection and 10 pM of three different miRNAs in the nasopharyngeal carcinoma family (miRNA-21, miRNA-155, let-7c, let-7f). Under both solution environments, the fluorescent intensities obtained from the target miRNA group are both significantly higher than those obtained from other miRNA groups (Figure 3c,d). The result proved that the proposed method could identify the target miRNA from other homologous miRNA both in experimental and clinical conditions.

Clinical Application of the Method for Nasopharyngeal Carcinoma Diagnosis. To demonstrate the potential clinical use of the RCA-integrated CHA system for miRNA detection, we then applied our scheme on the serum samples from nasopharyngeal carcinoma patients and healthy volunteers to detect the circulating target miRNA. It has been reported by many research studies that let-7b-5p is upregulated in nasopharyngeal carcinoma patients and thus been regarded as a potential biomarker for nasopharyngeal carcinoma detection. From the result in Figure 4, the obtained concentration of the target miRNA in the nasopharyngeal carcinoma patient group was much higher than that in the healthy volunteers, which is consistent with the former reports.

**CONCLUSIONS**

We have developed an ultrasensitive miRNA detection method via garland RCA-based signal amplification and CH-based signal generation. In the method, the target miRNA could first be amplified into the garland RCA products, which contain a *Nb.BbvCI* enzyme nicking site. With the cleavage of the *Nb.BbvCI* enzyme, a nicking site was generated and thus induce the following CHA process between the H probe, target miRNA, and garland RCA products. Therefore, two signal application processes were included in the whole sensing system and thus ensure a favorable miRNA detection sensitivity both in experimental conditions and clinical applications. From the brief comparison between the proposed method with some former reported methods (Table 1), the highlights of the method could be calculated as: (i) the amplification process can be carried out at a constant temperature and does not require precise temperature control; (ii) avoid the carry-out contamination; and (iii) high sensitivity from the signal amplification process. Lastly, we believe that the proposed method could provide a new method for miRNA detection and greatly contribute to the early diagnosis of nasopharyngeal carcinoma.
**Table 1. Brief Comparison of the Method with Former Reported Mirna Detection Methods**

| Method                        | Signal Amplification | Carry-out Contamination | Sensitivity       | Signal         | Refs   |
|-------------------------------|----------------------|--------------------------|-------------------|----------------|--------|
| **the method**                | RCA + SDA            | no                       | <10 fM            | florescence    |        |
| CRISPR-Cas9 based             | RCA                  | no                       | pM                | colorimetry    | 19     |
| CRISPR-Cas12a based           | RCA + Cas12a         | no                       | >10 fM            | florescence    | 15     |
| Lateral flow strip            | RCA                  | yes                      | pM                | chip           | 20     |

*RCA, rolling circle amplification; SDA, strand displacement amplification.

**EXPERIMENTAL SECTION**

Chemical Regents. The dumbbell probe and the related nucleic acids were purchased and purified from Shanghai Sango Biotechnologies Co., Ltd. (Shanghai, China). The obtained nucleic acids from Shanghai Sango Biotechnologies Co., Ltd. (Shanghai, China) were then rehydrated into a concentration at 10 μM. The experimental enzymes, including Phi29 polymerase, Nb.BbvCl enzymes were purchased from Thermo Fisher Scientific (Waltham, MA), dNTP mix, bovine serum albumin (BSA), and DNA ladder were brought from TaKaRa Biotech Company (Dalian, China). Twelve percent PAGE gel related reagents were all brought from Bio-Rad (Shanghai) Life Science Research and Development Co., Ltd. (Shanghai, China). Clinical samples were from Zhiu Affiliated Hospital of Shaoxing University.

Fluorescence Analysis of RCA and SDA. The RCA reaction was conducted with the following steps: we first mixed 1 μL of the prepared probe and 1 μL of the target miRNA and heated them to 90 °C. After cooling to room temperature, 1 μL of the T4 DNA ligase and 1 μL of the buffer were added into the mixture and incubated for about 1 h at 30 °C. Afterward, we have added 1 μL of phi29 enzyme and 1 μL of the related reagents to the mixture and incubated for another 2 h. After that, 2 μL of Nb.BbvCl enzymes and 4 μL of the corresponding buffer were added to the system. After reacting for 30 min, H probe 5 μL (10 μM) was added to the system and incubated for another 30 min. Then, the reaction product was used for electrophoresis/fluorescence analysis. In the PAGE gel electrophoresis, 10 μL of the obtained RCA product was mixed with 2 μL of the 6× loading buffer and loaded in 12% PAGE gel under 120 V for 90 min. Finally, the image was obtained through the gel imaging system. The fluorescence signal in the reaction system was detected by a fluorescence spectrophotometer from Hitachi F-4700 (Beijing, China).

Sensitivity and Specificity Analysis of the Method. We apply the above research methods to the detection of different concentrations of target miRNA and detect the fluorescence signal. After removing the background fluorescence from the obtained fluorescence signal, we used Graphpad to study the relationship between fluorescence intensity and target miRNA concentration and calculated the correlation coefficient and calibration equation. In addition, we apply this method to the detection of different miRNAs to determine their specificity.

**ASSOCIATED CONTENT**

1. **Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c00154.

Experimental section and details of the sequences (Table S1) (PDF)
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