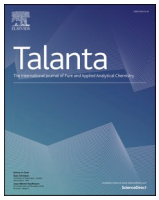
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Proximity hybridization-regulated CRISPR/Cas12a-based dual signal amplification strategy for sensitive detection of circulating tumor DNA

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ABSTRACT

Circulating tumor DNA (ctDNA) is regarded as an ideal candidate biomarker for the non-invasive diagnosis of cancer. However, the lack of convenient and reliable detection methods for ctDNA restricts its clinical application. Herein, we developed a dual signal amplification strategy for sensitive detection of ctDNA based on hybridization chain reaction (HCR) and proximity hybridization-regulated CRISPR/Cas12a. The ctDNA initiates HCR through the continuous hybridization of two hairpin probes (H1 and H2), yielding long nicked double-stranded DNA nanowires composed of numerous split segments, which are successively connected to activate the *trans*-cleavage activity of CRISPR/Cas12a. In this case, the doubly labeled single-stranded DNA reporter can be cleaved to produce a strong fluorescent signal. Owing to the dual amplification of HCR and CRISPR/Cas12a, this strategy exhibits high sensitivity toward ctDNA with a low detection limit of 5.43 fM. Moreover, the proposed method was successfully applied for ctDNA detection in serum samples with satisfactory results, which has great potential in the clinical diagnosis of cancer.

1. Introduction

Circulating tumor DNA (ctDNA), a small DNA fragment in peripheral blood, is released from apoptotic and necrotic tumor cells [1,2]. It was found that the level of ctDNA in the blood is highly correlated with tumor burden in cancer patients, thus making it a promising biomarker for monitoring tumor progression and evaluating therapeutic effects [3,4]. Compared with tissue biopsies, ctDNA-based liquid biopsy is a more promising tool for cancer diagnosis due to its superior advantages of high sensitivity, accuracy, and minor invasion [5]. The traditional techniques for ctDNA detection are mainly classified into two categories: one is based on polymerase chain reaction (PCR) [6], the other is based on DNA sequencing [7]. However, these methods suffer from several limitations, such as time-consuming procedures, high cost and the requirement of sophisticated instruments, which restricts their clinical applications [8]. Therefore, it is essential to develop a simple and efficient method for sensitive detection of ctDNA.

Recently, various isothermal nucleic acid amplification strategies have been developed for ctDNA detection, such as rolling circle amplification (RCA) [9], strand displacement amplification (SDA) [10], hybridization chain reaction (HCR) [11], and catalytic hairpin assembly (CHA) [12]. Among them, HCR is an enzyme-free and efficient isothermal amplification technique, which involves the cross-opening of two hairpin probes into long nicked double-stranded DNA (dsDNA) copolymers [13]. Due to its easy operation, low cost and high amplification efficiency, HCR has been widely applied in the detection of various targets, including nucleic acids, proteins, small molecules and even cells [14–17]. For example, Zou et al. developed a label-free light-up fluorescent sensing strategy based on HCR amplification and DNA triplex assembly for the detection of avian influenza A (H7N9) virus DNA and thrombin [18]. Luo et al. designed a ratiometric fluorescent DNA nanostructure for mitochondrial adenosine triphosphate imaging in living cells based on HCR [19]. Nevertheless, these HCR-based fluorescent methods suffer from high background signals due to the use of DNA hairpins labeled with fluorophore or DNA-intercalating dyes as indicators. To circumvent this problem, HCR has been further developed by combining it with other signal amplification strategies.

Recently, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas12a system has been widely utilized as an efficient signal amplification tool to constitute sensing platforms for sensitive detection of both nucleic acid and non-nucleic acid targets [20–24]. The

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CRISPR/Cas12a system exhibits *trans*-cleavage activity against non-target single-stranded DNA (ssDNA) after being activated by target ssDNA or dsDNA [25,26]. To achieve greater sensitivity, several CRISPR/Cas12a-based detection platforms have been developed by coupling with HCR amplification [27–30]. For example, Xing et al. developed an ultrasensitive HCR-amplified CRISPR/Cas12a aptasensor for the detection of tumor-derived extracellular vesicle proteins [31]. However, in these methods, the protospacer adjacent motif (PAM) sequence is introduced into the HCR products to activate the Cas12a-crRNA complex, which increases the difficulty of designing two hairpin probes for HCR. To address this issue, we herein develop a dual signal amplification strategy for sensitive detection of ctDNA by using the proximity sequence of HCR products as the DNA activator for CRISPR/Cas12a without PAM sequence. In this study, the target-triggered HCR converts each ctDNA into polymeric dsDNA nanowires composed of numerous split segments, which are successively connected to activate the Cas12a-crRNA complex. As a result, the doubly labeled ssDNA fluorescence reporter (ssDNA-FQ) is cleaved by the activated CRISPR/Cas12a system, leading to a significant increase in fluorescence signal. The proposed dual signal amplification strategy exhibited high sensitivity and selectivity for the detection of ctDNA, which has great potential for the early diagnosis and treatment of cancer.

2. Experimental section

2.1. HCR-CRISPR/Cas12a detection

The hairpin probes (H1 and H2) were separately heated to 95 °C for 5 min and then cooled to room temperature for 1 h before use. Subsequently, 10 μL of different concentrations of ctDNA, 20 μL of 100 nM H1, and 20 μL of 100 nM H2 were mixed in reaction buffer (20 mM HEPES, 200 mM NaCl and 20 mM $MgCl_2$, pH 7.4), and incubated at room temperature for 1 h to conduct HCR. Meanwhile, 10 μL of 100 nM LbCas12a and 10 μL of 100 nM crRNA were mixed 1 × Reaction Buffer and incubated at 37 °C for 30 min to form Cas12a-crRNA complex. Subsequently, the preassembled Cas12a-crRNA duplex and 10 μL of 600 nM ssDNA-FQ were added to the HCR product with a final volume of 200 μL and incubated at 25 °C for 30 min to trigger the *trans*-cleavage of CRISPR/Cas12a. Finally, the resulting solution was subjected to fluorescence measurements.

2.2. Gel electrophoresis analysis

15% native polyacrylamide gel electrophoresis (PAGE) was used to verify the formation of HCR products in the proposed strategy. Gel electrophoresis was carried out in 0.5 × TBE buffer at a constant voltage of 120 V for 100 min. After that, the gel was stained with 4S Red Plus Nucleic Acid Stain for 15 min, and finally scanned with the gel imaging system.

2.3. Detection of ctDNA in human serum

Human serum from healthy adults was diluted 200-fold with HEPES buffer (20 mM, pH 7.4), and the diluted serum samples were then spiked with different concentrations of ctDNA. The spiked samples were detected under conditions identical to those described above.

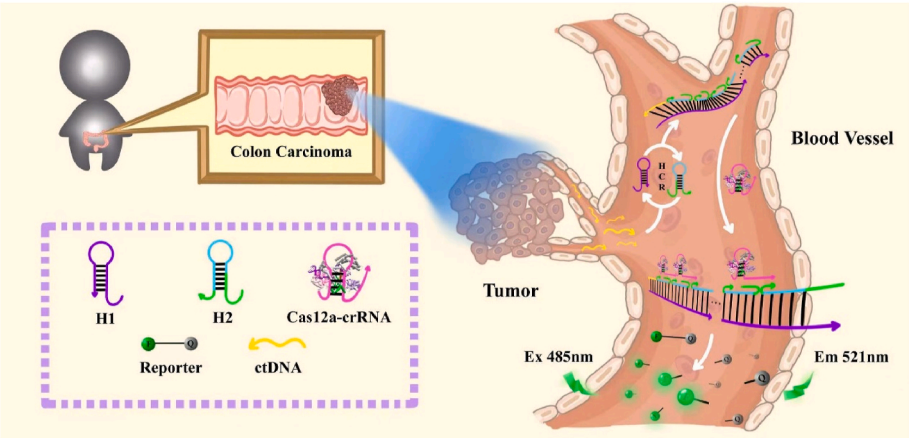
3. Results and discussion

3.1. Principle of the HCR-CRISPR/Cas12a strategy

The principle of CRISPR/Cas12a-based dual signal amplification strategy for ctDNA detection is illustrated in Scheme 1. The sensing system is composed of two parts: the target ctDNA-triggered HCR of H1 and H2, the proximity hybridization-regulated activation of CRISPR/Cas12a. H1 was designed to have one sticky end complementary to the target ctDNA. H2 was designed to have two domains at its 3′ and 5’ ends, respectively. The presence of target ctDNA can trigger the HCR between H1 and H2, which brings the domains of two different adjacent H2 into close proximity, yielding long nicked dsDNA nanowires composed of numerous split segments. The adjacent segments can be specifically recognized by Cas12a-crRNA complex, which activates the *trans*-cleavage activity of CRISPR/Cas12a to split the ssDNA reporter, leading to a significant increase in fluorescence signal. Therefore, ctDNA can be quantitatively detected by measuring the fluorescence intensity of the reporter.

3.2. Feasibility of the proposed strategy

The feasibility of the HCR-CRISPR/Cas12a strategy for ctDNA detection was verified by fluorescence experiments under different conditions. As shown in Fig. 1, a weak fluorescent signal was observed when the ssDNA reporter was mixed with Cas12a-crRNA (curve a). The introduction of H1 did not generate enhanced fluorescence (curve b), suggesting that H1 was unable to activate the *trans*-cleavage activity of CRISPR/Cas12a. A slightly increased fluorescence signal was observed upon the addition of H2 (curve c), possibly due to the low cleavage activity of CRISPR/Cas12a activated by the free domains in H2. The additions of H1 and H2 had no pronounced effect on the fluorescence signal (curve d), indicating that there was no amplification reaction between H1 and H2 in the absence of the initiator. However, with the addition of target ctDNA, a strong fluorescence signal was obtained (curve e). These results demonstrated that the target ctDNA can



Scheme 1. Schematic illustration of CRISPR/Cas12a-based dual signal amplification strategy for ctDNA detection.

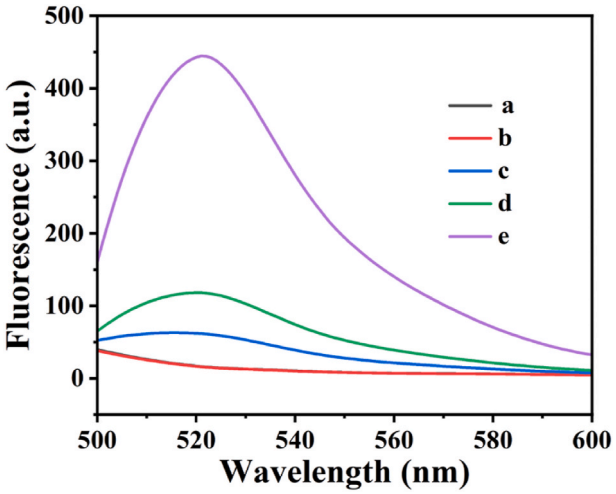


Fig. 1. Fluorescence spectra of the sensing system under different conditions: (a) ssDNA-FQ + Cas12a + crRNA, (b) a + H1, (c) a + H2, (d) a + H1 + H2, (e) d + ctDNA. Concentrations of ssDNA-FQ, Cas12a, crRNA, H1, H2 and ctDNA were 30 nM, 5 nM, 5 nM, 10 nM, 10 nM and 5 nM, respectively.

effectively trigger the hybridization of H1 and H2 to form nicked dsDNA nanowires composed of adjacent segments complementary to crRNA, which then activate the *trans*-cleavage activity of CRISPR/Cas12a.

Furthermore, PAGE analysis was performed to validate whether target ctDNA can effectively initiate the HCR process. As shown in Fig. 2, a new bright band with slower migration appeared when the target ctDNA mixed with H1 (lane 4), which was in agreement with our design. In addition, the bands corresponding to H1 and H2 remained intact in the absence of target ctDNA (lane 5), indicating that the mixture of H1 and H2 did not cause HCR process. In contrast, the bands of H1 and H2 attenuated and even disappeared upon addition of target ctDNA to the mixture of H1 and H2. Meanwhile, multiple characteristic ladder-like bands with higher molecular weight appeared for the prolonged DNA products (lanes 6–8), confirming that the target ctDNA can efficiently trigger the HCR between H1 and H2. The above results proved that the CRISPR/Cas12a-based dual signal amplification strategy for ctDNA detection is feasible.

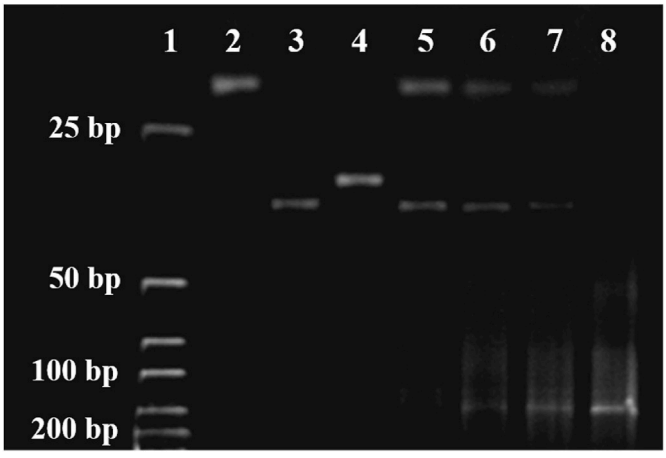


Fig. 2. PAGE analysis of the HCR products. Lane 1: DNA marker; Lane 2: 1 μM H1; Lane 3: 1 μM H2; Lane 4: 1 μM H1 + 1 μM ctDNA; Lane 5: 1 μM H1 + 1 μM H2; Lane 6: 1 μM H1 + 1 μM H2 + 0.1 μM ctDNA; Lane 7: 1 μM H1 + 1 μM H2 + 0.2 μM ctDNA; Lane 8: 1 μM H1 + 1 μM H2 + 0.5 μM ctDNA.

3.3. Optimization of experimental conditions

To achieve the best analytical performance of the HCR-CRISPR/Cas12a strategy for ctDNA detection, some experimental conditions including the lengths of two domains in H2, the concentration of ssDNA-FQ, HCR time, CRISPR/Cas12a cleavage reaction temperature and time were optimized. The background signal depended mainly on the lengths of the two H2 domains, with their total length fixed at 20 nt. As shown in Fig. S1, the sensing system exhibited the optimal background signal when the length of 5′-domain in H2 did not exceed 14 nt. Thus, 14 nt for 5′-domain and 6 nt for 3′-domain were chosen for preparation of H2. Then, the effect of the ssDNA-FQ concentration on the sensing performance was investigated. Fig. S2A showed that the value of F/F_0 increased obviously with the increase of ssDNA-FQ concentration from 10 to 30 nM, followed by a decrease beyond 30 nM (F_0 and F is the fluorescence intensity of the sensing system without and with target ctDNA, respectively). Therefore, the optimal concentration of ssDNA-FQ was 30 nM. In addition, the effect of HCR time on the sensing performance was explored. It could be observed that the value of F/F_0 increased significantly with an increase in reaction time from 0.1 to 1 h but decreased after 1 h (Fig. S2B). Hence, 1 h was selected as the optimal reaction time of HCR. The CRISPR/Cas12a reaction temperature and time are important parameters for efficient cleavage of the ssDNA-FQ by activated Cas12a. As depicted in Figs. S2C and S2D, the maximum value of F/F_0 was obtained when the reaction temperature was 25 °C and the time was 30 min. So, 25 °C and 30 min were chosen as the optimal reaction temperature and time for CRISPR/Cas12a.

3.4. Analytical performance for ctDNA detection

Under the optimal conditions, the analytical performance of the proposed strategy was investigated by detecting different concentrations of target ctDNA. As presented in Fig. 3A, the fluorescence signal gradually increased along with the increase of ctDNA concentration from 0 to 8 nM. Fig. 3B and C shows the relationship between the fluorescence intensity at 521 nm and the logarithm of ctDNA concentration, which exhibited two good linear correlations in the range of 0.05–1000 pM and 1000–6000 pM, respectively. The linear regression equations are $F = 163.9 + 25.32\lg C$ ($R^2 = 0.9970$) and $F = -674.7 + 305.1\lg C$ ($R^2 = 0.9968$), where F and C represent the fluorescence intensity at 521 nm and the ctDNA concentration (pM), respectively. The limit of detection (LOD) was calculated to be 5.43 fM with a signal-to-noise ratio of 3, which is comparable to or more sensitive than those of previous assays for ctDNA detection (Table S2). Herein, the high sensitivity of the proposed strategy is due to the dual signal amplification of HCR and CRISPR/Cas12a.

Furthermore, to evaluate the specificity of the proposed strategy, single-base (M1), two-base (M2), three-base (M3) mismatched DNA, and non-complementary (NC) DNA were detected at a concentration of 5 nM. As illustrated in Fig. 4, compared with target ctDNA, the fluorescence signal dramatically decreased in M1 and M2 mismatched DNA. In addition, the fluorescence signal was very weak in M3 and NC, which was comparable to as that of the blank. These results indicated that the HCR-CRISPR/Cas12a strategy exhibited high specificity in discriminating target ctDNA from other DNA sequences, which might be ascribed to the strict principle of complementary base pairing and the proximity hybridization-regulated activation of CRISPR/Cas12a.

3.5. Serum samples analysis

To further validate the practical applicability of the proposed strategy, recovery experiments were performed by spiking four different concentrations of ctDNA (0.5, 50, 500 and 5000 pM) into 200-fold diluted human serum. The analytical results are shown in Table 1, the recoveries of the spiked samples were in the range from 94.3 to 105.5% and the relative standard deviations (RSDs) were between 4.65% and

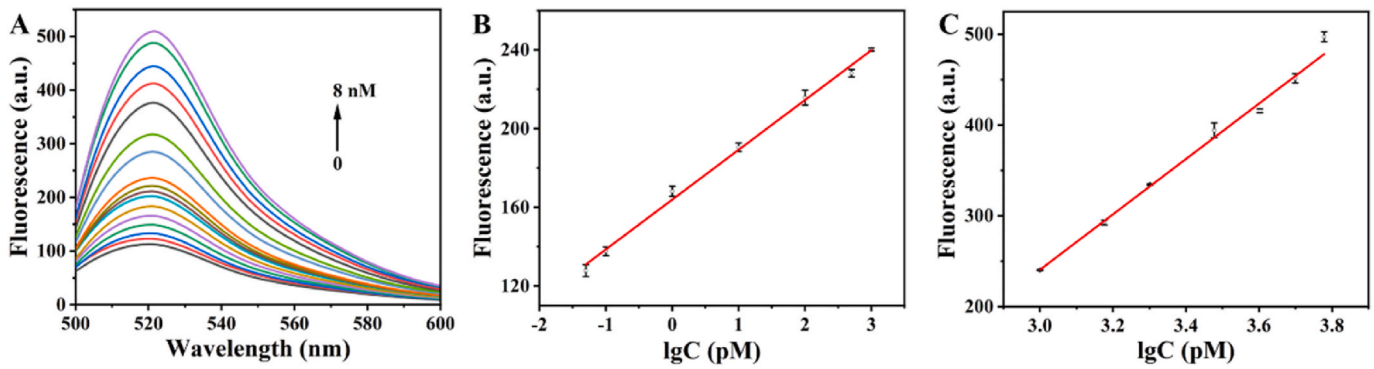


Fig. 3. (A) Fluorescence spectra of the sensing system with different concentrations of target ctDNA (0, 0.05, 0.1, 0.5, 1, 10, 50, 100, 500, 1000, 1500, 2000, 3000, 4000, 5000, 6000, 8000 pM). (B) and (C) Linear relationships between fluorescence intensity and the logarithm of ctDNA concentration. Error bars indicated the standard deviations of three measurements.

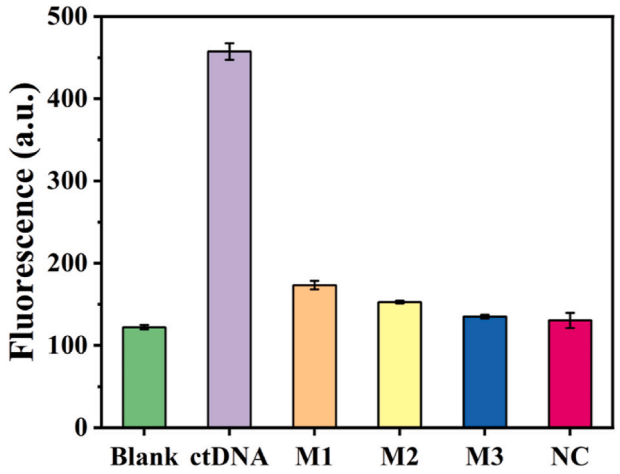


Fig. 4. Specificity of the proposed strategy against blank, target ctDNA, single-base (M1), two-base (M2), three-base (M3) mismatched DNA, and non-complementary (NC) DNA. The concentrations of all the DNA sequences were 5 nM. Error bars were estimated from three replicate measurements.

Table 1
Detection of target ctDNA in human serum samples[a] (n = 3).

Sample	Added	Found	Recovery (%)	RSD (%)
1	0.50 pM	0.52 pM	104.5	5.95
2	50.0 pM	48.7 pM	97.4	4.65
3	0.50 nM	0.53 nM	105.5	4.73
4	5.00 nM	4.71 nM	94.3	5.81

[a] The serum samples were diluted 200-fold.

5.95%. These results demonstrated that the proposed method can be applied for the analysis of target ctDNA in human serum samples.

4. Conclusion

In summary, we have developed a sensitive fluorescent sensing platform for ctDNA detection based on a dual signal amplification strategy of HCR and CRISPR/Cas12a. The HCR process can be triggered by target ctDNA, yielding long nicked dsDNA nanowires composed of numerous adjacent segments, which hybridizes with crRNA to activate the cleavage of ssDNA-FQ by Cas12a, producing a significant fluorescence signal. The proposed strategy exhibits several advantages as follows: (1) comparing with reported CRISPR-based methods, the sensitivity of this method is satisfactory due to the dual signal amplification; (2) our method has high specificity among different DNA sequences, indicating its potential applications in liquid biopsy of cancer; (3) the universality of the proposed approach is likely to be good, allowing it to detect different target DNAs without changing the spacer sequence of crRNA. However, we note that the sensing system can only be used for the detection of nucleic acids, which might be a limitation. We will devote ongoing efforts to extend the application for the detection of non-nucleic acid targets by means of functional DNA molecules such as aptamers and DNAzymes. In addition, this work will likely open the door to joint nucleic acid amplification techniques and CRISPR/Cas12a by proximity hybridization.

Credit author statement

Mengyan Li: Methodology, Investigation, Formal analysis, Visualization. **Nian Luo**: Validation, Investigation. **Xiaofei Liao**: Investigation, Data curation. **Li Zou**: Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2023.124395.

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