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Ultrasensitive biosensing of low abundance BRAF V600E mutation in real samples by coupling dual padlock-gap-ligase chain reaction with hyperbranched rolling circle amplification

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ABSTRACT

BRAF V600E mutation is an important drive gene mutation and biomarker for tumor diagnosis, monitoring and target treatment. In order to meet the needs of identifying low abundance BRAF V600E mutation from real clinical specimens, a real time fluorescent biosensing strategy was developed by perfectly integrating dual padlock-gap-ligase chain reaction (DP-gLCR) with hyperbranched rolling circle amplification (HRCA). A pair of padlock probes was designed to induce DP-gLCR by using single-base gap coupling with matched deoxyribonucleotides substrates, which significantly increased the specificity of mutation discrimination. A large amount of circularized padlock probes were produced and triggered subsequent HRCA, generating numerous dendritic double-stranded DNA (dsDNA) products for highly sensitive real time fluorescent biosensing. The designed biosensing strategy could detect as low as 200 zM mutation target and distinguish as low as 0.01% genomic DNA with B-type Raf kinase (BRAF) V600E mutation in 40 ng wild-type genomic DNA, which was equal to almost one copy of mutant genomic DNA. Moreover, the proposed method was successfully applied in the detection of BRAF V600E mutation from real clinical samples, proving great potential for ultrasensitive biosensing of low abundance somatic mutation for early cancer diagnosis and treatment.

1. Introduction

B-type Raf kinase (BRAF) gene V600E mutation, a single base transition from thymidine-to-adenine resulting in a valine to glutamic acid substitution at amino acid 600, leads constitutive activation of the BRAF kinase for tumorigenesis and is one of the most common pathogenic mutations in human cancers, especially in primary cutaneous melanoma and papillary thyroid carcinoma (PTC) [1,2]. Somatic BRAF V600E mutation has been proved to be a highly specific molecular biomarker for tumor diagnosis [3], a prediction of aggressive clinicopathological characteristics [4], a poor prognostic factor for clinical recurrence [5] and a therapeutic target of Vemurafenib [1,6]. Accordingly, it is increasingly important to detect BRAF V600E mutation in clinical samples for accurate diagnosis and personalized therapy of cancers. However, due to tumor heterogeneity and sampling variability, BRAF V600E mutation is likely to present at ultra-low levels in some clinical samples, such as fine needle aspiration biopsy (FNAB) samples of thyroid and circulating free DNA.

At present, Sanger sequencing is the golden standard for mutation detection while lack at sensitivity [7–9]. Amplification refractory mutation system (ARMS) polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) are extensively used to distinguish the single nucleotide polymorphism and gene mutation in clinical and research laboratories. But PCR and LAMP can not detect the gene mutation lower than the mutation ratio of 1% or 50 copies mutant genomic DNA [7–10], so they can not meet the demands for detecting low abundance somatic mutation in specific clinical samples. Next-generation sequencing [11] and digital PCR [12,13] have improved their sensitivity to satisfy the needs for lower abundance mutation detection. But the need of stringent laboratory conditions, professional staff and expensive cost make them hard for popularized clinical application. Thus, it is still urgent to develop a low-cost, simple, and

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ultrasensitive methodology for the detection of BRAF V600E mutation with ultra-low level.

In recent years, ligase chain reaction (LCR) has been applied to identify gene mutation due to its highly specific discrimination of single-base mismatch [14–18]. And the modified method, gap-LCR [19], added an extension reaction to minimize target independent ligation process by designing a gap with one or a few bases between the ligation probes. Gap-LCR has shown superior specificity than PCR and general LCR owing to its ligase and polymerase-based dual discrimination of mutation. Nevertheless, the conventional methods for the detection of LCR or gap-LCR products, such as electrophoresis and immumoassay-liked method are of low sensitivity [14,19–21]. Many works have explored new biosensing strategies to improve the sensitivity of LCR-based methods. These strategies mainly employed exonucleases to purify the ligation products [22–24] and/or used various micro/nano particles to indicate the ligation products [25–29]. The above mentioned biosensing strategies demonstrated to be sensitive and specific enough to detect down to femto-molarity of mutation DNA molecules. However, they mostly used synthetic oligonucleotides as targets. The capability of these methods to detect real clinical sample is not proved, which may contain ultra-low levels mutation copies within high abundance normal genomic DNA.

Attempting to meet the requirements of identifying low abundance BRAF V600E mutation from real clinical specimens, a highly sensitive and specific real-time fluorescent biosensing strategy is developed by perfectly integrating dual padlock-gap-LCR (DP-gLCR) with hyperbranched rolling circle amplification (HRCA). Compared with the above mentioned LCR-based biosensing strategy and previous method using single padlock LCR (SP-LCR) coupling RCA strategy [30], our strategy significantly improve the biosensing performance with three innovations. (i) Dual padlock probes was designed to ensure more uniform melting temperature (Tm) of ligation probes hybridized with templates and reduce the nonspecific ligation. (ii) Our DP-gLCR system further increased the specificity by using single-base gap coupling with matched deoxyribonucleotides substrates. (iii) Subsequent HRCA can produce more plentiful of double stranded amplification products than linear RCA for SYBR Green-based real-time fluorescent biosensing. Accordingly, the proposed strategy can accurately detect as low as 200 zM mutation DNA targets and 0.01% of mutant genomic DNA. Finally, the developed assay was successfully applied to analyze BRAF V600E mutation in real clinical samples, indicating a pragmatic platform toward highly sensitive and specific detection of low abundance BRAF V600E mutation from real clinical specimens, and a potential tool for improving accurate molecular diagnosis and personalized therapy of cancers.

2. Experimental section

2.1. Materials and reagents

All oligonucleotides were synthesized with solid phase phosphoramidite chemistry and purified high performance liquid chromatography (HPLC) by Sangon Biotechnology Co. Ltd. (Shanghai, China). The sequences were illustrated in Table S1, and their relevant characterization and purification information were shown in Figure S1–S4. Taq DNA polymerase, taq DNA ligase, Bacillus stearothermophilus (Bst) DNA polymerase and their buffer were obtained from New England Biolabs (Ipswich,MA, U.S.A.). Deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP) solution and deoxyribonucleoside triphosphate (dNTP) mixture were purchased from BBI Life Scicences Corporation (Toronto, Canada). Proteinase K was obtained from Solarbio Life Sciences (Beijing, China). SYBR Green I was purchased from Generay Biotech Co. Ltd. (Shanghai, China).

2.2. Sample preparation

The human colon cancer cell line HT29 with BRAF V600E mutation [31] was cultured in dulbecco's modified eagle medium (DMEM) containing high glucose and 10% fetal bovine serum with 5% CO_2 and 37 °C. Genomic DNA of cell line and human white blood cells were extracted with BloodGen Mini Kit (CW Biotechnology Co., Ltd., Jiangsu, China) according to the manufacturer's instruction. The tissue and FNAB samples of PTC were collected from The First Affiliated Hospital of Chongqing Medical University. And the genomic DNA was extracted by AmoyDx@FFPE DNA Kit (Amoy Diagnostics Co., Ltd., Xiamen, China) according to the manufacturer's instruction. Their DNA concentrations were quantified according to the absorption at 260 nm with a NanoDrop One Microvolume UV–vis Spectrophotometer (Thermo Fisher Scientific, Co., Ltd., Shanghai, China).

2.3. DP-gLCR

The DP-gLCR reaction was performed in 40 μL of mixture solution containing 20 mM Tris-HCl, 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM ammonium sulfate, 1 mM nicotinamide adenine dinucleotide (NAD), 10 mM dithiothreitol (DTT), 0.1% Triton X-100, 100 u M dGTP, 100 uM dCTP, 1 μM padlock probe 1, 1 μM padlock probe 2, 0.2 U taq DNA polymerase, 10 U taq DNA ligase and a certain amount of target DNA. Firstly the reaction mixture was heated for denaturation at 95 °C for 3 min, then the gap-LCR reaction was carried out with thermal cycle I (15cycles at 95 °C for 30 s, 65 °C for 30 s, and 68 °C for 1 min), and thermal cycle II (30 cycles at 95 °C for 30 s, 56 °C for 30 s, and 68 °C for 1 min) on T100™ Thermal cycler (Bio-Rad, U.S.A.). Following DP-gLCR, 2 μL of proteinase K was added to reaction products, and the mixture solution was heated for 10 min at 65 °C. The reaction was ultimately terminated by incubation for 15 min at 95 °C.

2.4. Real time HRCA (RT-HRCA)

8 μL of 2.5 μM RCA primers mixture was added to the DP-gLCR products above, then heated at 95 °C for 5 min and annealed at 50 °C for 2 min to hybridize RCA primers with the circular DNA produced with DP-gLCR. Then 30 μL of the reaction mixture was taken out and mixed with 20 μL of the solution containing 5 μL of 10 × isothermal amplification buffer, 12 U Bst 2.0 DNA polymerase, 5 μL of 1 μM branch primer 1, 2.5 μL of 1 μM branch primer 2, 2.5 μL of 1 μM branch primer 3, 1 μL of 10 mM dNTP and 2.5 μL of 20 × SYBR Green I. Next, the HRCA reaction was performed in ABI 7500 real-time PCR instrument (Applied biosystems, U.S.A) with 90 cycles at 63 °C for 1 min, and the fluorescence signals were collected at the Carboxyfluorescein (FAM) channel at the end of every cycle.

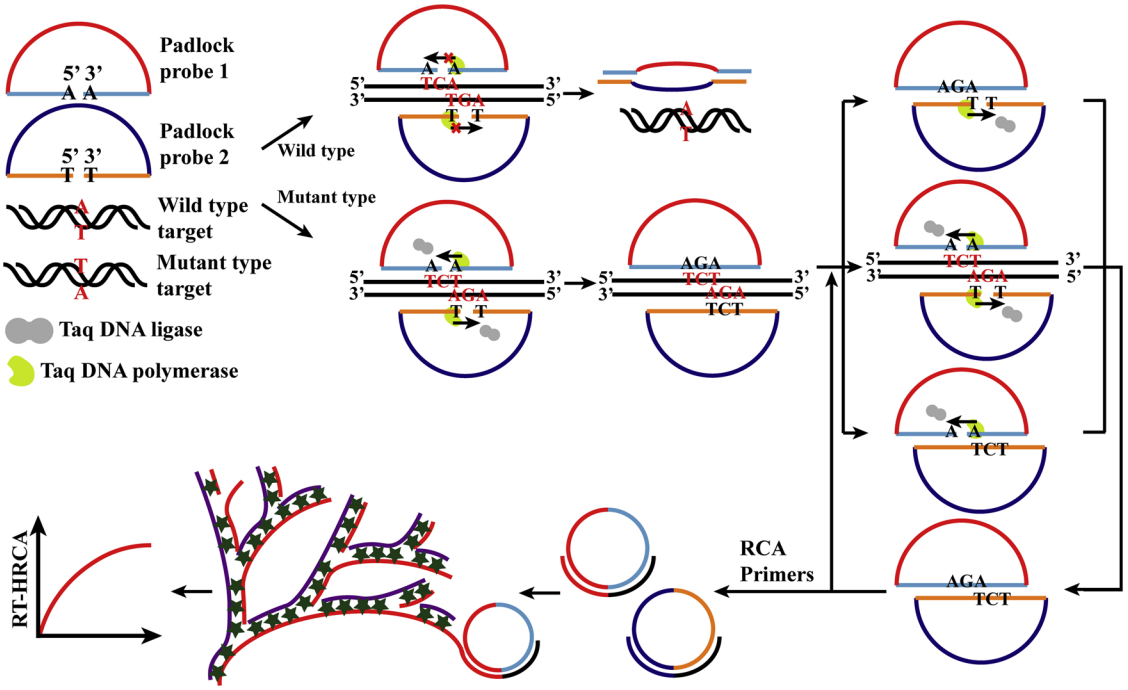
2.5. Agarose gel electrophoresis

The DP-gLCR and HRCA products were analyzed with 5% agarose gel electrophoresis (AGE) in 0.5 × TBE buffer (4.5 mM Tris–HCl, pH 7.9, 4.5 mM boric acid, 0.2 mM ethylene diamine tetraacetic acid) at 110 V for 20 min at room temperature. The results were visualized by a ST4-1100 Imaging System (Daoyi Medical Equipment, Co., Ltd., Chongqing, China).

3. Results and discussion

3.1. Principle of the biosensing strategy

The schematic of the proposed strategy for the detection of BRAF V600E mutation is illustrated in Scheme 1. In our DP-gLCR, a pair of



Scheme 1. Schematic illustration of the real time fluorescent biosensing strategy based on DP-gLCR and HRCA for the detection of BRAF V600E mutation.

padlock probes was hybridized with the template at the region cover the BRAF V600E mutation site. Their 3′ terminus uniquely matched with the mutant adenine at V600E mutation site. After annealing with the mutant templates, the gaps between 5′ and 3′ terminal of padlock probes would be filled by the matched deoxyribonucleotides substrates (dCTP or dGTP) with the catalysis of taq DNA polymerase which owns high fidelity and lacks 5′-3′ exonuclease activity. Then the extended padlocks were ligated to form circularized padlock probes which can act as the circular templates for subsequent LCR cycles. The exponential LCR amplification resulted in a large amount of circularized padlock probes for next HRCA.

For eliminating the influence of heat tolerant DNA polymerase and ligase on next HRCA, proteinase K was then added into DP-gLCR products to inactivate taq DNA polymerase and ligase. Then circularized padlock probes were hybridized with two RCA primers and triggered HRCA with Bst polymerase, dNTP and branch primers to generate numerous HRCA products containing dendritic double-stranded DNA (dsDNA). SYBR Green I can bond the minor grooves of double helix structure of dsDNA with high affinity and produce significant fluorescence enhancement with maximal excitation wavelength at 497 nm and maximal emission wavelength at 520 nm (Figure S5) [32]. So, all the real time fluorescent curves of HRCA were obtained by monitoring SYBR Green I fluorescent signal with FAM-channel of real-time PCR instrument at 470 nm excitation wavelength and 520 nm emission wavelength. Thus, the BRAF V600E mutation from genomic DNA was discriminated with high specificity and sensitivity depending on the real time fluorescent signals.

3.2. Feasibility of the DP-gLCR and HRCA

5% agarose gel electrophoresis was carried out to prove the validity of the DP-gLCR and subsequent HRCA. As shown in Fig. 1A, in presence of mutation targets DNA, DP-gLCR products exhibited a distinct band with slower migration than that of linear padlock probes (lane 1), indicating the ligation of padlock probes to produce abundant circularized padlock probes. After subsequent HRCA, RCA products present a remarkably bright band stagnating in the sample slot (lane 4), suggesting circularized padlock probes could further trigger efficient HRCA reaction to produce a mass of dendritic RCA products with large molecule weight. The fluorescent signals of HRCA for mutation target significantly increased with the increasing of HRCA reaction time (Fig. 1B curve a), indicating that the HRCA products can be real time detected with the fluorescent signal of SYBR Green I. Meantime, there is not any nonspecific ligation product and HRCA product found in the reactions with the wild targets and blank controls (Fig. 1A lanes 2, 3, 5 and 6, and Fig. 1B curve b). These results indicated that only in the presence of mutation target, the DP-gLCR would be carried out, generated the circular ligation products and triggered subsequent HRCA to form a number of dendritic dsDNA products for real time fluorescent detection, demonstrating the feasibility of the designed strategy for BRAF mutation assay.

Furthermore, as control experiments, single padlock-LCR (SP-LCR) and linear RCA (LRCA) were implemented. As shown in Fig. 1B, the system of SP-LCR coupling with HRCA showed lower fluorescent signals for mutation target than DP-gLCR and obvious nonspecific amplification signals for wild target (curves c and d). This control experiment proved the excellent specificity of our proposed DP-gLCR compared with SP-LCR, due to its more uniform Tm for target hybridization and its ligase and polymerase-based dual discrimination of one base mutation. On the other hand, according to the agarose gel electrophoresis analysis, the quantity of reaction products showed little difference between HRCA and LRCA which only applied single RCA primer to trigger RCA without branch primers (Fig. 1C). However, the real time fluorescent signals of HRCA were obviously higher than that of LRCA, owing to more dendritic dsDNA products were obtained with HRCA (Fig. 1D). These results further demonstrated the advantage of our designed strategy for mutation discrimination based on DP-gLCR and HRCA.

3.3. Optimization of the DP-gLCR strategy

To obtain optimized DP-gLCR with high specificity and efficiency, different DP-gLCR strategies with different base numbers in the gap and different deoxyribonucleotides substrates were explored. As shown in Fig. 2A, many consecutive bands with lower mobility, indicating aberrant nonspecific amplification products, can be seen when we employed dNTP mixture as substrates (lanes 1–6). It demonstrated that

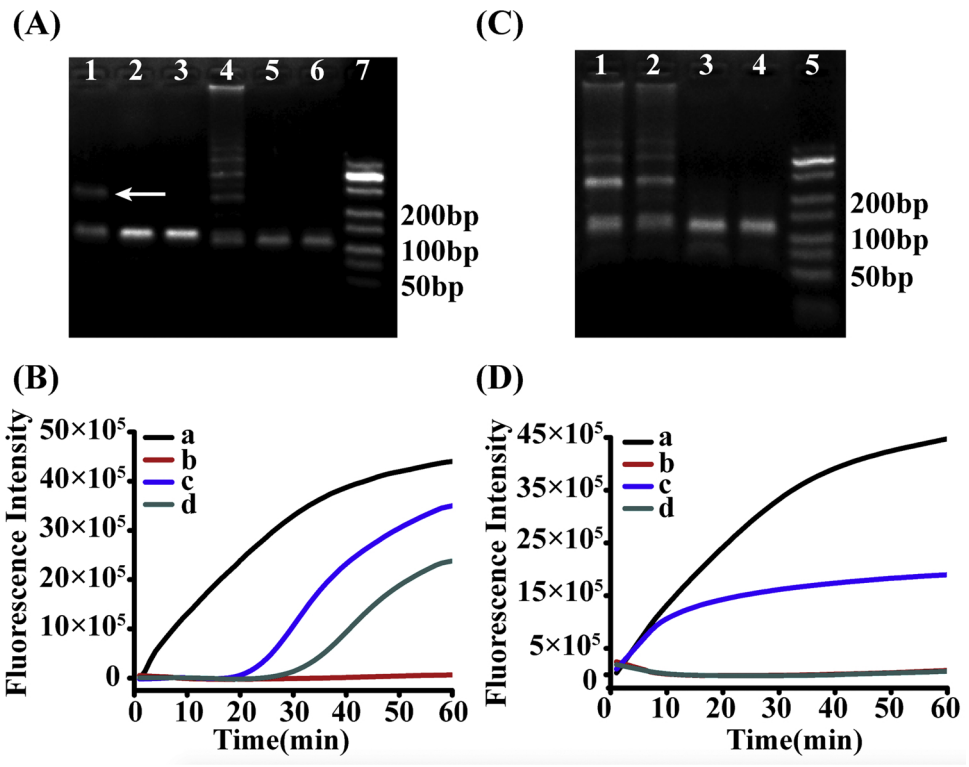


Fig. 1. (A) Agarose gel electrophoresis of the products of DP-gLCR (Lanes 1–3) and subsequent HRCA (Lanes 4–6) for the mutant targets (Lanes 1 and 4), wild targets (Lanes 2 and 5) and blank controls (Lanes 3 and 6), respectively. Lane 7 is DNA marker. (B) Real time fluorescent curves of DP-gLCR + HRCA (a, b) and SP-LCR + HRCA (c, d) for the mutant targets (a, c), wild targets (b, d), respectively. (C) Agarose gel electrophoresis of DP-gLCR + HRCA (Lanes 1 and 3) and DP-gLCR + LRCA (Lanes 2 and 4) products for the mutation targets (Lanes 1 and 2) and wild targets (Lanes 2 and 4), respectively. Lane 5 is DNA marker. (D) Real time fluorescent curves of DP-gLCR + HRCA (a, b) and DP-gLCR + LRCA (c, d) for the mutant targets (a, c), wild targets (b, d), respectively.

nonspecific hybridization and polymerization reactions were subsistent with both two-base gap and one-base gap, if using dNTP substrates. When we adopted one-base gap and matched deoxyribonucleotides (dGTP and dCTP) as substrates, the nonspecific amplification products disappeared and the DP-gLCR kept greatly specific (Fig. 2A lanes 7–9), owing to specific polymerization with corresponding deoxyribonucleotides. Real time fluorescent curves also proved that only DP-gLCR based on one-base gap and two deoxyribonucleotides can specifically produce circularized padlock probes to induce HRCA successfully (Fig. 2B).

On the other hand, the annealing temperature was also important for the performance of DP-gLCR. Two thermal cycles was designed in our DP-gLCR by using 65 °C and 56 °C as the annealing temperatures for thermal cycle I and II, respectively. The thermal cycle I was designed to hybridize the padlock probes with target gene and circularize them. So its annealing temperature is the same as the Tm of the target gene and two padlock probes (65 °C) for keeping their specific hybridization and ligation. Afterwards, the thermal cycle II is to ligate padlock probes by using circularized padlock probes as templates. To obtain high amplification efficiency, the annealing temperature is 5 °C lower than the Tm of them (61 °C). As shown in Fig. S6, our designed DP-gLCR with two thermal cycles proved much higher amplification efficiency than those controls with only single thermal cycle, regardless of 65 °C or 56 °C.

3.4. Performance analysis of the biosensing strategy

Fig. 3A shows the real time fluorescent curves of synthetic mutation target DNA with different concentrations in the range from 200 zM to 2 pM. The fluorescence signals increased over time due to continuously producing dendritic dsDNA products of HRCA. Moreover, the velocity of the fluorescence increase proportionally increased upon the concentration of mutant target DNA. As low as 200 zM mutant DNA can be measured with a distinct increase of fluorescence after 50 min (Fig. 3A curve h), compared with the curve of 2 pM wild-type DNA (Fig. 3A curve i). To quantify the mutant target, we inspired by the quantitative method of RT-PCR to defined signal threshold as the tenfold standard deviation of average signal intensity in 3–15 min (threshold = 10 × $SD_{min\ 3–15}$), and Rt as the time when the fluorescence signal raises above the threshold. As shown in Fig. 3B, a good linear relationship between R_t and concentration of mutation targets in widely dynamic range from 200 zM to 200 fM was exposed with the limit of detection (LOD) of 200 zM. In other words, the proposed method was able to detect about 5 copies of mutation target DNA in the 40 μL of reaction system. The ultrahigh sensitivity was attributed to the cascade

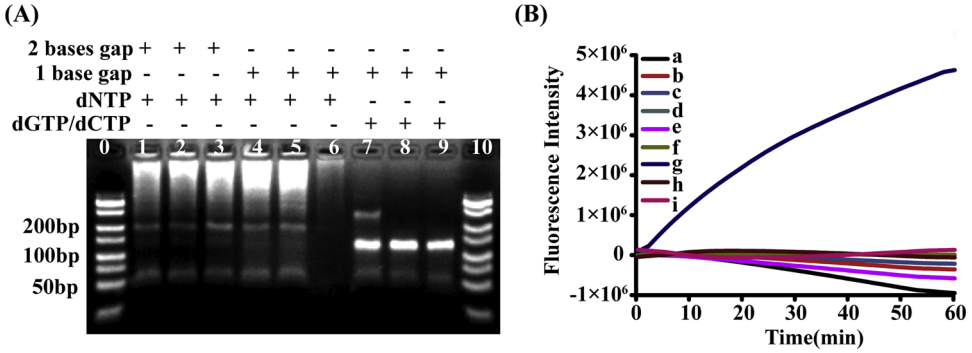


Fig. 2. (A) Agarose gel electrophoresis of different DP-gLCR strategies with different base numbers in the gap and different deoxyribonucleotides substrates for the mutant targets (Lanes 1, 4 and 7), wild-type targets (Lanes 2, 5 and 8) and blank (Lanes 3, 6 and 9). (B) Real time fluorescent curves of HRCA coupled with different DP-gLCR strategies. The curves from a to i are corresponding to the lanes from 1 to 9, respectively.

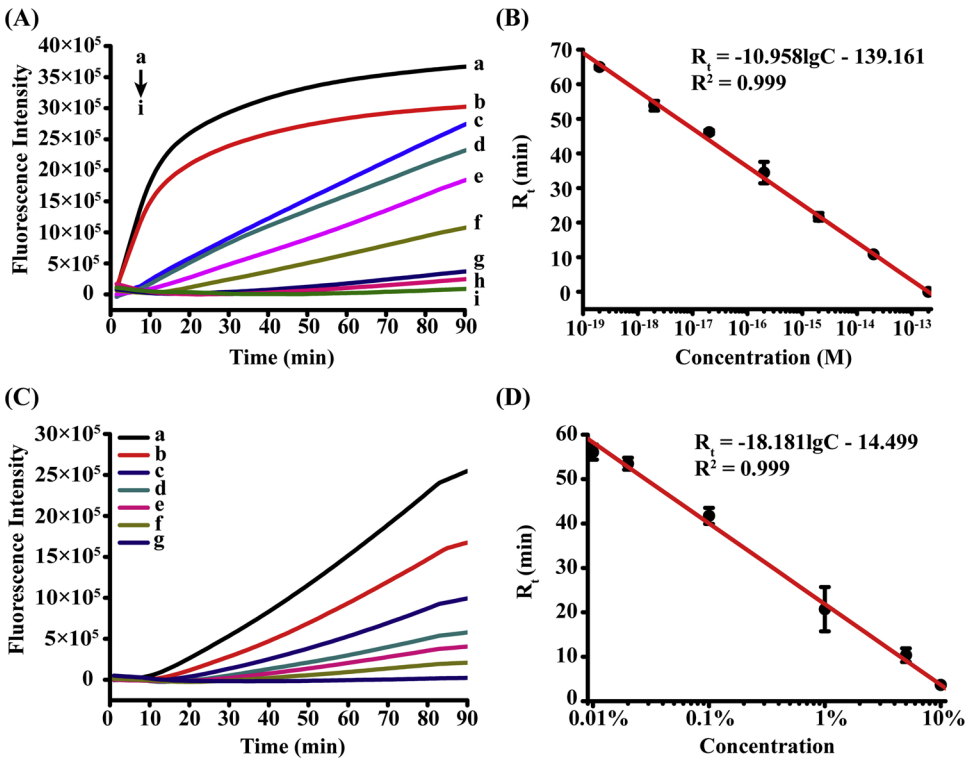


Fig. 3. (A) the real time fluorescent curves of 2 pM, 200 fM, 20 fM, 2 fM, 200 aM, 20 aM, 2 aM, 200 zM synthetic mutant DNA and 2 pM wild-type DNA (from a to i). (B) The calibration curve between R_t and mutant DNA concentrations. (C) The real time fluorescent curves of 10% (a), 5% (b), 1% (c), 0.1% (d), 0.02% (e) and 0.01% (f) BRAF mutant genomic DNA in 40 ng total genomic DNA, and 40 ng normal human leukocyte genomic DNA (g). (D) The calibration curve between R_t and different mutation ratios. The error bars show the standard deviation of three replicate experiments.

amplification of LCR and RCA, high specificity of our designed DP-gLCR, and low background signal.

In real clinical samples, BRAF V600E mutation may present with ultra-low abundance and be accompanied by abundant normal genomic DNA. To further demonstrate the applicability and specificity of the system for the detection of low ratio BRAF mutation, the real time fluorescent signal was evaluated in the presence of different mutation ratio by spiking various amounts of mutant genomic DNA into wild-type genomic DNA. Mutant genomic DNA extracted from HT 29 cell line and wild-type genomic DNA acquired from normal human leukocyte were mixed together with different mutation ratios of 10%, 5%, 1%, 0.1%, 0.02%, and 0.01% with a total 40 ng genomic DNA to serve as mimetic samples. The blank control contained 40 ng of the wild-type genomic DNA did not show any fluorescent signal change in 90 min (Fig. 3C curve g), indicating very high specificity. More importantly, as low as the ratio of 0.01%, which contained 4 pg mutant type genomic DNA, nearly one copy genomic DNA, can be clearly distinguished with the blank curve (Fig. 3C curve f). The proposed method illustrated significantly higher sensitivity than Sanger sequencing, ARMS-PCR and other LCR-based biosensing methods [18,28,29]. Notably, R_t also manifest a good linear relationship with different mutation abundances ranging from 0.01% to 10% (Fig. 3D). These results proved that our method had outstanding performance for the detection of much lower abundance BRAF V600E mutation in complex matrix, compared with ARMS-PCR and LAMP [7–10]. Furthermore, lower cost and simpler operation make the method more pragmatic than next-generation sequencing and digital PCR in the setting where sophisticated instruments are not available.

3.5. Detection of BRAF V600E mutation gene in real clinical samples

To further validate the feasibility of the proposed strategy in clinical application, 20 cases of clinical specimens with certain pathological diagnose, containing 14 cases of tissue and 6 cases of FNAB samples of PTC, were analyzed by our proposed method and a commercial ARMS-PCR assay. As shown in Fig. 4, different from the standard S-shaped curves of RT-PCR, the typical RT-HRCA curves for real clinical samples displayed linear increase of fluorescence signal over time due to that RCA is not exponential amplification like PCR, but a linear amplification. However, the fluorescence signal of our proposed RT-HRCA significantly increased at about 8 min and 30 min for the detection of BRAF V600E mutation from the PTC tissue and FNAB samples, respectively, which showed faster increase than those of matched ARMS-PCR assays (35 min for 10 cycles, and 42 min for 15 cycles, respectively).

The analysis results and detailed information of 20 cases of clinical specimens are revealed in the Table 1. Among the 16 cases of PTC samples, 15 cases were identified as BRAF V600E mutation and all 4 cases of benign lesions were wild type with our proposed method, showing 93.75% of sensitivity and 100% specificity. The results agree with the results of ARMS-PCR completely and accord with that the prevalence of BRAF V600E mutation in PTC is not 100% [33]. These results indicated high accuracy and practicability of the designed strategy for the detection of BRAF V600E mutation from multiple clinical samples.

4. Conclusion

In conclusion, the present study has developed a real time fluorescent biosensing system for the detection of low abundance BRAF V600E mutation by coupling DP-gLCR with HRCA. By taking advantage of the ingenious design of DP-gLCR and remarkable amplification efficiency of HRCA, our strategy shows ultra-high sensitivity and specificity for the mutation discrimination. The proposed assay can detect as low as 200 zM mutant targets and 0.01% mutant genomic DNA, and is applicable for distinguishing BRAF V600E mutation from multiple types of clinical specimens. This proposed strategy presents a pragmatic and low cost platform toward ultrasensitive and specific detection of somatic mutation with very low level and shows great potential for clinical application in early diagnosis and treatment of cancer.

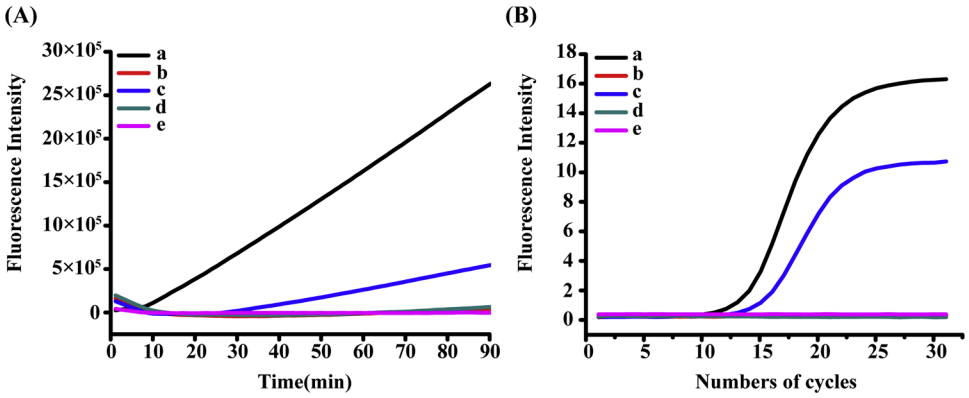


Fig. 4. The typical (A) RT-HRCA curves and (B) ARMS-PCR curves for the detection of BRAF V600E mutation from the PTC tissue (a), benign thyroid tissue (b), PTC FNAB sample (c), benign thyroid FNAB sample (d) and blank control (e).

Table 1
The comparison of our proposed assay and a commercial ARMS-PCR assay for detecting BRAF V600E mutation in different real clinical specimens.

Methods	Results	Pathological diagnose		Sensitivity	Specificity
		PTC	Benign		
D-gLCR	Positive	15(10)[a]	0	93.75%	100%
	Negative	1(1)	4(3)		
ARMS-PCR	Positive	15(10)	0	93.75%	100%
	Negative	1(1)	4(3)		

[a] The sum of samples and the number of FNAB samples in the parenthesis.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.snb.2019.01.125.

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