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Direct detection of circulating free DNA extracted from serum samples of breast cancer using locked nucleic acid molecular beacon

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ABSTRACT

As an emerging noninvasive blood biomarker, circulating free DNA (cfDNA) can be utilized to assess diagnosis, progression and evaluate prognosis of cancer. However, cfDNAs are not "naked", they can be part of complexes, or are bound to the surface of the cells via proteins, which make the detection more challenging. Here, a simple method for the detection of Ubiquitin-like with PHD and ring finger domains 1 (UHRF1) DNA exacted from serum of breast cancer (BC) has been developed using a novel locked nucleic acid molecular beacon (LNA-MB). In order to enhance the stability and detection efficiency of the probe in biofluids, we design a shared-stem molecular beacon containing a 27-mer loop and a 4-mer stem with DNA/LNA alternating bases. The fluorescence is released in the presence of target. The detection procedure is simple and can be completed within 1 h. This method shows a sensitive response to UHRF1 DNA with a dynamic range of 3 orders of magnitude. The limit of detection is 11 nM (S/N=3) with excellent selectivity. It can discriminate UHRF1 DNA from three-base mismatched DNA with a high specificity. More importantly, this method can distinguish the expression of serum UHRF1 DNA among 5 breast cancer patients and 5 healthy controls. The mentioned superiority may suggest that this assay can be served as a promising noninvasive detection tool for early BC diagnosis and monitoring.

1. Introduction

Circulating free DNA (cfDNA) refers to 500 bp to 30 kb double-stranded DNA present in circulating body fluids [1–3], which has been proven to be resistant to RNase and pronase, but digestible with DNase I. The dysregulation of cfDNA could be in response to a number of factors, including mutation, DNA hypermethylation, microsatellite instability and loss of heterozygosity [4]. In recent years, many studies suggest that cfDNA in blood samples has a potential to become new biomarkers for cancer diagnosis and prognosis [5–7]. So it is of high-importance to develop a method for rapid detection of cfDNA in serum with high sensitivity and specificity.

The detection of cfDNA is facing challenge due to the unique characteristics of cfDNA: (1) the low abundance of cfDNA that requires the method to be highly sensitive, (2) the double-stranded structure of cfDNA that requires the technology to be specific to the duplex, (3) the combination with complexes or cells of cfDNA aggravates the difficulty in cfDNA detection. Some earliest studies employed fluorometric assays to quantify total cfDNA in serum or plasma [8–11], which did not require DNA extraction and amplification, and DNA was measured directly in the diluted samples using a fluorescent dye. Recently, Li's group tried to detect total cfDNA in human serum using a novel electrochemical sensor. The biosensor exhibited excellent sensitivity and high specificity [12]. But then, it makes little sense in clinic because the elevated total cfDNA has no relationship with certain diseases. So it is imperative to focus the research on certain DNA sequence amongst the millions of nucleotides, which is correlated with a particular disease. Ubiquitin-like with PHD and ring finger domains 1 (UHRF1) is exactly a potential tumor marker. It is a multi-domain protein that consists of an N-terminal ubiquitin-like domain, a tandem Tudor domain, a plant homeodomain, a set and ring-

Abbreviations: cfDNA, circulating free DNA; PCR, polymerase chain reaction; UHRF1, Ubiquitin-like with PHD and ring finger domains 1; BC, breast cancer; LNA-MB, locked nucleic acid modified molecular beacon; PNA, peptide nucleic acid; NCBI, National Center for Biotechnology Information.

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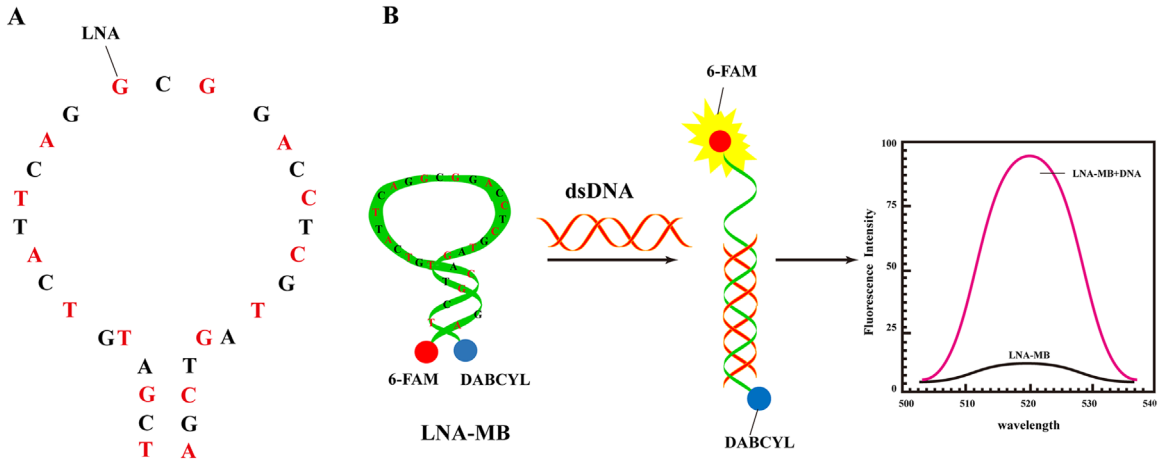

Fig. 1. (A) Structure of LNA-MB. The LNA bases are colored in red. (B) Schematic illustration of DNA detection with the LNA-MB. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

associated domain, and a C-terminal really interesting new gene [13]. Our previous study suggested that UHRF1 DNA levels in serum of breast cancer were significantly higher than healthy controls [14].

Among the conventional molecular techniques for the detection cfDNA sequences, polymerase chain reaction (PCR) is the most widely used, but it is labor-intensive, time-consuming, expensive, and not suitable for large-scale quantification of clinical samples. To overcome the drawbacks, several strategies have been adopted, including quantum-dots modified probes [15], nanomaterials-based sensors [16–18], and novel bimolecular beacons [19]. However, these methods are restricted to centralized laboratories because of the structure of cfDNA and the complexity of human serum. In this work, we tried to use molecular beacon (MB) to detect UHRF1 DNA. MB is a short hairpin oligonucleotide probe that has a fluorophore at 5′ end and a quencher at 3′ end. The loop portion of an MB is a target-recognition region and the two stem sequences are complementary to each other. In the absence of a target, the hybridization between two stem sequences brings the quencher and fluorophore into close proximity, the fluorescence is quenched effectively due to the energy transfer. When MB hybridizes to its target, the stem helix is opened, and the fluorophore and quencher are separated, resulting in the restoration of fluorescence [20]. MBs are therefore used for the real-time detection of nucleic acids. However, MBs are lack of stability in biofluids, including conformational stability and nuclease resistance, which definitely cannot satisfy its further clinical application in non-invasive biomarkers detection and is greatly required to be improved.

Currently, there are two strategies for constructing of stable and nuclease-resistant probes. One is to use nanomaterials or polymer macromolecules to protect probes from nuclease cleavage by forming probe-nanomaterial or probe-polymer complexes [21,22]. This strategy can effectively prolong retention time of probes in biofluids, but the size of probe-based complexes is much bigger than the original one, which may restrict the application of probes. Another major strategy is to incorporate nuclease-resistant building blocks into probes, such as peptide nucleic acid (PNA) [23], locked nucleic acid (LNA) [24], phosphorothioate [25], and so on. This strategy can commonly improve probe stability to enzyme degradation on the promise of maintaining its function and size [26]. Among these candidates, LNA is a most promising for its increased helical thermostability, high binding affinity, excellent mismatch discrimination capability, nuclease resistance and non-toxicity [27]. It is a conformationally restricted nucleic acid analogue, which connects the 2′-O atom with the 4′-C atom and generates a locked 3′endo conformation, thus reducing the conformational flexibility of the ribose [28]. Furthermore, LNA can associate with double-stranded DNA to form triple-helix model [29], which is the main reason we chose LNA as a modification. By using LNA-MB to detect nucleic acids, the biofluids stability is promised. The high binding affinity of LNA can contribute to the lower background in the absence of target and higher signal in the presence of the complementary sequences. In other word, LNA-MB has a higher signal-to-background ratio than DNA-MB.

Herein, we aim to develop a new method to recognize UHRF1 DNA in serum of BC patients. In order to enhance the stability and detection efficiency of the probe in biofluids, a novel locked nucleic acid (LNA)/DNA chimeric molecular bacon was developed through proper LNA incorporation. To make full use of the hairpin structure as well as LNA modification, Tan et al. systematically designed a series of LNA modified MBs and then studied the thermodynamics, hybridization kinetics, protein binding affinity and enzymatic resistance of the MBs to find the best DNA/LNA ratio in MBs [30]. Refer to Tan's conclusion, we designed a shared-stem molecular beacon containing a 27-mer loop and a 4-mer stem with DNA/LNA alternating bases. The hybridization of LNA-MB and dsDNA is shown as Fig. 1. Our method can selectively distinguish target DNA and the mismatch segment, with a detection limit of as low as 11 nM and a large dynamic range of 3 orders of magnitude. Moreover, the experimental results are in agreement with those previously reported by PCR, which indicating the method hold a great potential for further applications in the clinical diagnosis of breast cancers.

2. Methods

2.1. Selection of DNA sequence

The sequence of the UHRF1 DNA was obtained from the nucleotide database on the website of the National Center for Biotechnology Information (NCBI). A 27-base DNA recognition sequence was chosen for target and studied by extensive BLAST search analysis to evaluate specificity as the serum DNA target. The loop section of the hairpin DNA was designed according to the complementary base-pair specificity. All sequences were synthesized from Sangon Biological Engineering Technology & Co. Ltd. (Shanghai, China) and purified using high-performance liquid chromatography. All oligonucleotides were suspended in 20 mM Tris–HCl (pH 7.4) buffer and further diluted to appropriate concentrations. The oligonucleotides were shown in Table 1.

Table 1
Sequences of oligonucleotides in this work.

Oligonucleotide	Sequence (5′-3′)
UHRF1	TCGACTACGAGGTCCGCCTGAATGACA
TM-UHRF1	TCGAC**T**TCGAGGT**G**CGCCTG**T**ATGACA
LNA-MB	*TCGATGTCATTCAGGCGGACCTCGTAGTCGA*
DNA-MB	TCGATGTCATTCAGGCGGACCTCGTAGTCGA

Italic letters represent LNA bases. Underlined letters represent MB stem. Bold letters indicate mismatched bases. All MBs are labeled with DABCYL at 3′ ends and FAM at 5′ ends.

2.2. Equipment

A RF-5301PC spectrofluorometer (Shimadzu Co., Japan) equipped with a xenon lamp was used for recording the regular fluorescence data. The spectra were recorded from 500 to 650 nm with a step of 1 nm at the excitation wavelength of 480 nm, and the excitation bandwidth was set to 5 nm.

2.3. Optimization of hybridization time

To determine the optimal hybridization time, the experiments were conducted with 10 nM LNA-MBs, 10 nM complimentary segments in 20 mM Tris–HCl (pH 7.4) buffer containing 100 mM NaCl, 5 mM KCl, and 5 mg $MgCl_2 \cdot 6\ h_2O$ with a total volume of 200 μL for 5 min, 15 min, 30 min, 60 min, respectively.

2.4. DNase I sensitivity

To verify the nuclease sensitivity of MBs, the fluorescence of a 200 μL solution including 20 mM Tris–HCl (pH 7.4), 100 mM NaCl, 5 mM KCl, 5 mg $MgCl_2 \cdot 6\ h_2O$, and 10 nM LNA-MBs (or DNA-MBs), with or without one unit of ribonuclease-free DNase I, was measured at room temperature for 30 min separately.

2.5. Selectivity of the LNA-MB Probe

To test the selectivity of the probe, UHRF1 DNA and TM-UHRF1 segment with final concentrations of 10 nM each were prepared and incubated with 10 nM LNA-MB probe in 20 mM Tris–HCl (pH 7.4) buffer containing 100 mM NaCl, 5 mM KCl, and 5 mg $MgCl_2 \cdot 6\ h_2O$ with a total volume of 200 μl at optimal conditions.

2.6. Sample analysis

5 serum samples were collected from Jiangsu Cancer Hospital, 5 were used as healthy controls. All of them were processed for serum at 3000 rpm for 10 min at 4 °C to completely remove cellular components after 20 minutes of collection, and then were stored at −80 °C prior to use without further modification. cfDNA was extracted from 200 μL serum using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The extracts were resuspended in 40 μL of nuclease-free water. Subsequently, PCR amplification was performed using sense primer CGTGGTCCAGATGAACTCC antisense primer CACGTTGGCGTAGAGTTCC. The reactions were incubated at 95 °C for 2 min, and then 45 cycles circulating at 95 °C for 3 s and 60 °C for 30 s. Ct values were achieved using LightCycler Software version 3.5. The study was approved by the Ethics Committee of Jiangsu Cancer Hospital. Written informed consents were provided by all patients and healthy volunteers.

2.7. Detection of target DNA

10 nM (2 μL) of LNA-MB and 5 μL of extracted DNA were added in 193 μL 20 mM Tris–HCl (containing 5 mM $MgCl_2$, 50 mM NaCl, pH 7.4), keeping the mixture to hybridize for 30 min at room temperature.

3. Results and discussion

3.1. Design of LNA-MB

MBs are lack of the stability in biofluids, including conformational stability and nuclease resistance, which cannot definitely satisfy its further clinical application in noninvasive biomarkers detection [20]. In consideration of the excellent properties of LNA, we introduced LNA into MB to improve its capability for in clinical applications. In order to design a proper LNA-MB, several strategies were adopted for the project. Firstly, we describe a shared-stem molecular beacon, of which one of the stem sequences participates in both hairpin formation and target hybridization [31]. In contrast to conventional molecular beacons, it can prevent the formation of sticky-end pairs, which might result in a very low signal response and apparent slow hybridization kinetics [31]. Secondly, the incorporation of LNA bases in a MB sequence can greatly enhance the mismatch discrimination capability and make the probe resist nuclease degradation. Unfortunately, it also slows the hybridization rate. So the number of LNA bases in a MB sequence has a great significance during the design of LNA-MB. Use Tan's investigation as reference [30], we design a shared-stem molecular beacon containing a 27-mer loop and a 4-mer stem, with DNA/LNA alternating bases. Thirdly, the choice of quenching molecules is also of great importance, quenching efficiency has a direct relationship with the properties of the probe. In MB synthesis, the most commonly used quencher is Dabcyl, it possesses the quenching efficiency of 92.9% [32].

3.2. Stability of LNA-MB

For the detection of cfDNA in clinical application, one of the big challenges is the vulnerability of probes to enzymatic digestion. It has been reported that LNA modification within probes can improve its in vivo stability [33–35]. So in this experiment, we also investigated the enhanced stability of the LNA modification probe. The sensitivity of LNA-MB to nuclease digestion was test using DNase I. As shown in Fig. 2A, immediate increase in fluorescence signal was observed, when DNase I was added to a solution containing DNA-MB. Meanwhile, LNA-MB gave no observable signal change, indicating their resistance to DNase I. These results suggested that the incorporation of LNA bases in a MB sequence obviously enhanced the resistance ability to nuclease of the probe, making LNA-MB an ideal probe in clinical application.

3.3. Selectivity of LNA-MB

For the direct detection of cfDNA segments from serum samples, the selectivity of probe is as important as its in vivo stability. LNA has high-affinity to the complementary nucleic acids [36]. Many studies suggested that the melting temperature (Tm) of modified nucleic acid increases 4–8 °C per LNA monomer [37,38]. In other words, the Tm of LNA/DNA hybrids reduces greatly when the introduction of mismatched bases. Take advantage of this property, a lot of researches have focus on improving the mismatch discrimination of probe by using LNA [37,39]. In this work, in view of the stability of LNA-MB against DNase I, we investigated the selectivity of the proposed method using the synthesized UHRF1 and mismatched DNA. In order to eliminate the inference brought by instrumental factors which contributed to day-to-day variety of fluorescence intensity, the study was carried out

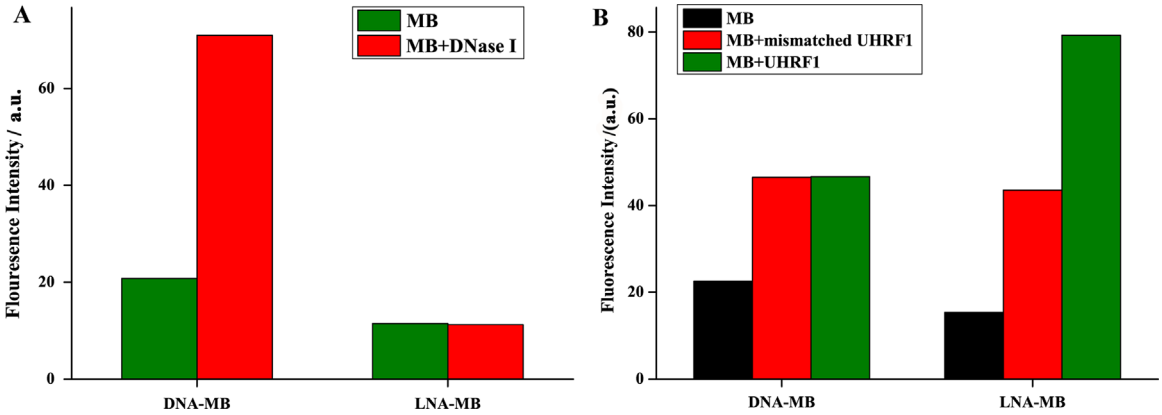

Fig. 2. (A) Stability of LNA-MB and DNA-MB against DNase I. The concentrations of MBs and DNase I are 10 nM and one unit, respectively. (B) Selectivity of LNA-MB and DNA-MB. The concentrations of LNA-MB, DNA-MB, UHRF1 DNA, and mismatched UHRF1 DNA are all 10 nM.

simultaneously and calibrated with Tris–HCl buffer. Additionally, parallel tests were used for the elimination of the accidental error. Considering that the fluorescence intensity is also proportional to concentration of target species, UHRF1 and mismatched DNA have a fixed concentration of 10 nM refer to the early work [40]. As shown in Fig. 2B, in the absence of target, the fluorescence intensity of DNA-MB is higher than LNA-MB, which suggests that LNA-MB has lower background fluorescence. The addition of UHRF1 resulted in significant increase in LNA-MB fluorescence. After adding mismatched DNA as control, the solution also shows a fluorescence increase. This signal can be distinguished from that of the fully complementary target because it is nearly 50% less intense. Furthermore, we found that the fluorescence of DNA-MB is also increase in the presence of target, but it cannot distinguish the complementary and mismatched DNA. As a contrast, LNA-MB is much better than DNA-MB in mismatch discrimination.

3.4. Optimization of experimental condition

As everyone knows, an optimal hybridization condition is very crucial to the assay because the detection was based on the association of the probe and the target. Here, we made a research on the hybridization efficiency of probe design, hybridization time and serum concentration. Recently, LNA has been widely applied because of its excellent biophysical characteristics [33–35]. Many studies indicated that Methylene Bridge of LNA analogues can lock the ribose ring into a rigid conformation and hence increased the melting temperature of the hybrid when introducing a number of LNA analogues into a sequence of DNA or RNA [36–38], which is associated with stability increasing of the probe. Besides, due to the high affinity of LNA, the modified segments usually have enhanced affinity toward target sequence and excellent mismatch discrimination capability to the similar sequences. As shown in Fig. 3, with and without target molecules, LNA-MB gives relatively higher signal-to-background compared to DNA-MB. Hence, LNA-MB was used as the detection probe for the rest of the experiment.

Incubation time is also important in the hybridization. As shown in Fig. 4, the hybridization times of 5, 15, 30 and 60 min were studied. It shows that the fluorescence intensity of hybrids increased while prolonging the incubation time and then reached a plateau at 30 min. Hence, the incubation time was set at 30 min for the entire experiment.

3.5. Extracted UHRF1 DNA detection in serum

The sensitivity of the proposed assay was investigated using different concentrations of UHRF1 DNA. As shown in Fig. 5A, in the presence of different-concentration UHRF1 DNA, the fluorescence intensity increases with the increasing concentration of UHRF1 DNA. In the logarithmic scales (Fig. 5B), the value of $(F-F_0)/F_0$ has a linear correlation with the concentration of UHRF1 DNA. The regression equation is $Y=0.10982+0.17134\,C$ ($R^2=0.99556$), where Y is the value of $(F-F_0)/F_0$ and C is the concentration of UHRF1 DNA, and F_0 and F are the fluorescence intensity in the absence and presence of UHRF1 DNA, respectively. This method shows a sensitive response to UHRF1 DNA with a dynamic range of 3 orders of magnitude. The limit of detection is estimated to be 11 nM based on the 3σ method. The improved sensitivity of the

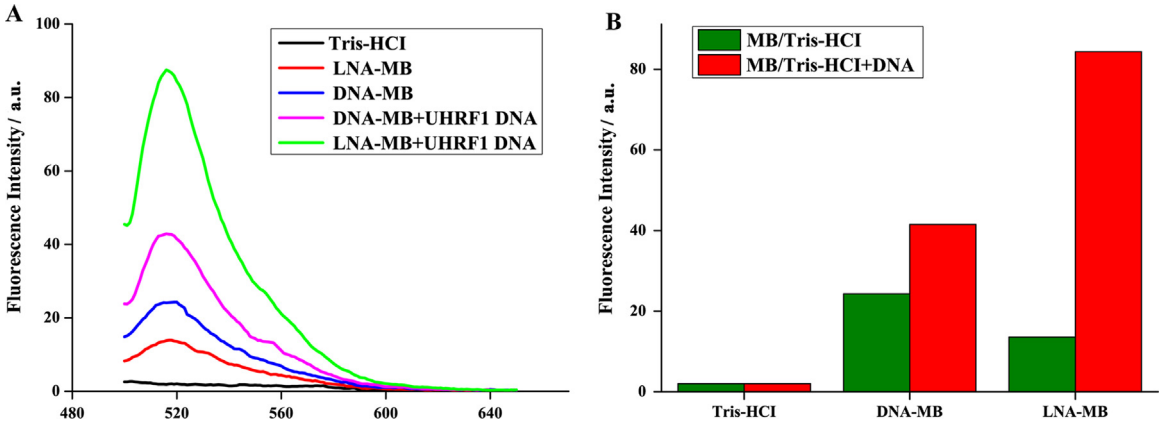

Fig. 3. (A) Fluorescence emission spectra of Tris–HCl, LNA-MB, DNA-MB, LNA-MB+UHRF1 DNA, and DNA-MB+UHRF1 DNA solutions. (B) Comparison of signals and backgrounds for DNA-MB and LNA-MB.

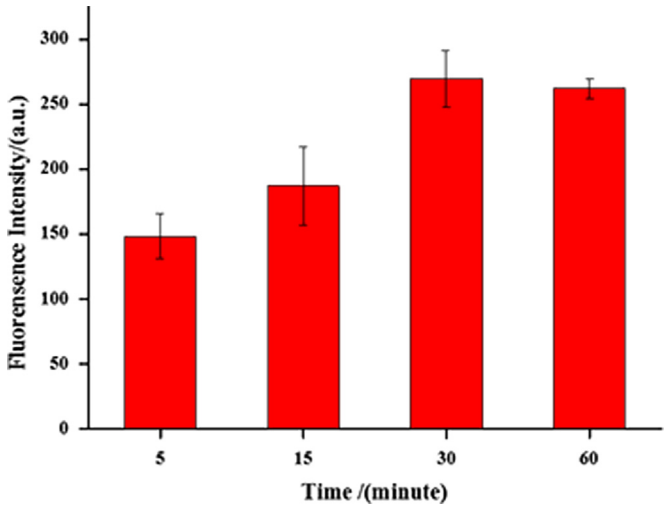

Fig. 4. Optimization of incubation time of 10 nM LNA-MB and 10 nM UHRF1 DNA.

proposed method can be attributed to both the unique design of hairpin probe (Fig. 1A) and the extremely superiority of LNA.

Our former research indicated that the expression levels of UHRF1 in plasma is highly associated with breast cancer, and might be a novel biomarker for both breast cancer and the survival of breast cancer patients [14]. In this research, we investigated the feasibility of the proposed method for detecting UHRF1 in serum. cfDNA is associated with protein complexes, which are benefit for their high stability but also are deterrent to the hybridization between DNA and hairpin probe. We first extracted DNA from serum using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The available DNA was profiled using real-time PCR and the proposed technology simultaneously. Thereupon, cycle number was determined, and the relative amount of UHRF1 DNA was described using $2^{-\Delta\Delta Ct}$ [41], here $\Delta\Delta Ct=(Ct_{target}-Ct_{\beta-actin})-(Ct_{reference}-Ct_{\beta-actin})$. The obtained histogram was shown in Fig. 6B. The relative amounts of UHRF1 DNA in patients are obviously higher than that in healthy controls. Meanwhile, as shown in Fig. 6A, the value of $(F-F_0)/F_0$ obtained from the BC patients is significantly (unpaired t test, $P<0.005$) higher than that obtained from the healthy controls. The concentrations of UHRF1 DNA is linear correlated with $F-F_0/F_0$, then the concentrations of UHRF1 DNA in serum can be further calculated according to the calibration curve in Fig. 5B. The results obtained by the proposed method have the same trend of those obtained by PCR, further confirming the accuracy of assay. However, as shown in Fig. 6, the relative fluorescence is not matched perfectly with the relative amounts of UHRF1 DNA in 5 patients and 5 healthy controls. For example, patient 5 has lower fluorescence signal than normal 2 and comparable to normal 1 and normal 3, but in Fig. 6B normal has much lower signal. This is possibly due to the potential molecules in the matrix and many unknown factors that may affect the fluorescence intensity. Our work is in exploratory stage, it may have a general tendency with PCR results rather than in agreement with PCR totally. These results demonstrate a possibility that the proposed method may directly detect cfDNA in serum with great reliability, which is proven to have a potential to become new biomarkers for cancer diagnosis and prognosis. In addition, compared to PCR, our method is time-saving, low-priced, and easy to be operated. Even though our results have a general tendency with PCR, it should be noticed that this method needs to be improved in the recognition of target, and more samples should be used to confirm the feasibility of test.

4. Conclusion

In summary, we have developed a rapid, sensitive and sample detection assay for the detection and quantification of cfDNA using a LNA-MB. This method shows a sensitive response to UHRF1 DNA with a dynamic range of 3 orders of magnitude. The limit of detection is estimated to be 11 nM based on the 3σ method. And it can discriminate UHRF1 DNA from three-base mismatched DNA with a high specificity. Moreover, this proposed assay is capable of distinguishing the expression of serum UHRF1 DNA among 5 breast cancer patients and 5 healthy persons, the results is agreed with PCR generally, and the whole procedure can be completed within 1 h, which indicated that the proposed method may have a great potential for further applications in the clinical diagnosis.

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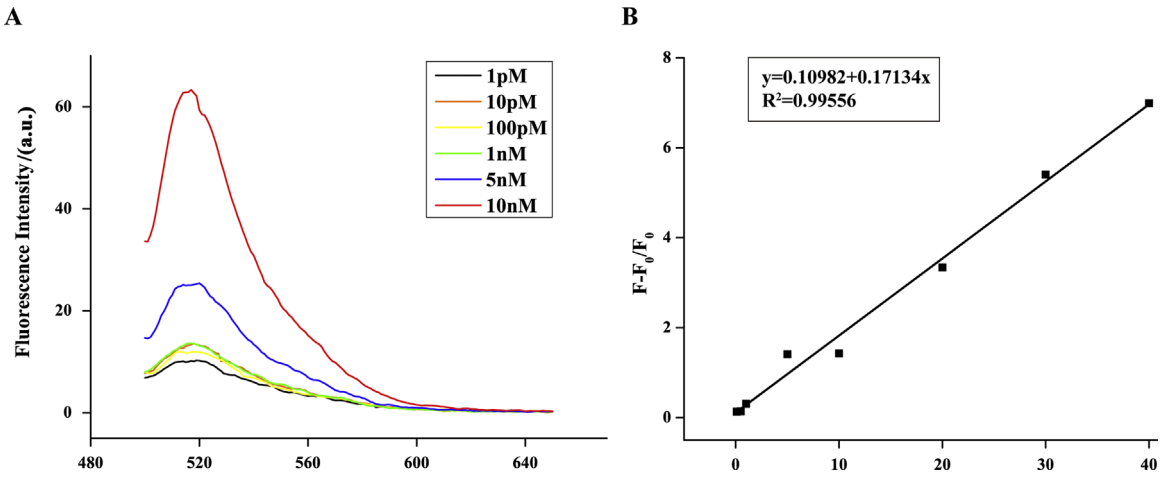

Fig. 5. (A) Fluorescence emission spectra of 10 nM DNA-MB solutions in presence of different concentrations of UHRF1 DNA. (B) Plot of $(F-F_0)/F_0$ vs concentration of UHRF1 DNA. F_0 and F are the fluorescence intensity in the absence and the presence of UHRF1 DNA, respectively.

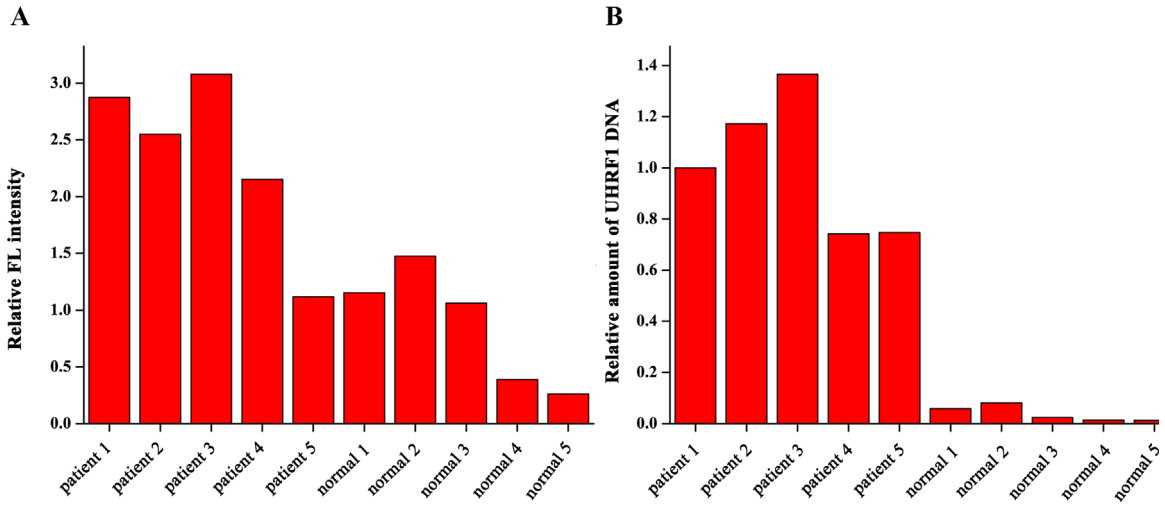

Fig. 6. Comparison of relative fluorescence and relative quantity of 5 patients and 5 healthy controls. (A) The relative fluorescence obtained by UHRF1 DNA exacted from serum (5 breast cancer patients and 5 healthy controls) described using $(F-F_0)/F_0$. (B) The relative quantification of UHRF1 in serum (5 breast cancer patients and 5 healthy controls) by PCR results described using $2^{-\Delta\Delta Ct}$.

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