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A new method to evaluate trinucleotide repeats length polymorphism

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ABSTRACT

Trinucleotide repeats (TNRs) are involved in a number of debilitating diseases and disorders, the length of which usually indicates disease severity at gene level. Herein we have developed a novel fluorescent method in this work to evaluate TNRs length polymorphism based on its DNA secondary structure with doxorubicin (Dox) as fluorescent probe. This new method makes use of the fact that TNRs rich in guanine (G) and cytosine (C) are susceptible to forming stable intramolecular structures, resulting in the formation of double-stranded 5'-GC-3' or 5'-CG-3' sequences. So, intercalating of these sequences by Dox, fluorescence quenching of Dox occurs. Consequently, the length polymorphism of TNRs can be evaluated. Taking the study of CAG for an example, a linear relationship between fluorescence intensity and the sequences ranging from 10 to 35 CAG repeats has been obtained, and the assay of the TNRs length polymorphism for PCR products has been realized. Therefore, without the necessity to introduce fluorophores and quenchers by chemical modifications, this new method is simple, cost-effective and convenient to be operated, so it may hold great promise in the diagnosis of diseases arouse from the triplet expansion.

1. Introduction

Trinucleotide repeats (TNRs) are involved in a number of debilitating diseases such as myotonic dystrophy and neurodegenerative disease [1–4]. The triplet expansions are usually classified into two main categories. Those occur within genes can be translated into toxic protein containing an expansion of a given amino acid, and those occur in noncoding regions can alter RNA functions or reduce transcription and translation [5]. TNRs often exhibit a strong negative guanine (G) and cytosine (C) skew [4], thus, due to the repetitive nature and highly biased nucleotide composition, they are suspected to form stable intramolecular structures that may play an important role in the mutational process. Therefore, the length of the repeat indicates disease severity and the diagnoses of these genetic disorders at the DNA level is important for confirming clinical diagnosis, for genetic screening of families at risk, and for early recognition of heterozygous carriers [6].

So far, PCR amplification of DNA regions spanning the repeat has been employed to study TNR length polymorphism by determining the amplicon length using gel electrophoresis [7–9]. In this work, we have developed a new method to evaluate TNR length polymorphism. The principle of this new method is based on the fact that the expansions are unrelated to the sequence of TNRs, but are related to the propensity of the sequence to form secondary structures [5]. So, we propose that new method to evaluate TNR length polymorphism could be developed by making use of the secondary structure other than the sequence. In the meantime, we propose using fluorescent technique to indicate the evaluation, since it is more simple and easily-operated, thus it may have great potential applications.

As is well known, fluorophores can work alone as a probe or indicator as its fluorescence can be affected by its environment. For example, the fluorescence of nucleic acid dyes is often nucleic acid-selective or base pair-selective. So, considering the fact that the repetitive CG-rich TNRs are susceptible to the formation of duplexes by self-folding, forming two Watson–Crick G–C pairs and one mismatch pair [10–13], we have introduced doxorubicin (Dox), a well-known anticancer drug [14], in this study as the probe to study TNR length polymorphism. On the one hand, Dox can preferentially bind to double-stranded 5′-GC-3′ or 5′-CG-3′ sequences [15,16], due to the presence of flat aromatic rings in this molecule [17]. On the other, the fluorescence of Dox can be quenched after intercalation into DNA [18–22]. Therefore, this

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fluorescence property of Dox has been utilized in this work to probe into the TNR length polymorphism.

2. Material and methods

2.1. Materials and chemicals

Doxorubicin (Dox) was purchased from Sigma. Blood genome DNA extraction kit and Takara LA PCR™ kit ver.2.1 were purchased from Takara Biotechnology (Dalian) Co., Ltd. All other chemicals were analytical grade.

All the DNA sequences used in this study were obtained from Shanghai Invitrogen Biotechnology Co., Ltd. The details of the DNA sequences were listed as follows:

$(CAG)_n$ repeats: $(CAG)_{10}$, $(CAG)_{15}$, $(CAG)_{20}$, $(CAG)_{25}$, $(CAG)_{30}$, $(CAG)_{35}$
Simulated PCR product DNA 10 (S_{10}): 5′-TCC CTC AAG TCC TTC $(CAG)_{10}$ CAA CAG CCG CCA-3′
Simulated PCR product DNA 20 (S_{20}): 5′-TCC CTC AAG TCC TTC $(CAG)_{20}$ CAA CAG CCG CCA-3′
Primer 1: 5′-TCC CTC AAG TCC TTC CAG CAG-3′
Primer 2: 5′-TGG CGG CTG TTG CTG CTG-3′

The buffer solution employed in this work were as follows. TE buffer solution: 10 mM Tris–HCl and 1 mM EDTA (pH 8.0). Fluorescence test buffer solution: 100 mM NaAc, 50 mM NaCl and 10 mM $MgCl_2$. All solutions were prepared with double-distilled water, which was purified with a Milli-Q purification system (Branstead, USA) to a specific resistance of 18 MΩ cm.

3. Evaluation of TNRs length polymorphism

All $(CAG)_n$ repeats solutions were heated at 90 °C for 10 min in order to dissociate any intermolecular interaction. They were then gradually cooled to room temperature to form stable intramolecular secondary structures. Afterwards, physical complexes of Dox and $(CAG)_n$ repeats were made by sequential addition of $(CAG)_n$ repeats to Dox (5 μM) in the test solution. Finally, the fluorescence intensity was monitored at excitation 480 nm and emission was recorded in the interval of 500–720 nm on a Hitachi F-7000 spectroflourophotometer.

To show the feasibility of our method, control experiments were introduced, in which $(CAG)_{n-1}$ were employed by cooling $(CAG)_n$ repeats solution immediately to 4 °C from 90 °C.

4. Evaluation of TNRs length polymorphism in PCR products

To show the feasibility of our method in measuring $(CAG)_n$ repeats in PCR products, simulated PCR product DNAs (S_{10} and S_{20}) were employed and the fluorescence intensity was recorded after they were added to the Dox in the test solution. After that the length polymorphism of $(CAG)_n$ in PCR products were evaluated. Firstly, genomic DNA was extracted from blood samples (provided by the First Affiliated Hospital of Nanjing Medical University) with blood genome DNA extraction kit. Then, the $(CAG)_n$ repeats were amplified by asymmetric PCR reaction. After that, the single-stranded DNA products were dissolved to be 0.05 μM with TE buffer. Finally, the sample was analyzed by using both the new method proposed in this work and the traditional method of capillary electrophoresis.

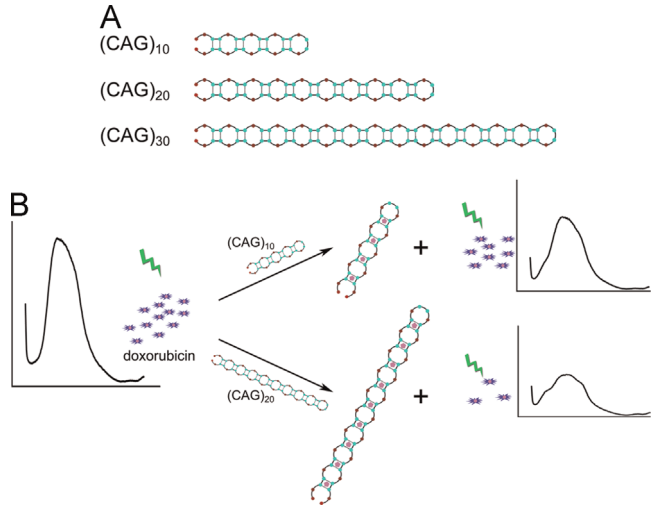
5. Results and discussion

In this work, we have taken $(CAG)_n$ repeats as the model triplet repeats to develop a new method for the evaluation of TNRs length polymorphism. CAG is the most frequent codon found in gene encoded TNRs. Expansions of $(CAG)_n$ repeats inside exons have been found in Huntington disease (HD) and several Spinocerebellar ataxia (SCAs). In these neurodegenerative disorders, the reading frame consists of an expanded CAG triplet that always encodes a toxic polyglutamine tract to cause neurodegeneration [5]. Meanwhile, the length of the polyglutamine repeat indicates disease severity irrespective of the gene affected, with the longest repeat lengths associated with the most disabling early-onset (juvenile) forms of these disorders. So detection of $(CAG)_n$ expansion has been used for the diagnoses of these genetic disorders.

Biophysical and biochemical analyses have shown that $(CAG)_n$ repeats can form hairpin structures in vitro, in which cytosines and guanines are paired and adenines are excluded [23–25]. So Dox should be able to intercalate into the double-stranded 5′-GC-3′ sequences, resulting in the fluorescence quenching of Dox, thus the length polymorphism of $(CAG)_n$ may be evaluated by using Dox as the probe to show the experimental results. Scheme 1A shows the intramolecular stem with several repetitions of a A–A mismatch sandwiched between 2G–C base pairs, which further results in the formation of double-stranded 5′-GC-3′ sequences. In these hairpins, the number of Watson–Crick G–C pairs is maximized in the stem through the formation of A–A mispairs flanked by G–C pairs. It can also be noticed that longer strands give rise to the formation of more double-stranded 5′-GC-3′ sequences. Consequently, more Dox will be fluorescently quenched after intercalating into longer $(CAG)_n$ repeats (Scheme 1B). So, the fluorescence intensity of Dox can be used to indicate the repetition number and a new method to evaluate the length polymorphism of $(CAG)_n$ can be developed.

5.1. Qualitative study of TNRs length polymorphism

As shown in Fig. 1A, decrease in the native fluorescence spectrum of Dox can be observed after $(CAG)_{10}$ is added in the test solution containing Dox, resulted from the intercalation of Dox into the DNA sequences. To further show the effect of the



Scheme 1. (A) The secondary structures of $(CAG)_{10}$, $(CAG)_{20}$ and $(CAG)_{30}$ analyzed by NUPACK software. Note that in the hairpins of the G- and C-rich strands, the central mismatched A–A pair in the stem (in red) is surrounded by two Watson–Crick G–C pairs (in green). (B) Schematic illustration of this new method to evaluate TNRs length polymorphism (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).