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Exploration of G-quadruplex function in c-Myb gene and its transcriptional regulation by topotecan

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ABSTRACT

Our bioinformatics research shows that there are four G-rich sequences (S1-S4) in the upstream region of the transcription start site of c-Myb gene, and we have proved that these sequences have the ability to form G-quadruplex structures. This work mainly focuses on G-quadruplex function, recognition and transcription regulation in c-Myb gene, revealing a novel regulatory element in c-Myb proximal promoter region, and its transcription regulation by G-quadruplex binder. The research has identified that the enhancer effect in c-Myb transcription was primarily affected by the G-quadruplex formed by S1 sequence, and the up-regulation effect may due to the removal of repressive progress of MZF-1 by stabilizing G-quadruplex. Attentions were being paid to the development of G-quadruplex binders for selective recognition, and topotecan was found to have high binding affinity *in vitro* and could effectively affect the c-Myb transcription activities in cells. The regulation of G-quadruplex with binders in transcriptional, translational levels by Q-RT-PCR and western blot was in expectation of providing a strategy for gene expression modulation. In conclusion, our study revealed a G-quadruplex structure in c-Myb proximal promoter region, which was of great importance in the regulation of c-Myb function.

1. Introduction

Bioinformatics analysis shows that there are about 300,000 G-rich sequences in human genome if searching with the consensus sequence ($G_{3+}N_{1-7}G_{3+}N_{1-7}G_{3+}N_{1-7}G_{3+}$) [1], and their localizations are non-random. These G-rich sequences always localize in functional regions, such as promoters [2,3], untranslated regions [4,5] and telomeres [6] to regulate the gene expression [7,8]. The unusual topologies of G-quadruplex make it a potential target, for example, the chiral metallo-supramolecule and the carvoxylated single-wall carbon nanotubes could selectively recognize human telomeric G-quadruplex and inhibit telomerase activity [9,10]. And expression of a given gene could be modulated by selective G-quadruplex binders. More convincingly, studies show that after the addition of G-quadruplex binder (For instance, TMPyP4), the expression of numerous genes showed differences, especially the genes which were enriched in G-rich sequences [11]. About 42.7% of human promoters have one or more G-rich sequences [2], which could affect the transcription progress in different ways. Their existences may impair the transcription by impending the RNA polymerase, or in other situations, may enhance gene expression through the removal of transcriptional suppressor, the recruitment of G-quadruplexes activators or facilitating the re-initiation of transcription by holding the DNA open [12].

c-Myb proto-oncogene is aberrantly expressed in leukemia and some other solid tumors, and it is an important target for cancer therapy [13,14]. The G-rich sequences in the downstream region of c-Myb transcription start site have been proved to fold into G-quadruplex structures and to regulate c-Myb transcription [15–17]. Recently, the bioinformatics analysis shows that there are other four G-rich sequences [-80∼ −49 (S1), −112∼ −94 (S2), −352∼ −318 (S3) and −809∼ −775 (S4)] [19] in the upstream region of the transcription start site of c-Myb gene. At the upstream close to the transcription start site, the transcription factor myeloid zinc finger 1 (MZF-1, −52∼ −64 and −162∼ −178) [18] was proven to be a suppressor in the transcription of c-Myb gene through the binding of specific sequence, which coincided with S1 sequence (Fig. 1). We have used ESI–MS and CD spectroscopy to probe the formation of G-quadruplexes from the S1, S2, S3 and S4 sequences and their recognition by small-molecules, revealing that the G-rich sequences (S1-S4) could form stable G-quadruplex structures

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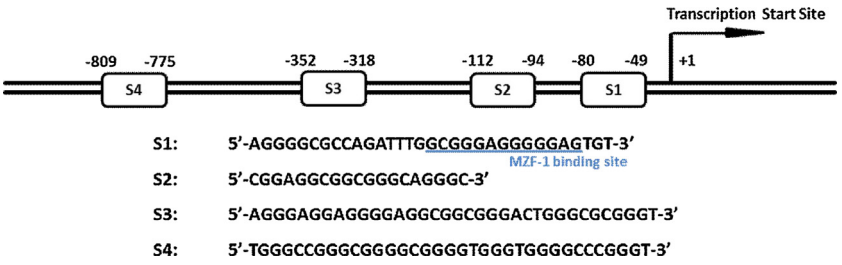

Fig. 1. The G-rich sequences in the upstream region of the transcription start site in c-Myb gene [19]. Transcription initiation site (+1) was indicated with an arrow. The binding site of transcription suppressor MZF-1 was underlined in blue line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Q1-Q4) [19] (Fig. S1), respectively. In this work, Q1 G-quadruplex was proved to function as an enhancer of c-Myb gene. In addition, topotecan was found to bind towards the G-quadruplex with high affinity, and to modulate the function of c-Myb in both mRNA and protein levels, which was based on the stabilization of the G-quadruplex structure. This study provided the possibility to modulate c-Myb transcription by G-quadruplex and topotecan.

2. Materials and methods

2.1. DNA oligonucleotides and ligands

All the guanine-rich oligonucleotides d[5′-$AG_4CGCCAGATTTG_2CG_3AG_5AGTGT$-3′] (S1), d [5′-$CG_2AG_2CG_2CG_3CAG_3C$-3′] (S2), d [$AG_3AG_2AG_4AG_2CG_2CG_3ACTG_3CGCG_3T$] (S3), d[5′-$TG_3CCG_3CG_4CG_4TG_3TG_4CCCG_3T$-3′] (S4) were synthesized by Sangon Biotech Co., Ltd. (Beijing, China) with HPLC purification and were dissolved in ultrapure water (18.3 MΩ·cm) to prepare 100 μM stock solutions. All ligands were purchased from BioBioPha (Yunnan Province, China) and dissolved in ultrapure water (18.3 MΩ ·cm) to prepare 1 mM stock solutions.

2.2. ESI mass spectrometry (ESI–MS)

The ESI–MS experiments were performed by a Finnigan LCQ Deca XP Plus ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA). The instrument was used in the negative ion mode, with the capillary voltage of 3.0 kV (2.7 kV for S2). The capillary temperature was set to 140 °C (120 °C for S2) with a sheath gas flow rate of 25 arb. The samples infusing rate was 2 μL/min. ESI–MS spectra were recorded for 5 min and the averaged spectra were obtained. The 10 μM DNA solutions were prepared by 100 μM DNA stock solutions, 25% CH_3OH (volume ratio, to improve electrospray efficiency) and 50 mM NH_4OAc. The mixture of the G-quadruplex and ligands were generated by adding 1, 2 and 4 equivalents ligands to the 10 μM DNA solutions.

The high-resolution mass spectra were carried out by a Bruker SolariX-XR Fourier transform ion cyclotron resonance mass spectrometer (Bruker, Billerica, MA, USA) with an ESI source. The samples were tested in negative ion mode with a capillary voltage of 3000 V and drying gas temperature of 140 °C. Syringe flow rate was 3.0 μL/min.

2.3. Circular dichroism spectrometry (CD)

CD experiments were performed by a J-815 CD spectrometer (JASCO, Tokyo. Japan) equipped with a Peltier junction temperature controlled cell holder. The scans were carried out in a 0.1 cm pathlength cuvette, and each sample was measured for three times to obtain the averaged spectra from 220 nm to 350 nm. For CD melting experiments, the rising temperature rate was 1 °C/min from 20 to 95 °C. Before the DNA samples were tested, they were annealed at 95 °C for 5 min and then programmed cooling to 4 °C.

2.4. Dual-luciferase reporter assay

The pGL4.10 vector, pRL-TK vector and Dual-Luciferase Reporter Assay Kits were all purchased from Promega Biotech Co., Ltd. (Beijing, China). The construction of the plasmids was completed by TaKaRa Biotechnology Co., Ltd. (Dalian, China). The wild type plasmids (WT) were constructed by inserting a 1500-bp fragment, −1100 to +400 relative to the transcription start site of c-Myb gene, between the Kpn I/Hind III sites of the pGL4.10 vector. The mutation plasmids (M1-1, M1-2, M2, M3 and M4) were constructed in the same method and specific guanines (G) were mutated to thymines (T) to destroy the G-quadruplex structure. A 200-bp fragment (-200 to −1) were inserted to create S1-wt and S1-mut plasmids in the same way.

The cultured MCF7 cells were split into 24-well plates (1×10^5 cells/well) one day before the transfection and cultured at 37 °C overnight to obtain 80% confluent at the time of transfection. 1 μg constructed plasmids were transfected into MCF7 cells via Invitrogen™ Lipofectamine® 2000 (Thermo Fisher Scientific Inc. Beijing, China), together with 20 ng of pRL-TK which served as the internal control. Medium was changed after 4 h and cells were then incubated at 37 °C in a CO_2 incubator for another 18 h prior to testing according to the manufacturer's instruction. To investigate the potency of topotecan on the transcriptional activities of plasmids, topotecan were added to the 24-well plates and the final concentration was 0, 0.1, 1, 5, 10 μM. The luciferase activities were measured using the Dual-Luciferase Reporter Assay Kit and following the manufacturer's instruction.

2.5. Real-time quantitative PCR (RT-qPCR)

The cultured MCF7 cells were split into 6-well plate (1×10^6 cells/well) and incubated at 37 °C overnight. 0–10 μM topotecan was added to each well. The cells were harvested 18 h after the addition of topotecan. Total RNA were extracted with Trizol reagent (Sangon Biotech Co., Ltd., Beijing, China) and converted to complementary DNA using the TransScript II All-in-One First-Strand cDNA Synthesis SuperMix kit for qPCR (Transgen Biotech., Beijing, China). Real-time quantitative PCR (qPCR) was performed in an Eppendorf Realplex Real-time PCR System (Eppendorf, Hamburg, Germany) using the TransStart Tip Green qPCR SuperMix kit (Transgen Biotech., Beijing, China).

The c-Myb primers were 5′-GAGGTGGCATAACCACTTGAA-3′ (forward) and 5′-AGGCAGTAGCTTTGCGATTTC-3′ (reverse), respectively. β-actin was used as a control, and the forward and reverse primers of β-actin were 5′-CATGTACGTTGCTATCCAGGC-3′, 5′-CTCCTTAATGTCACGCACGAT-3′, respectively.