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Effects of Triethylene Tetraamine on the G-quadruplex Structure in the Human c-myc Promoter

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The increasing data show that a parallel G-quadruplex structure formed by the element of nuclease-hypersensitivity element III_1 (NHE III_1) in the P1 promoter of c-myc functions as a transcriptional repressor. Triethylene tetraamine has exhibited interesting properties of stabilizing both inter- and intra-molecular G-quadruplex structures and telomerase-inhibitory potency. Here, we present evidences showing that triethylene tetraamine facilitates the formation of G-quadruplex structure by the NHE III_1 element, and inhibits the expression of c-myc in HeLa cells.

Key words: c-Myc, G-quadruplex, telomerase, triethylene tetraamine.

Abbreviations: TETA, triethylene tetraamine; CD, circular dichroism; NHE, nuclease-hypersensitivity element.

Human c-myc, a 65 kDa nuclear phosphorylated protein, is a central regulator of cellular proliferation and cell growth, and is an attractive target for anti-gene therapy (*1*). The aberrant overexpression of c-myc gene is associated with the progression of many cancers, including colon, breast, small-cell lung, osteosarcomas, glioblastomas, and myeloid leukaemia (*2*). There is, however, a major control element of human c-myc oncogene, located (−115)–(−142) bp upstream of the c-myc promoter P1. This element is termed nuclease-hypersensitivity element III_1 (NHE III_1) and controls 85–90% of the transcription activation of this gene, which has been the subject of considerable research over the past two decades(*3*). The NHE III_1 has unusual strand asymmetry; one strand is an almost perfect homopyrimidine tract with the sequence of 5′-TGGGGA GGGTGGGGAGGGTGGGGAAGG-3′. Using chemical probing, Simonsson *et al*. (*4*) proposed an intramolecular G-quadruplex structure for this G-rich strand. Hurley *et al*. have shown that the 12th G-to-A mutation destabilizes the G-quadruplex-forming unit, which results in a 3-fold increase in basal transcriptional activity. In contrast, agents that stabilize the specific G-quadruplex structure are able to suppress c-myc transcriptional activity (*5*).

So far, some telomerase inhibitors that induce and/or stabilize the G–quadruplex structures of c-myc promoter and inhibit the c-myc expression have been widely characterized *in vivo* and *in vitro* (*6*, *7*). The transcription of c-myc can be inhibited by ligand-mediated G-quadruplex stabilization (*8*, *9*) and transfection of the oligonucleotide encompassing this quadruplex into a Burkett's lymphoma cell line resulted in cell growth decrease (*10*).

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The emerging data show that NHE III_1 element, which can form quadruplex structure by some ligands, is a promising target for the design or screen of drug for antitumour. In previous studies, we reported that triethylene tetraamine (TETA), a small linear molecule, exhibited interesting properties of stabilizing both inter- and intra-molecular G-quadruplex structures and telomerase-inhibitory potency (*11*). Coupled with this inhibition, TETA also inhibited the cell proliferation and increases the proportion of cells in the G_1 phase of the cell cycle in a dose-dependent manner (*12*). Because c-myc is a core regulator of human telomerase reverse transcriptase, to explore the mechanism of TETA inhibiting telomerase activity, we demonstrate here a circular dichroism spectroscopy and polymerase chain reaction (PCR)-stop studies on the stability of G-quadruplex structure of NHE III_1 in the c-myc promoter; the results show that TETA enhances the stability of G-quadruplex formed by both the NHE III_1 and its mutated sequence. Accompanied with this, TETA also down-regulates the expression of c-myc in a dose-dependent manner in HeLa cell line.

MATERIALS AND METHODS

Oligonucleotide—All oligonucleotides were synthesized by a cyanoethyl- phosphoramidite method on an Applied Biosystems 394 DNA synthesizer and purified by anion exchange HPLC, desalted by HPLC in TaKaLa.

Cell Culture—The HeLa cervical adenocarcinoma cell line was obtained from the cell collection of the Shanghai Institute for Cell Research, Chinese Academy of Sciences. Cells were cultured in DMEM medium (Hyclone) supplemented with 10% heat-inactivated calf serum (Hyclone), 100 μg/ml streptomycin and 100 units/ml penicillin. Cells were maintained at 37°C, 100% humidity in an atmosphere of 95% air and 5% CO_2.

Quadruplex Formation—The oligonucleotides NHE III_1 (5′-TGGGGAGGGTGGGGAGGGTGGGGAAG G-3′) and its mutant (5′-TGGGGAGGGTG**A**GGAGGGTGGGGA AGG-3′) were synthesized in TaKaLa (Japan), which was dissolved in double-distilled water, and kept at −20°C. DNA solutions were prepared in a 10 mM Tris–HCl buffer, pH 7.4, with or without KCl or TETA. DNA samples were heated to 85°C for 10 min. Before the DNA cooled slowly to room temperature, TETA was added into the samples to produce a specified concentration at a total volume of 200 μl. The DNA samples were stored at 4°C.

Circular Dichroism (CD) Spectroscopy—CD spectra were recorded on a Jasco J-820 spectropolarimeter (Jasco, Easton, MD). For each sample, at least four spectrum scans were accumulated over the wavelength range of 200–320 nm in a 0.1 cm path length cell at a scanning rate of 20 nm/min. The scan of the buffer alone was subtracted from the three averaged scans for each sample.

PCR-stop Assay—The stabilization of G-quadruplex structures by TETA was investigated by a PCR-stop assay using a test oligonucleotide and a complementary oligonucleotide that partially hybridize to the last G-repeat of the test oligonucleotide. Sequences of the test oligonucleotides (P22 and M22) and the corresponding complementary sequence (RevP22) used here are presented in Fig. 1. Assay reaction were performed in a final volume of 25 μl, in a 10 mM Tris, pH 8.3, buffer with 50 mM KCl, 1.5 mM $Mg(OAc)_2$, 7.5 pmol of each oligonucleotide, 1.5 U Taq polymerase and the indicated amount of TETA. Reaction mixtures were incubated in a thermocycler with the following cycling conditions: 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. Amplified products were resolved on 15% non-denaturing polyacrylamide gels in 1 × TBE and stained with SYBR Green I (Roche). Fluorescence was scanned with the software of quantity one (Bio-Rad). Results represent means ± SE of three independent experiments, except as indicated.

Semi-quantitative reverse Transcription-polymerase Chain Reaction—Total RNA was extracted from HeLa cells after treatment with the specified concentration of TETA using Catrimox-14™ RNA isolation Kit Ver 2.11 (TaKaLa, Japan). The total RNA (2 μg) was reversed transcribed and amplified using a TaKaLa RNA PCR kit Ver 3.0 (Japan). Expressions of c-myc and GAPDH were examined by PCR method. The primers are 5′-AAGTCCTGCGCCTCGCAA-3′(c-*myc* sense), 5′-GCTG TGGCCTCCAGCAGA-3′ (c-*myc* antisense), 5′-CATCACC ATCTTCCAGG AGCG-3′ (GAPDH sense) and 5′-TGAC CTTGCCCACAGCCTTG-3′ (GAPDH antisense), respectively; the elongation was finished at 42°C for 30 min and 94°C for 2 min, and amplification was undertaken for 22 cycles with 30 s at 95°C, 30 s at 58°C, and 60 s at 72°C. The intensities of the genes expressions were semi-quantified using Quantity One (Bio-Rad, America).

Protein Analysis by Western Blotting—After HeLa cells were harvested by two centrifugations at 1000 × *g* for 5 min at 4°C, cell extracts were prepared by homogenization in lysis buffer (50 mM Tris, pH 7.4, 0.1% SDS, 1 mM EDTA, 150 mM NaCl, 1 μg/ml aprotinin and

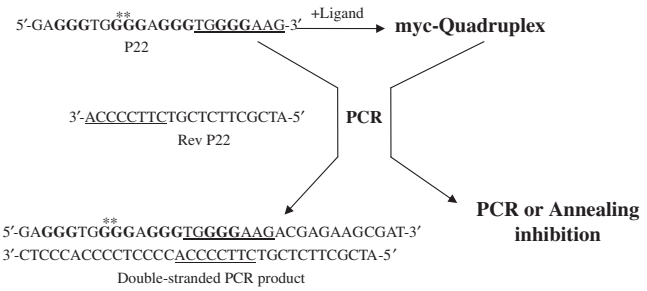

Fig. 1. **Principle of PCR-stop assay.** Test oligomer (P22 or M22) was amplified with a complementary oligomer (RevP22) overlapping the last G-repeat. Taq polymerase extension resulted in the formation of a final 34 base pair double-stranded PCR product. In the presence of a ligand that stabilizes P22 into a G-quadruplex structure, annealing of oligomer and therefore Taq polymerase extension is inhibited. Underlined sequence corresponds to overlap between the two oligomers. Asterisks in P22 corresponded to the mutations (G → A) that replace guanine by adenine in M22.

1 μg/ml PMSF). Protein concentration was determined using the absorbance assay with a UV spectroscopy (Shimadzu, Japan). Ten micrograms of proteins were separated using SDS–PAGE on 8% gel and transferred to PVDF membrane (Millipore, USA). Blots were first blocked in 5% de-fat milk in Tris-buffered saline Tween-20 (TBST, containing 8 g of NaCl, 12.5 g of Tris base, and 0.1% Tween-20) and incubated for 2 h with c-myc antibody at a 1:5000 dilution (mouse monoclonal antibody, Santa Cruz Biotech) at room temperature. Then the blots were washed three times for 15 min with TBST and incubated for 1 h with HRP labelled secondary antibody at a 1:10,000 dilution; after the second washing, proteins were visualized using an enhanced chemiluminescence detection system (ECL advance, Amersham).

Statistical Analysis—All data are presented as mean ± SE. Data were analysed by a one-way ANOVA, followed by Dunnett's test and an unpaired Student's *t*-test. Differences among groups were considered statistically significant at $P < 0.05$.

RESULTS

Effect of TETA on the Stabilization of G-quadruplex Structure formed by NHE III1 Element of c-myc Promoter—To explore the effect of TETA on the stabilization of G-quadruplex formed by the NHE III_1 element in c-myc promoter, CD spectra were recorded on a Jasco J-820 spectropolarimeter (Jasco, Easton, MD). The results are showed in Fig. 2. It is obvious that TETA itself, have only a little effect on the stability of G-quadruplex DNA formed by the sequence of c-myc NHE III_1 promoter. But, in the presence of low concentration of K^+ (1.0 mM), TETA can enhance this progress evidently (Fig. 2A). In this study, we also observe an interesting phenomenon that TETA remarkably facilitates the formation of G-quadruplex structure with the mutated NHE III_1 sequence in the presence of low-dose K^+ (Fig. 2B). With the non-denaturing gel electrophoresis analysis, we also find that the

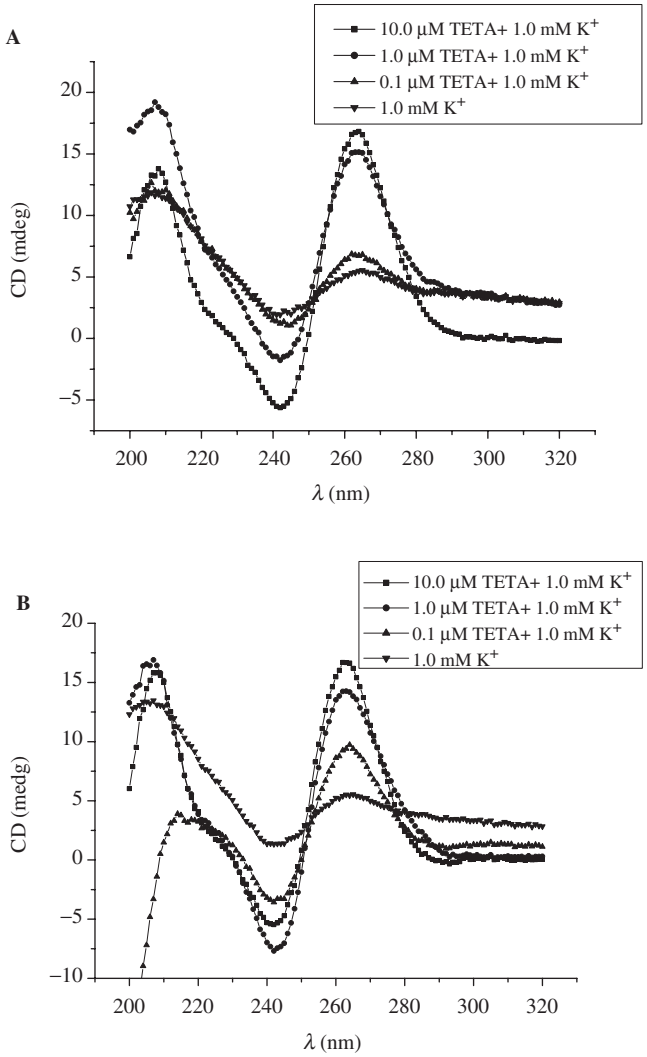

Fig. 2. **Effects of TETA on the stability of G-quadruplex DNA formed with the NHE III_1 sequence in c-myc promoter (A) and its mutated sequence (B).** The specific procedure of this experiment is shown in '*Materials and Methods*'. The NHE III_1 and its mutated sequences are 5′-TGGGGAGGGTGGGGGGGTGGGGAAGG-3′ and 5′-TGGGG AGGGTGAGGAGGGTGGG GAAGG-3′.

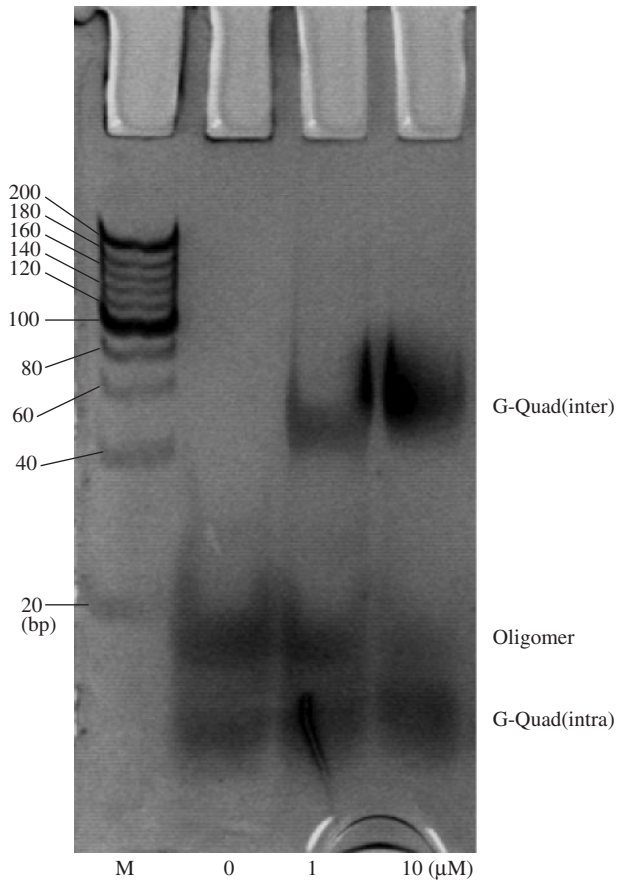

Fig 3. **Determination of the structures of the unimolecular G-quadruplexes formed after incubation of the mutated NHE III_1 sequence (5′-TGGGGAGGGTG AGGAGGGTGGGGAAGG-3′) and indicated concentration of TETA in the presence of 1.0 mM K^+.** After the G-quadruplexes formed under the specified conditions, the samples were collected to non-denaturing gel analysis with 20% polyacrylamide gel, the electrophoresis results was stained with sliver-staining Kit (Promega) to develop the bands in different sites.

G-quadruplex formed by the mutated sequence in the presence of TETA maybe a mixture of intra- and intermolecular structure (Fig. 3).

PCR-Stop Assay on G-quadruplex Oligonucleotide—The role of TETA in the stabilization of G-quadruplex structures from the NHE III_1 element was investigated by a PCR-stop assay using a test oligonucleotide and a complementary oligonucleotide that partially hybridizes to the last G-repeat of the test oligomer. The results showed that in the presence of TETA, the P22 oligomer was stabilized into a G-quadruplex structure that blocked hybridization with a complementary strand overlapping the last G-repeat. In that case, 5′ to 3′ extension with Taq polymerase was inhibited in a dose-dependent manner by TETA and the final double-stranded DNA PCR product was not detected (Fig. 4A). The IC_{50} value which indicates the concentration of TETA required to achieve 50% inhibition of the reaction was about 28 μM. To further demonstrate that inhibition induced by TETA was due to G-quadruplex stabilization of P22 oligomer, a parallel experiment with an oligomer that contains two mutations in one of the guanine repeats (M22, 5′-GAGGGTG**AA**GAGGGTGGGGAAG-3′) was performed. In that case, no significant inhibition was observed at the highest concentration of TETA in this experiment (Fig. 4B).

TETA Causes a Decrease in c-myc Expression—To verify the effects of TETA on c-myc expression, after HeLa cells were treated with TETA at indicated concentration for 72 h, western blot analysis was performed for the c-myc protein level. Treatment with TETA dramatically reduced the expression of the c-myc protein compared with control. Densitometric quantification of the protein bands showed significant reduction

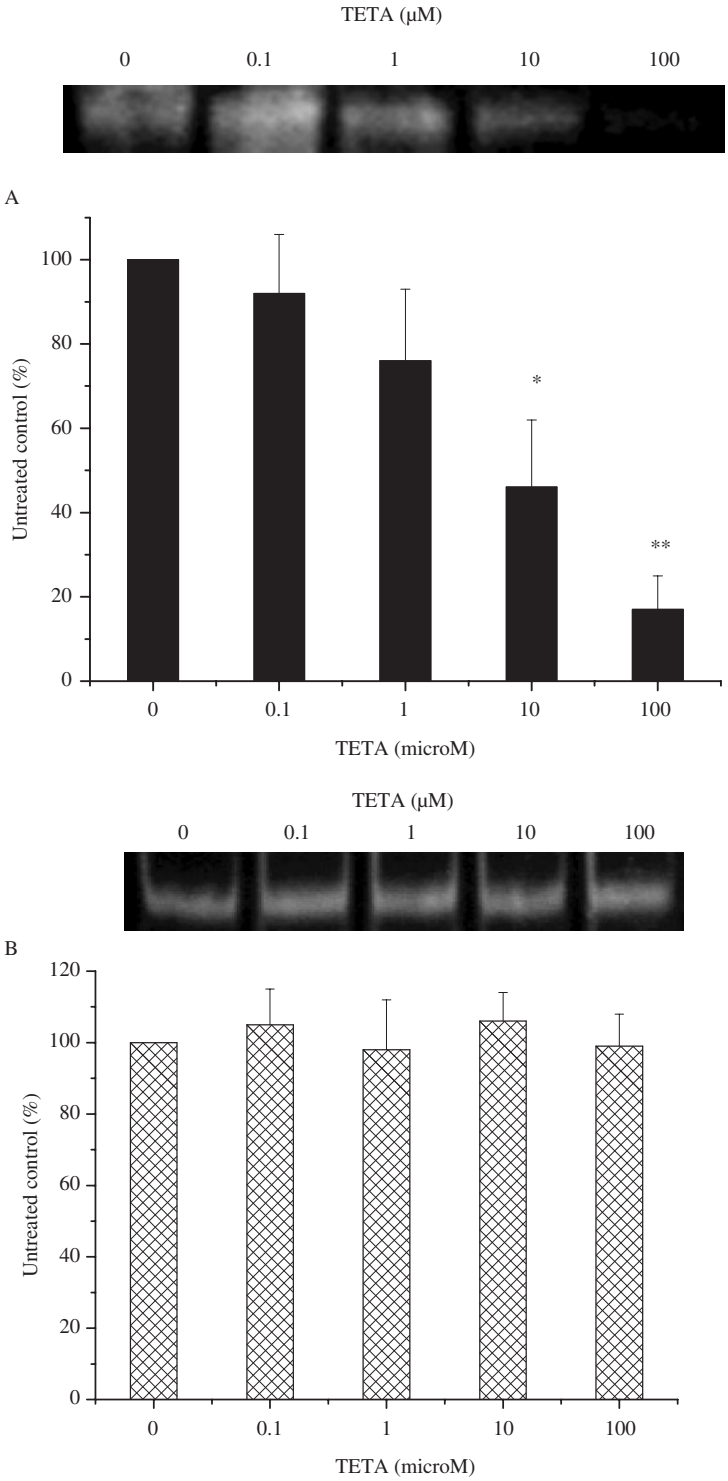

Fig. 4. **PCR-stop assay for the effect of TETA on the G-quadruplex forming Pu22 myc (P22) oligomer (A) or mutated Pu22mu (M22) oligomer (B).** All data are mean ± SE of three independent repeats. $^{*}P<0.05$, and $^{**}P<0.01$ compared with control.

for treatment with over 20 μM TETA relative to control (Fig. 5, $^{**}P<0.01$), which appeared to be dose-dependent.

We also detected the influence of TETA on the transcription of c-myc. After treated with indicated concentration of TETA for 72 h, RT–PCR was conducted to check the mRNA levels of c-myc. The results show that TETA can inhibit the transcription of c-myc in a dose-dependent manner, Fig. 6 shows that there are

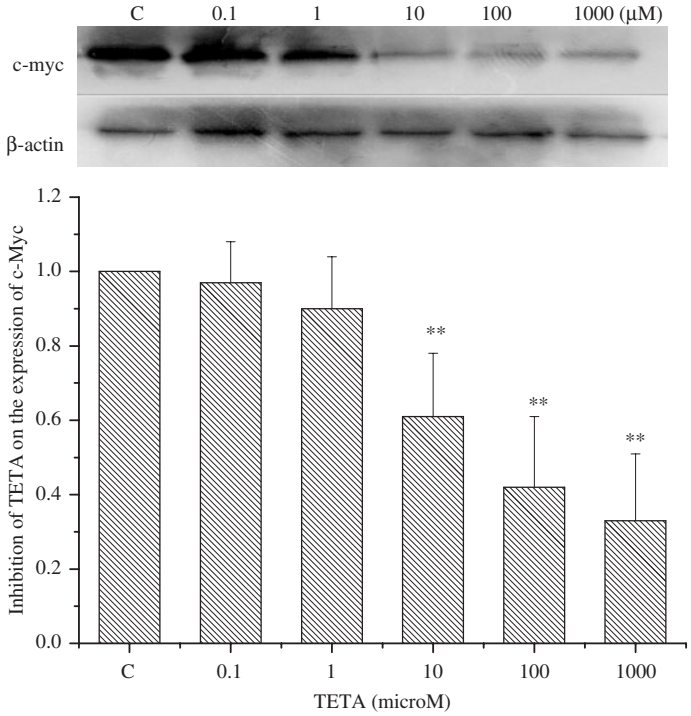

Fig. 5. **TETA decreases the protein level of c-myc in a dose-dependent manner.** After HeLa cells were treated with TETA in an indicated concentration for 72 h, the protein of c-myc was analysed by western blot according the introduction in '*Materials and Methods*'. The data are mean ± SE of three independent repeats. $^{**}P < 0.01$ compared with control.

significant band near the 250 bp site in the presence of low concentration of TETA (the target fragment is 248 bp), but, when the concentration of TETA is over 10 μM, the target band gets faint (Fig. 6).

DISCUSSION

Normally, the human c-myc gene is tightly regulated and alterations in its expression are a key step in the progression of many cancers. The major control element of the human c-myc oncogene, locates (−115)–(−142) bp upstream of the c-myc promoter P1 (*13*). It is reported that the purine-rich strand of the NHE III_1 in the c-myc promoter P1 in the form of G-quadruplex is a silencer element, which accounts for over 85% of total c-myc transcription (*7*), so, compounds that enhance the stabilization of G-quadruplex formed by NHE III_1 sequence serve to inhibit c-myc gene expression (*14*).The increasing compounds, which can increase the stabilization of G-quadruplex structure formed by the NHE III_1 sequence, have been reported (*15*).

On the basis of new molecular insights gained through the experiments described in this article, it is evident that TETA is a novel enhancer of G-quadruplex structure of NHE III_1 in human c-myc promoter. Although TETA itself have very little influence on the formation of G-quadruplex with the NHE III_1 sequence, it can enhance the stabilization of G-quadruplex structure formed by this sequence in the presence of low concentration of potassium ion. Furthermore, the results

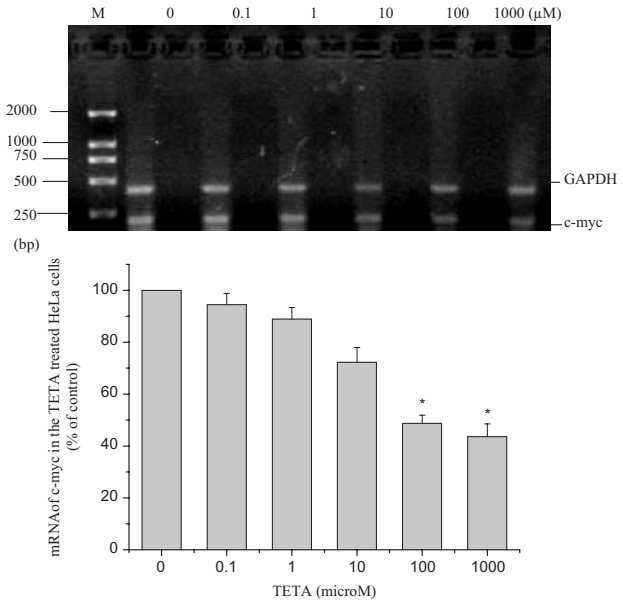

Fig. 6. **Effect of TETA on the c-myc mRNA synthesis in HeLa cells.** RT–PCR was collected to identify the change of c-myc mRNA (218 bp) in HeLa cells after treatment with indicated concentration of TETA, and GAPDH (443 bp) was taken as internal reference. The primers for c-myc are 5′-AAGTCCTGCGCCTCGCAA-3′ (c-myc sense), 5′-GCTGTGGC CTCCAGCAGA-3′ (c-myc antisense), and the primers for GAPDH are 5′-CATCACCATCTTCCAGGAGCG-3′ (sense) and 5′-TGACCTTGCCCACAGCCT TG-3′ (antisense), separately. The results represent at least three independent experiments and are expressed as mean ± SE.

obtained from the PCR-stop test show that TETA have almost no effect on the mutation of NHE III_1 element (5′-GAGGGTG**AA**GAGGGTGGGGAAG-3′), suggesting to us that the unique effects of TETA on c-myc expression may be mediated through an interaction with such a secondary structure.

It is reported that the 12th G-to-A mutation could disrupt sequence-specific repressor binding interactions and likewise result in transcriptional activation in the NHE III_1 of the c-myc promoter, which exists in some human colorectal tumour (a 1 in 196, if this were random) (*16*). In the present work, we also observe that TETA can significantly facilitate the formation of G-quadruplex formed by the mutated sequence of NHE III_1, so the ability to reinstate the G-quadruplex silencer element with TETA, and thereby lower c-myc expression, establishes the principle of drug intervention to reverse the consequences of this late mutational event.

To better understand the mechanistic basis of the observed effects of TETA on the G-quadruplex of c-myc promoter, we have examined change in c-myc expression after treatment with TETA in HeLa cells. The results confirm that TETA decreases c-myc expression at the RNA and protein levels in HeLa cells. Of considerable interest to us now is the mechanism by which TETA mediates its effects on c-*myc* expression and telomerase activity and whether this is related to the binding of TETA to G-quadruplex structures in the NHE III_1. The inhibition of c-myc expression *in vitro* by TETA accounts for at least part of the reduction of telomerase activity by this molecule, and TETA, as a G-quadruplex enhancer, might also potentially be used to concomitantly inhibit c-myc gene expression in tumour cells.

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