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Stabilization of G-quadruplexes in Intronic Hematopoietic-specific Enhancer of *WT1* gene with G-quadruplex targeting ligands: *in silico* and *in vitro* techniques

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Wilms' tumor 1 (*WT1*) gene was identified as a tumor-suppressor gene in childhood renal neoplasm, Wilms' tumor, and has an important impact on cell growth and differentiation. So, mutations in *WT1* and its over-expression related to not only Wilms' tumor but also other tumors and leukemias. *WT1's* intronic hematopoietic-specific enhancer is GC-rich and could form G-quadruplex structures. Herein, we study the effect of three G-quadruplex stabilizing ligands on the formation and stability of G-quadruplex structures in *WT1* intronic hematopoietic-specific enhancer. We designed a truncated sequence of *WT1* intronic enhancer (named *WT1-I33*) with the highest likelihood score of G-quadruplex formation and study the effect of different concentrations of TMPyP4, daunorubicin, and mitoxantrone on G-quadruplex formation in *WT1-I33* oligonucleotide using *in silico* and cell-free assays. The results revealed that *WT1-I33* has the potential to form G-quadruplex structures and these structures in *WT1* hematopoietic-specific enhancer could be stabilized by the ligands. According to the role of enhancers in transcription, stabilizing of G-quadruplex structures could make the enhancer inaccessible for the transcription factors and transcription of the *WT1* gene might be downregulated. These data could help emerge novel gene-specific therapeutic strategies and selective targeting of G-quadruplex structures.

Keywords: Daunorubicin, G-quadruplex, Mitoxantrone, TMPyP4, *WT1* intronic hematopoietic-specific enhancer

The occurrence of cancers is tightly associated with mutations or attenuated expression of tumor suppressor genes and oncogenes. One of these cancer genes is the Wilms' tumor 1 (*WT1*) gene which is responsible for the most prevalent children's solid tumor, known as a pediatric nephroblastoma or Wilms' tumor[1]. Mutation or deletion of both alleles of *WT1,* as a tumor suppressor gene, have been reported in Wilms' tumor and other solid tumors[2-4]. Additional reports specified that *WT1* overexpression happens commonly in acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL) chronic myelogenous leukemia (CML), and likewise myelodysplastic syndrome (MDS) [5].*WT1* expression is tissue-specific and reported in the mesothelium, kidney, uterus, gonad, and the progenitor cells in different types of tissues[5-8] which revealed that besides *WT1* GC-rich promoter, additional regulatory elements are required for *WT1* tissue-restricted gene expression pattern. One of these regulatory elements in the *WT1* gene is an intronic hematopoietic-specific enhancer in the third intron of the gene and it increased the basal transcription level of the *WT1* promoter in the erythroleukemia cell line K562[9]. Enhancers are auxiliary regulatory elements thatemploy large protein complexes including transcription factors and cofactors to get precise control of the expression of target genes. They enhance the proficiency of RNA polymerase II to begin and retain transcription[10]. Consequently, even though promoters are necessary to attain a basal expression level in target genes, by utilization of the enhancers, 100-fold increases in transcription level could be gained[10].

One of the important features of these regulatory elements is that they are guanine-rich regions with the ability to form G-quadruplex structures. G-quadruplexes are unusual and stable secondary structures in DNA or RNA which formed by the pairing of four guanine bases[11,12]. Lots of studies reported *in vitro* G-quadruplex formation using synthetic oligonucleotides which are derived from the guanine-rich regions in the human genome, especially from oncogenes[13]. Moreover, the G-quadruplexes stability could be increased by some ligands with

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special structures which these stable structures could interrupt the fundamental cellular pathways and consequently contribute to cellular toxicity. Therefore, these findings emphasize the therapeutic potential of G-quadruplexes structure and ligands[14,15]. Consequently, by the stabilization of G-quadruplexes in the regulatory regions of oncogenes, they are considered as developing new targets in oncology and regarded as novel anticancer therapeutic agents[16].

Previously we reported three G-quadruplex stabilizing ligands, TMPyP4 as the strongest one and daunorubicin and mitoxantrone as anti-leukemia drugs, with special structures and the ability to stabilize G-quadruplex structures in *WT1* gene promoter[17,18]. Herein, we study if these ligands could have a significant influence on the conformation and stability of intramolecular G-quadruplex structures in *WT1* intronic hematopoietic-specific enhancer in a cell-free system.

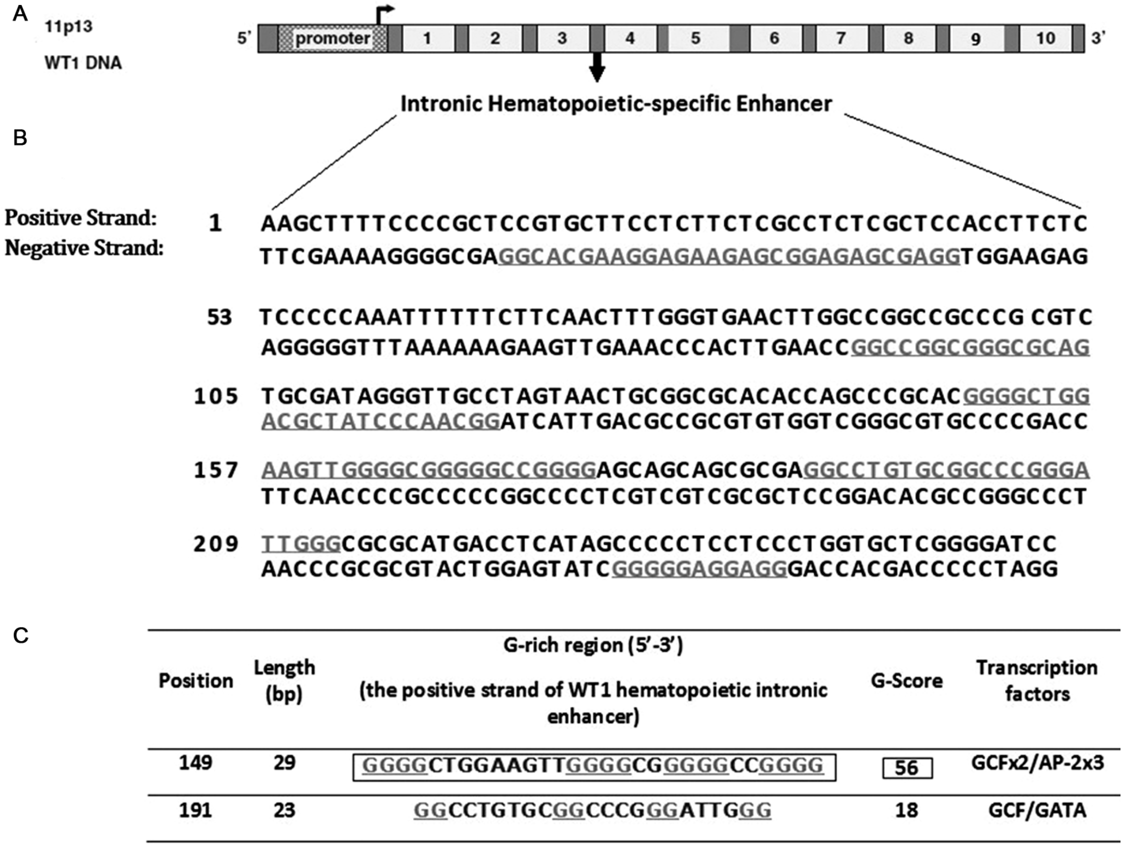
Materials and Methods

Bioinformatics

The G-quadruplex forming sequences are considered as four distinct sub-motifs, including 2–5 ‘‘Gs’’ split by 1–7 other nucleotides (N) termed loop length: $G_{2–5}N_{1–7}G_{2–5}N_{1–7}G_{2–5}N_{1–7}G_{2–5}$. Two web servers: QGRS Mapper (http://bioinformatics.ramapo.edu/QGRS/)[19] and Quadfinder (http://miracle.igib.res.in/ quadfinder/)[20] were used to analyze the G-quadruplex forming sequences in 258 bp *WT1* intronic hematopoietic-specific enhancer region. These web servers have not checked the antisense strand of the enhancer sequence; thus, extra analysis of the reverse complement sequence was required. We have reported the non-overlapping motifs.

Oligonucleotides and drugs

We designed a truncated sequence of *WT1* intronic enhancer (named *WT1-I33*) from the guanine-rich region with the highest probability of forming G-quadruplexes (G-score) (Fig. 1). The oligonucleotide sequences are



Position	Length (bp)	G-rich region (5′-3′) (the positive strand of WT1 hematopoietic intronic enhancer)	G-Score	Transcription factors
149	29	GGGGCTGGAAGTTGGGGCGGGGGCCGGGG	56	GCFx2/AP-2x3
191	23	GGCCTGTGCGGCCCGGGATTGGG	18	GCF/GATA

Fig. 1 — DNA sequences with the potential of forming G-quadruplex structures in the *WT1* intronic hematopoietic-specific enhancer. (A) Schematic diagram of the *WT1* gene on 11p13 and its intronic hematopoietic-specific enhancer; (B) The DNA sequence of negative and positive strand of *WT1* intronic hematopoietic-specific enhancer region; and (C) Analysis results of the *WT1* intronic hematopoietic-specific enhancer using a G-quadruplex algorithm. G-tracts with the potential of forming stacked G-quartets are underlined. These G-tracts act as binding sites for transcription factors

Table 1 — The oligomers and primers used in this study

Name	Sequences 5′ to 3′	Length
WT1-I33	ACGGGGCTGGAAGTTGGGGCGGGGGCCGGGGAG	33
WT1-I33-rev	ATCGATCGCTTCTCGTCTCCCCGGC	25
WT1-I33-com	CTCCCCGGCCCCCGCCCCAACTTCCAGCCCCGT	33
WT1-I33m	ACGAGGCTGGAAGTTGAGGCAAGAACCAAGGAG	33
WT1-I33m-rev	ATCGATCGCTTCTCGTCTCCTTGGT	25
MYC-P31	GGGAGGGTGGGGAGGGTGGGGAAGGGTGGGG	31

The mutation sites are shown in bold and the complementary sequences are underlined.

provided in (Table 1) and were purchased from Bioneer Co. (Korea). *WT1-I33-com* is a complementary sequence of *WT1-I33* and is used to make a dsDNA. *MYC-P31*was used as a positive control which was a G-quadruplex structure-forming sequence in the human *MYC* promoter[21]. The HPLC-purified oligonucleotides were analyzed by denaturing polyacrylamide gel electrophoresis (PAGE), and the concentrations were tested at 260 nm using a spectrophotometer(NanoDrop). TMPyP4 (5,10,15,20-tetra-(N-methyl-4-pyridyl) porphine), was purchased from Porphyrins Systems, Lubeck Co., Germany, and daunorubicin and mitoxantrone drrugs were purchased from Sigma-Aldrich (Sigma Chemical Co, USA. Ltd). All the ligands were dissolved in dimethyl sulfoxide (DMSO) at the concentration of 10 mM as the stock solution and conserved at –20°C. Both two drugs are used in the management of AML and have acceptable safety profiles. The final solutions have been prepared by dissolving specified amounts of the ligands into the appropriate oligonucleotides. All the assays with ligands were done under minimum exposure to light.

Electrochemical assay

Differential pulse voltammetry (DPV) assay were accomplished by an AutolabPGstut electrochemical analysis system controlled by GPES 4.9 software (Eco Chemie,Netherlands). A conventional two-electrode system with a modified carbon paste electrode (CPE) as the working electrode and an Ag/AgCl/KCl (3 M) as a reference electrode was used. DPV measurements were recorded up to the exhibition of a steady-state baseline voltammogram. Technical accuracy of the assay was established by very reproducible experimental results.

CD spectra measurement

We used *WT1-I33* as *w*ild-types and *WT1-I33m* asmutant-type oligonucleotides with 5 guanines to Adenine substitutions for circular dichroism (CD) measurements. Before use, all the oligonucleotides were diluted to 15 µM in TK buffer (50 mM Tris–HCl, 100 mM KCl, pH 7.5). All of the oligonucleotides were heat-treated as follows: they denatured at 95°C for 3 min and later cooled down to room temperature (RT) for 30 min. Subsequently, the oligonucleotides were diluted to 10 µM in the presence or absence of each G-quadruplex ligand and then incubated for 10 min before measurement. The measurement of CD spectra was performed in the presence of different concentrations of the ligands, with a J-1500 CD Spectrometer (JASCO, Tokyo, Japan) at 220-320 nm *via* a 1 mm path-length cuvette. The correction of each spectrum baseline was performed for signal contributions by the buffer with and without each ligand.

Electrophoretic mobility shifts assay (EMSA)

The wild type and mutant oligonucleotides which diluted to 10 µM in Tris–HCl buffer (10 mM, pH = 7.4) in the presence of 100 mM KCl, annealed by heating to 95ºC for 5 min, then were cooled down slowly to RT. Then different concentrations (0.1–10 µM) of each ligand were added to the oligonucleotide solutions and then were incubated overnight at 4°C. The products were checked on native 15% polyacrylamide gel at 70 V for 2 h at 0°C. The silver-stained gel was pictured as described before[22].

PCR-stop assay

The PCR-stop assay was done on wild wild-type oligonucleotide (*WT1-I33*) and a one-end complementary oligonucleotide (*WT1-I33-rev*) by a modified protocol which we have described previously[17]. Additionally, mutated *WT1-I33m* oligonucleotide, and it's complementary, *WT1-I33m-rev* were examined to study the effect of the ligands on non-G-quadruplex forming DNA. The amplified products were resolved on 1.3% agarose gel and visualized by GelRed stain.

Results

The WT1 intronic hematopoietic-specific enhancer contains a G-rich region that is capable of forming G-quadruplex structures

We analyzed the 258-bp intronic hematopoietic-specific enhancer of the *WT1* gene in the region between the 3′ end of exon 2 and the 5′ end of intron

3 and identified a high G/C content in this region (65.9%) with 6 G≥3-tracts, signifying its potential for G-quadruplex structures formation. (Fig. 1). G-quadruplexes might be set up in both strands of double-stranded DNA (dsDNA) once fitting into the conserved motifs and the G content in the negative strand of the intronic enhancer is 65.8% and could be relevant for the formation of these structures (Fig. 1B), So we analyzed the sequence of both strands of intronic hematopoietic-specific enhancer region by a website-based algorithm (http://bioinformatics.ramapo.edu/QGRS/analyze.php)[19]and Quadfinder (http://miracle.igib.res.in/quadfinder/)[20]. We identified more fragments that can potentially form G-quadruplexes (Fig. 1B) but only one of them (*WT1-I33)* showed a higher G-score (Fig. 1C), than of a region in the MYC promoter (*MYC-P31*) which was known as a positive control (G-score:42) and previously reported to form G-quadruplex structures[21]. *WT1-I33* was used to evaluate whether the G-tracts of the intronic enhancer could form G-quadruplex. Another oligonucleotide was a control mutant oligonucleotide, *WT1-I33m*, in which all 3 G-tracts in *WT1-I33 were* disrupted by G-to-A substitutions to reduce the possibility of canonical G-quadruplex formation in this region (Table 1).

Voltammetric and Circular dichroism studies indicated G-quadruplex formation by the G-tracts of the WT1 intronic hematopoietic-specific enhancer

Voltametric behavior of the oligonucleotides was analyzed, in the absence and the presence of different concentrations of TMPyP4 as the strongest G-quadruplex ligand and two AML drugs. Figure 2 showed that in the absence of different concentrations of the ligands, one main anodic peak specific for guanine at Epa = +0.97 V was detected as a result of the oxidation of guanine residues. In the presence of the stabilizing ligands, the G oxidation peak shifted to the right due to the oxidation of guanine residues in a single-stranded oligonucleotide, and the G-quartets peak occurred, at Epa = + 1.05 V. (Fig. 2). The shift of G oxidation peak might be detected because of formation and stabilization of the G-quadruplex structures and other higher-order nanostructures[23]. In the presence of higher concentrations of the ligands, in a dose-dependent manner, the G oxidation peak decreased and the G-quartets oxidation peak shifted to more positive potentials (Fig. 2). These findings proposed a greater stability for the G-quadruplexes and it occurred as a result of a decrease in the concentration of free guanine residues which are available for oxidation and it might result in the formation of G-quadruplex structures in the *WT1-I33*in presence of different concentrations of the ligands.

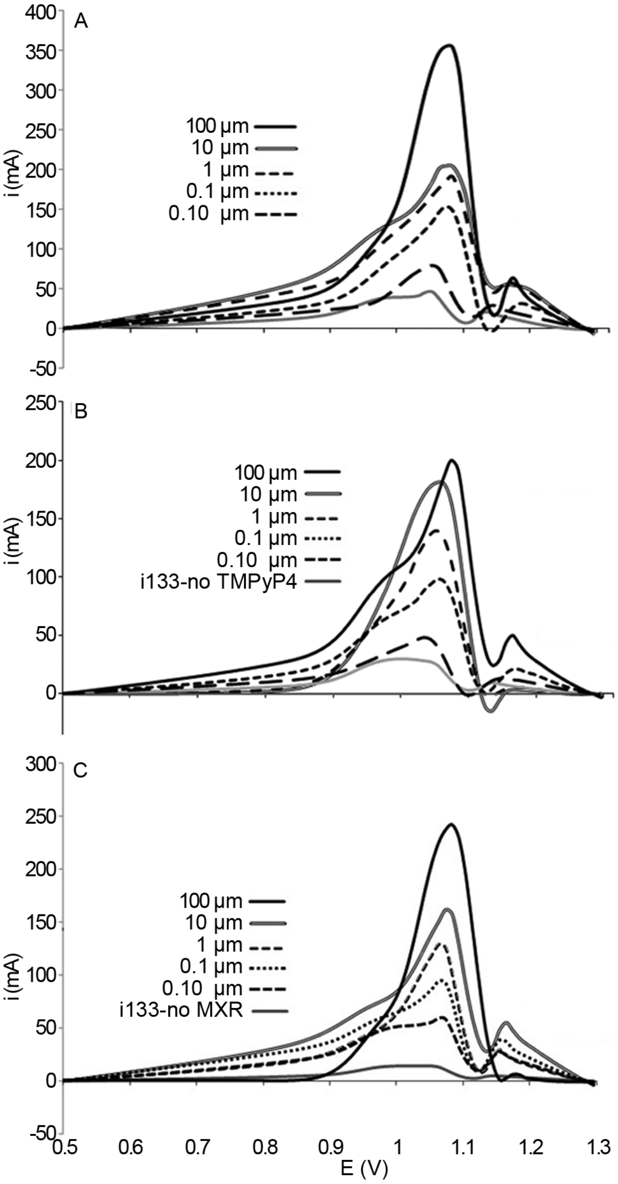


Fig. 2 — Volumetric behavior of *WT1* intronic hematopoietic-specific enhancer-derived oligonucleotide. DP voltammograms baseline corrected in 1 Mm*WT1-I33*, in 0.1 M Tris-Cl, pH = 7.0, in the presence of 100mM KCl and different concentrations of, (A) daunorubicin; (B) mitoxantrone; and (C) TMPyp4

To evaluate the conformational changes in *WT1-I33* and G-quadruplex formation, circular dichroism (CD) spectra were assessed for these oligonucleotides in the presence of 50 mM KCl and different concentrations of the ligands at 25°C.

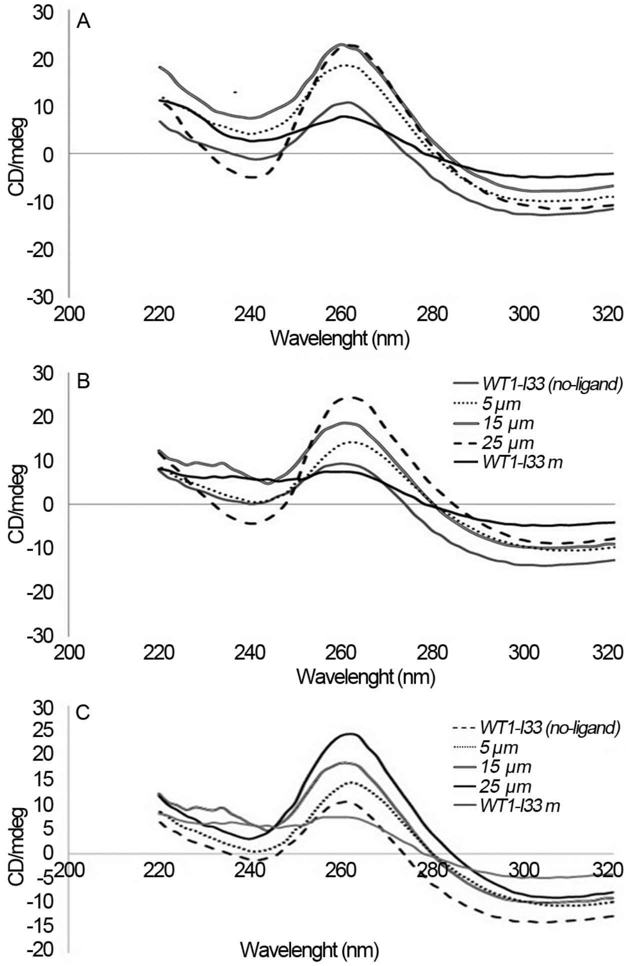


Fig. 3 — Circular dichroism spectroscopy analyses of *WT1-I33* and *WT1-I33m* oligonucleotides. Circular dichroism analyses at 25 °C to determine the G-quadruplex formation of *WT1* intronic hematopoietic-specific enhancer-derived oligonucleotides. Circular dichroism analyses in the presence of different concentrations of (A) daunorubicin, (B) mitoxantrone, and (C) TMPyP4

WT1-I33 showed both positive and negative molar ellipticity peaks at 262 nm and 240 nm respectively, which were known as a signature for the establishment of parallel G-quadruplexes (Fig. 3). Interestingly, the *WT1-I33m*, showed very different CD spectra, suggesting guanine mutations in *WT1-I33m* were sufficient to abolish the G-quadruplex forming potential of the oligonucleotide. In all CD experiments, the *MYC-P31* oligonucleotide exhibited characteristic molar ellipticity peaks at 262 nm and 240 nm, signifying the formation of parallel G-quadruplex structures. For *WT1-I33*, the presence of a higher concentration of the ligands led to stronger molar ellipticity at 262 nm, proposing that a higher concentration of the stabilizing ligands was favorable to G-quadruplex formation. The *WT1-I33m* showed a broader molar ellipticity peak around 268 nm and its molar ellipticity at 262 nm was not comparable to the *WT1-I33* CD spectra which showed the partial formation of G-quadruplexes (Fig. 3).

WT1-I33 oligonucleotide formed G-quadruplexes in native polyacrylamide gels

In the next step, the ability of *WT1-I33* oligonucleotide for G-quadruplex formation was assessed by EMSA. We analyzed the annealed *WT1-I33* and *WT1-I33m* *via* 12% native polyacrylamide gel electrophoresis (PAGE), followed by silver staining to visualize the DNA bands. It was revealed that G-quadruplex-forming oligonucleotides exhibited slower migration than ssDNA[24]. According to the EMSA results, effective oligonucleotide folding and slow migration in a dose-dependent manner were detected in the presence of higher concentrations of the ligands. Therefore, we proposed that *WT1-I33* oligonucleotide could form intramolecular G-quadruplex (intra-G4) structures in the presence of KCl and different concentrations of the ligands, and, *WT1-I33m* did not display a striking DNA band in that region (Fig. 4A1-A3).

The ligands block the replication machinery at the G-quadruplex forming motifs

Afterward, we planned to examine if the ligands binding to the potential G-quadruplex motif prevents DNA replication or not. To undertake this, we applied a PCR stop assay, in which a G-region overlapping primer was utilized for PCR amplification. In the absence of the ligands, a double-stranded product formed because of the primer annealing and extension[17]. The presence of ligands leads to the stabilization of G-quadruplex in the template oligonucleotides resulting in the unavailability of the template for primer annealing, and thus PCR got inhibited (fully inhibited at higher concentration and partially inhibited at a lower concentration). As shown in Figure 4B1-B3, for all the ligands, increasing concentrations of the ligands have a strong impact on PCR amplification. On the contrary, when *WT1-I33m* was used as a DNA template, the ligands did not inhibit DNA synthesis leading to full product generation.

Discussion

Recently, lots of reports have focused on G-quadruplexes epigenetic features and their effect on chromatin structure and function along with interactive

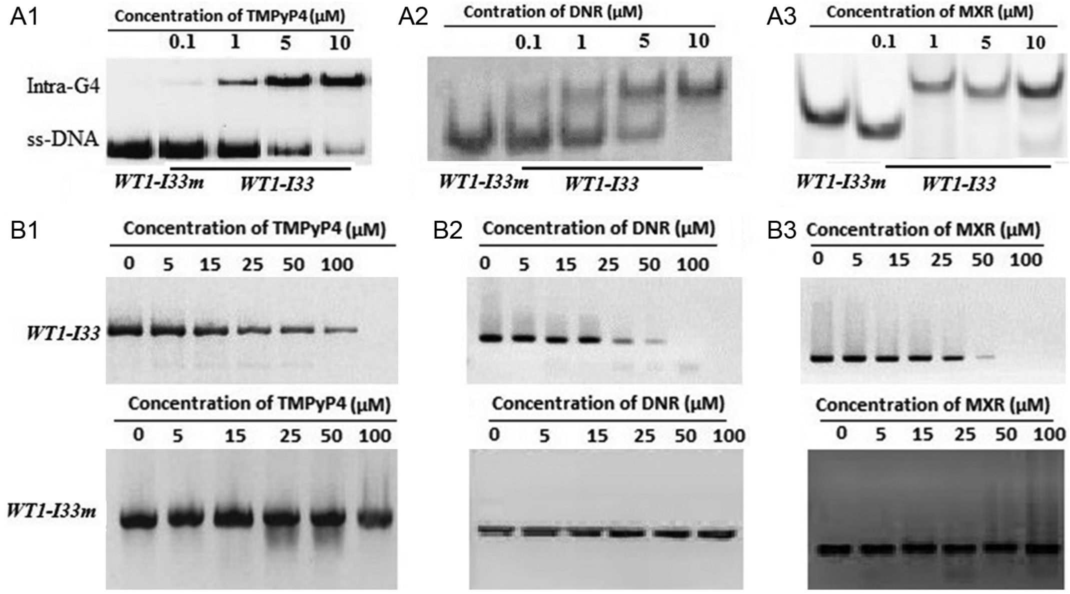


Fig. 4 — Detection of G-quadruplex formation in the sequence of the *WT1* intronic hematopoietic-specific enhancer-derived oligonucleotides by (A) Native PAGE analyses; and (B) PCR-stop assay. The migration of wild-type oligonucleotides annealed in different concentrations of, (A1) TMPyP4, (A2) danunorubicin, and (A3) mitoxantrone and mutant oligonucleotides as labeled were analyzed. The positions of ssDNA and the regions of oligonucleotides with G-quadruplex structures were marked on the left. Dose-dependent effect of different concentrations of, (B1) TMPyP4, (B2) daunorubicin, and (B3) mitoxantrone on the formation of the PCR product were checked in wild-type oligonucleotides and mutant one

proteins[25]. G-quadruplex formation in promoter regions and their special impact on gene expression regulation[17,26-28]. But distal cis-regulatory elements including enhancers and silencers are important in transcriptional regulation and the probable effect of G-quadruplex formation in these regions should be investigated more[29]. Both the promoters and these regulatory regions showed a considerably higher incidence of G-quadruplex forming motifs. One of these motifs was reported in *cis-regulatory* elements of MECP2 mRNA, a gene associated with autism which proposed that it could have a key role in regulating of post-transcriptional expression of the *MECP2* gene[30]. In other studies, functions of the G-quadruplex forming motifs of the *Ccnb1ip1* enhancer were investigated. They specified that the enhancer activity increased by the deletion that occurred in G-quadruplex forming regions revealed that these motifs negatively regulate the *Ccnb1ip1* enhancer[31]. In addition, seven species of human pathogenic bacteria are reported to have G-quadruplex forming motifs in their *cis*-regulatory regions. The occurrence of these G-quadruplex motifs in the *cis*-regulatory regions were significantly higher in comparison to their intragenic regions[32,33]. It proposed that G-quadruplexes had an impact on the cellular physiology of human pathogenesis. In this report, we present the *in silico* and experimental evidence, that the *WT1* intronic hematopoietic-specific enhancer-derived oligonucleotide could form G-quadruplex structures. Voltametric behavior, CD spectra, EMSA, and PCR-stop assay were assessed in the presence of different concentrations of the G-quadruplex stabilizing ligands. Our data revealed that *WT1* intronic hematopoietic-specific enhancer could form G-quadruplex structures and it might function as a negative regulator of the *WT1* gene expression. In this study, the *WT1* mRNA expression was not studied because, in our previous report, a down-regulation in the *WT1* gene expression was detected after treatment of the K562 cells with TMPyP4, daunorubicin, and mitoxantrone[17,18]. This negative regulation might happen due to the stabilization of G-quadruplex structures even in the *WT1* promoter or its intronic enhancer.

The tissue-specific manner of *WT1* expression revealed that not only the promoter but also a 258-base pair intronic hematopoietic-specific enhancer in *the WT1* gene are essential for its tissue-specific expression and 8–10-fold increase in transcriptional level of the *WT1* in K562 cell line was reported as a result of intronic enhancer function[9]. Enhancers as cis-regulatory elements, recruit several transcription factors to regulate the gene expression. To identify transcription factors that bind to the *WT1* intronic hematopoietic-specific enhancer, we refer to the enhancer transcription factor binding profile which was mentioned in Zhang *et al*., report[9]. Three types of transcription factor binding sites, were predicted in the two regions with the potential to form a G-quadruplex structure at positions 149 and 191 (Fig. 1C). These data raise the possibility that these transcription factors, could be required for activation of the *WT1* intronic enhancer. They could make a protein bridge to link several sequence-specific transcription factors to the basic machinery of transcription; or they might contribute as a protein scaffold to form a multicomponent complex for transcription-stimulating activity. One of these proteins is GATA-1 whose binding site was discovered in the *WT1* promoter and its significant impact on the activation of the promoter was reported earlier[9]. GATA-binding sites have also been reported in the *WT1* intronic enhancer and its interaction with GATA-binding proteins could establish a direct contact between the *WT1* promoter and the enhancer. So, GATA-1 and other proteins such as GCF might interact with other transcription factors in the *WT1* promoter and facilitate the transcription of *WT1* gene[9]. According to our previous reports, the transcription level of the *WT1* gene is down-regulated in the presence of the stabilizing ligands. As well as the formation of G-quadruplex structures, the recognition sites of these transcription factors in the promoter or intronic enhancer of *WT1* might be blocked and the *WT1* transcription level was decreased[17,18]. This could happen as a result of the inhibition of RNA polymerase movement by G-quadruplex structures or the recruitment of G-quadruplex-binding proteins which might inhibit the transcription machinery. Therefore, G-quadruplex stabilization of *WT1* intronic enhancer by TMPyP4 or AML drugs would likely interfere with GATA-1 and other transcription factors recruitments and down-regulate the expression of *WT1* gene.

Conclusion

Based on the previous knowledge, as well as the findings of this study, we propose that the *WT1* intronic enhancers have the potential to form G-quadruplexes, which these structures showed more stability in the presence of some stabilizing ligands and could down-regulate transcription of the *WT1* gene might be decreased. These data develop the realization respecting the function of enhancer G-quadruplexes and could help emerge novel gene-specific therapeutic strategies using particular drugs that target G-quadruplex structures.

Conflict of interest

All authors declare no conflict of interest.

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