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Folate modulates guanine-quadruplex frequency and DNA damage in Werner syndrome

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ABSTRACT

Guanine-quadruplexes (G4) are stable tetra-stranded DNA structures that may cause DNA replication stress and inhibit gene expression. Defects in unwinding these structures by DNA helicases may result in telomere shortening and DNA damage. Furthermore, due to mutations in WRN helicase genes in Werner syndrome, G4 motifs are likely to be key elements in the expression of premature aging phenotypes. The methylation of DNA plays a significant role in the stability and occurrence of G4. Thus, G4 frequency and DNA methylation mechanisms may be affected by excesses or deficiencies in methyl donors such as folate. B-Lymphocytes from Werner patients (n = 5) and healthy individuals (n = 5) were cultured in RPMI medium under condition of folate deficiency (20 nM) or sufficiency (200 nM) for 14 days. Cells were fixed on microscope slides for immunofluorescent staining to measure G4 frequency and γH2AX (a marker of DNA strand breaks) intensity, using automated quantitative imaging fluorescent microscopy. There was a significant increase ($p < 0.05$) in G4 levels in Werner syndrome patients compared to healthy controls. Werner and control cells grown in 20 nM folate media also showed significant increases in G4 ($p < 0.001$) and γH2AX ($p < 0.01$) signals compared with the same cells grown in 200 nM folate. Control cells grown in 20 nM folate also showed a significant reduction in DNA methylation levels ($P < 0.05$). The results of this study suggest that the occurrence of DNA G4 structures can be modulated *in vitro* via nutrients with important roles in methylation.

1. Introduction

In addition to the Watson-Crick double-helix structure, DNA can adopt numerous alternative conformations [1]. For instance, within guanine-rich sequences in the genome, four guanines can fold into a quartet (tetrad), a square-planar arrangement stabilized by Hoogsteen hydrogen bonding. To form a guanine-quadruplex (G4), at least two of those quartets must stack together [2]. Unresolved G4 motifs can block replication, trigger telomere dysfunction and extensive telomere loss, and induce DNA strand breaks and chromosomal instability [3]. One of the primary cellular responses to DNA double-strand breaks is the alteration of the core histone protein H2AX into its phosphorylated form, γH2AX [4]. These possible threats to genome stability reinforce the necessity of unravelling G4 structures immediately before/during DNA replication, transcription, and DNA repair. For instance, it has been shown that observed defects in some premature aging disorders, such as Werner syndrome, are caused by deleterious mutations in genes encoding DNA helicases, such as WRN [5]. Werner syndrome is an autosomal recessive premature aging disorder characterized by acceleration of normal aging phenotypes and occurs as a consequence of mutation in the WRN gene [6,7]. Previous studies have verified the ability of WRN helicase to bind and resolve G4 motifs, thus indicating the possibility of G4 involvement in the phenotype of aging and premature aging disorders such as Werner syndrome [5,8,9]. DNA methylation at various locations within a G4 forming sequence can have a stabilising effect on G4 structures. DNA methylation in cells depends on the presence of methyl donor nutrients such as folate [3,10]. Furthermore, folate deficiency as well as inhibition of DNA-methyltransferase could modulate the frequency of G4 structures [11]. Nutrients affecting methylation pathways may reduce genome instability in progeroid syndrome patients by modulating G4 formation. However, the extent to which folate can affect G4 frequency is still unknown. This study aimed to address the hypothesis that the prevalence of DNA G4 structures and G4-induced DNA damage may be increased in Werner syndrome, and could be modulated by folate, possibly via alterations in DNA methylation.

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2. Materials and methods

2.1. Reagents

Bovine serum albumin (BSA) was purchased from AusGeneX (Molendinar, QLD, Australia). Pyridostatin, 4′,6-diamidino-2-phenylindole (DAPI), Roswell Park Memorial Institute (RPMI) 1640 medium, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium pyruvate, l-glutamine-penicillin-streptomycin solution, and formaldehyde were purchased from Sigma (Castle Hill, NSW, Australia). Phosphate-buffered saline (PBS), ProLong Gold antifade mounting medium and Alexa Fluor antibodies were obtained from Life Technologies (Mulgrave, VIC, Australia). Triton X-100, tris(hydroxymethyl)aminomethane-buffered saline (TBS), blocking milk protein, and Tween 20 were obtained from BioRad (Gladesville, NSW, Australia). 20× stock saline sodium citrate (SSC) solution was prepared from 3 M NaCl and 0.3 M sodium citrate. Mouse anti-γH2AX antibody (clone JBW301) was purchased from Millipore (Bayswater, VIC, Australia). Antibody (rabbit anti-Flag, 2368S) was obtained from Genesearch (Arundel, QLD, Australia). The genomic tip 100/G midiprep kit was purchased from Qiagen (Chadstone Centre, VIC, Australia). Fetal bovine serum (FBS), Qubit dsDNA HS Kit, and MethylFlash Global DNA methylation (5-mc) ELISA Easy Kit were obtained from Thermo Fisher Scientific (Scoresby, VIC, Australia) and Epigentek (Redfern, NSW, Australia), respectively. An expression construct for the BG4 antibody was kindly provided by Professor S. Balasubramanian, Department of Chemistry, University of Cambridge, UK [7].

2.2. Plasmid expression, G4 antibody production and purification

The BG4 plasmid construct [12] was used to produce the G4-recognizing antibody. This plasmid construct consists of the BG4 antibody, a Hexa-histidine affinity tag, and a Flag-tag, which were used, respectively, for purification and independent detection of G4. G4 antibody expression and purification were successfully performed as described previously [11].

2.3. B-Lymphocyte cell culture and treatment with folate

In this study, B lymphocytes were selected, since they were previously shown to be sensitive to the effect of folate concentrations in a dose-dependent manner over the physiological range, on their genome stability and G4 structure levels [13,14]. B-Lymphocytes from five Werner patients and five age- and gender-matched healthy individuals were obtained from the Coriell Institute (Camden, NJ, USA) (Table 1). These lymphocytes have been transformed with the Epstein-Barr virus. Affected individuals of both sexes were carrying point mutations within the RECQL2 gene. Before each set of experiments, cells were cultured in complete RPMI medium supplemented with 15% FBS, 25 mM HEPES, 5 mM pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 20 mM L-glutamine. In experiments where folate concentration was tested, cells were grown in RPMI medium containing either 20 nM or 200 nM folate; these concentrations represent deficient and normal-high folate levels, respectively [11]. These are the concentrations used in many previous studies to represent deficient and sufficient conditions of folic acid status [11,15,16]. All WRN and control B-lymphocytes were cultured for 14 days and cell viability and concentration were checked every 3–4 days, using a Luna Cell Counter (Logos Biosystems, VIC, Australia). Cells were passaged at the desired concentration using standard cell culture techniques. There were no significant differences in cell growth between WRN and control lymphocytes. Cells were used for microscopy (imaging analysis) or for DNA extraction and global methylation analysis.

Table 1
Catalogue number and characteristics.

	Samples	Gender	Age	Race	RECQL2 Mutation
WRN	AG04103	Female	37	Caucasian	ARG368TER
	AG14424	Male	45	Caucasian	Loss of exons 19–23/ ARG889TER
	AG14426	Female	48	Caucasian	ARG368TER
	AG14425	Male	57	Asian	G > C, −1, FS1061TER
	AG07896	Female	57	Caucasian	PHE1074LEU/ CYS1367ARG
CONTROL	GM12593	Female	34	Caucasian	n/a
	GM14667	Male	45	Caucasian	n/a
	GM23246	Female	48	Not Available	n/a
	GM13068	Male	56	Caucasian	n/a
	GM14820	Female	57	Caucasian	n/a

B-lymphocytes from Werner patients and age- and gender-matched healthy individuals used in this study were obtained from the Coriell Institute. Abbreviations: WRN, Werner.

2.4. Slide preparation

B-Lymphocyte cell suspensions were collected, centrifuged at 120 x g for 10 min, and the supernatant was discarded. Cell pellets were resuspended in 5 ml PBS and centrifuged at 120 × *g* for 10 min. Collected supernatant was replaced with 5 ml 1% formaldehyde in PBS, incubated at room temperature for 5 min and then centrifuged at 300 × *g* for 5 min. Cell pellets were resuspended in 1 ml of PBS and diluted to 400,000 cells/ml. Cell suspensions (100 µl) were cytocentrifuged on microscope slides using a Shandon Cytospin 4 (Thermo Scientific, USA) (600 rpm) and stored in sealed microscope slide boxes at −20 °C prior to staining.

2.5. Immunofluorescence imaging of lymphocytes

Microscope slides of cytospin cell preparations were defrosted at room temperature, washed in PBS for 5 min, cells were permeabilized in 0.1% Triton X-100 in PBS for 10 min, and slides were incubated with 3% milk protein in TBST (blocking solution A) for 40 min in a humidified box to avoid drying at 37 °C. G4-recognizing antibody, diluted in blocking solution A at 1:20, was then added to the cytospots and incubated for 2 h at 37 °C. Before the addition of secondary antibodies, slides were rinsed twice in TBST (TBS with 0.1% Tween). Rabbit anti-Flag (1:100) and mouse anti-γH2AX (1:400), mixed and diluted in blocking solution A, were added to the cytospots and incubated in a humidified box overnight at 4 °C. After washing the slides twice in TBST, Goat Alexa Fluor 488 Anti-Rabbit (1:200) and Goat Alexa Fluor 568 Anti-mouse (1:800) diluted in 3% BSA in TBST blocking solution (blocking solution B) was added to each cytospot, covered with parafilm and incubated at 37 °C for 1.5 h. Following these steps, cells were counter-stained for 5 min with 0.4 µg/ml DAPI diluted in 4 x SSC buffer and the excess was then rinsed with 2 x SSC. Each cytospot was treated with antifade mounting medium prior to applying coverslips and sealing the edges of the slides with nail polish. For quantitative measurements, an automated imaging fluorescence microscope (Zeiss Axio Imager M1) with MetaSystems software (Metafer 4 V3.113122) was used and the same settings were applied to all cytospots to ensure consistency. Slides were scanned using 63× oil objective and G4, γH2AX, and DNA signals were collected using FITC (green), Texas red (red), and DAPI (blue) filters, respectively. Parameters measured were DNA content (DAPI integral), γH2AX intensity, nuclear size (area), and number and intensity of G4 foci for each nucleus. A minimum of 500 nuclei were scanned per condition and the obtained images for all nuclei were visually confirmed. A similar protocol has been successfully used in this laboratory for previous studies to detect G4 structures [11,13,14].