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Revealing G-quadruplex DNA structures in HPV16 using quantitative PCR stop and ligands

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Motivation and Aim: G-quadruplex helical structures (G4) represent non-canonical conformations that can emerge in guanine-rich regions of nucleic acids under specific intracellular conditions, such as the presence of monovalent cations, pH fluctuations, temperature changes, and cellular crowding. Additionally, they can be modulated by small molecules, also known as G4 ligands [1]. The non-random occurrence of G4 structures within viral genomes accentuates their relevance in various biological contexts. The identification of G4 structures in diverse viral genomes of medical importance suggests their potential roles as pivotal actors in infectious mechanisms, positioning them as promising therapeutic targets [2]. Recently, over 100 putative G-quadruplex-forming sequences (PQS) have been predicted in the genomes of three high-risk Human Papillomaviruses using bioinformatic tools [3]. HPV are primary causative agents of the most prevalent sexually transmitted infection worldwide. Genotype 16 is highlighted due to its high risk for oncogenicity; however, it lacks direct antiviral treatments [4, 5]. Accordingly, exploring the structural features of the HPV 16 genome and understanding their role could reveal strategies for designing antiviral treatments against this infection. Therefore, this research aims to confirm the *in vitro* presence of G-quadruplex DNA structures to lay the foundation for further studies of their functions and possible application as molecular targets. These findings could provide significative insights in virology and contribute to the search for new therapeutic approaches for viral infections, including HPV.

Methods and Algorithms: First, we utilized data from the G4-forming sequences or PQS database, as reported by [3], and classified it into clusters based on their genomic position, DNA strand, and G4 score prediction. Subsequently, specific primer sets were designed to amplify G4 clusters located within the E6, E7, E1, E2, L1, and L2 genes, as well as within the long control region or promoter (LCR) of the HPV16 genome. Additionally, primer pairs were designed to flank non-G4 regions, which were predicted by bioinformatic programs in the HPV16 genome, serving as a control region for qPCR stop application. To validate the technique, plasmid DNA (pDNA) containing a known DNA-G4 sequence reported for HPV52 (ID PDB: 5O4D) in the LCR region was utilized as a reference. For this purpose, the HPV52 LCR region (1,163 bp) was cloned in DH5-α cells, as it contains a confirmed G4 by NMR (23 bp), providing a reference for standardization purposes. Primer pairs were designed to flank the reference G4 sequence in HPV52 and a predicted non-G4 sequence in the same genotype, serving as an amplification control. Optimal thermocycling conditions were determined for each primer pair for both HPV16 and 52 by qPCR. Once established, the concentration of KCl was standardized and applied in reactions with different concentrations of the ligands

PhenDC3, PDS, and BRACO-19 to assess their stabilizing effect on G4-DNA, after which three ligand concentrations were selected. These same conditions were applied for G4-DNA detection in HPV 16. The qPCR stop reactions were performed using the GoTaq SYBR PCR master mix (PROMEGA) and ran in a CFX96 Deep Well PCR detection system (BIORAD). A clone containing the complete genome of HPV 16 was used as template to detect the presence of G4-DNA in almost all evaluated regions of HPV 16 genome, except for the ORF L1, which was cloned to supplement the existing biological material. The obtained C_t values were collected and processed with the modified $2^{-\Delta\Delta Ct}$ formula to obtain the relative inhibition degree exerted by the stabilizing effect of ligands on the G4s. Subsequently, a one-way analysis of variance (ANOVA) was conducted to determine significant differences between the ligand concentrations and their effect on each evaluated G4 region. Finally, Dunnett's multiple comparison test was performed to determine differences between the assessed concentrations.

Results: PQS data were classified into 23 clusters distributed in the HPV16 genome, with 12 on the plus strand and 11 on the minus strand. The most abundant genes with PQS were L2 (62), followed by E2 (47) and E1 (36). Al least five PQS clusters were selected for their *in vitro* validation, with PQS scores ≥0.7. Two bacterial clones were obtained: one with the LCR region of HPV52, containing a confirmed G4, and another holding a fragment of the L1 gene of HPV16, serving as DNA templates for the qPCR stop assay. Annealing temperatures ranging from 62 to 66 °C were established for the HPV16 primer pairs corresponding to G4 regions in the early and late genes, the LCR region, non-G4 regions, and those designed for standardization with HPV52. Regarding the technique, the chosen concentration of KCl was 35 mM, based on the evidence obtained during the standardization process, demonstrating its ability to induce the formation of the confirmed G4 of HPV 52 without affecting the detection system.

Additionally, other studies identifying G4s use of concentrations between 25 to 50 mM, supporting its use within this range. Notably, the selected potassium value is lower than that reported in normal cellular environments (100–150 mM) and in carcinogenesis conditions (60 mM), where the formation of a greater quantity of G4s is promoted [6]. Ligand concentrations were determined, resulting in 25, 50 and 100 nM for PhenDC3, while 10, 50 and 100 nM were chosen for PDS and BRACO-19. The assay successfully confirmed the presence of G4-DNA in the E6, E7, E2, E1, L1, and L2 genes of HPV16, with the mentioned ligands exhibiting differential reactivity.

Conclusions: A qPCR Stop assay was standardized at 35 mM KCl and various concentrations of the ligands PhenDC3, PDS, and BRACO-19 to confirm G4-DNA structures in HPV16, resulting in different responses when exposed to PhenDC3, PDS and BRACO-19 ligands. The G4 region located into E6 ORF stood out for its sensibility towards the evaluated ligand concentrations. The confirmed G4 structures could play a relevant role in viral infection cycles and pose an interesting opportunity for future research and antiviral treatment development.

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