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Identification of G-quadruplex-forming Sequences in Nucleocapsid Gene of SARS-CoV-2 Variants of Concern: An *In Silico* Analysis

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ABSTRACT

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), as an enveloped RNA virus, has resulted in a global health threat. Recent studies emphasized that G-quadruplex structures are intrinsic obstacles to genome replication and targeting them in viral genomes could be a novel antiviral strategy to develop antiviral agents. The genomic RNA of SARS-CoV-2 codes for 29 proteins. One of them is the nucleocapsid protein with multiple functions, which is crucial for several steps of the viral life cycle. Here, we have analyzed putative G-quadruplex sequences (PQSs) in the Nucleocapsid gene of SARS-CoV2 and its variants of concern using a bioinformatics tool. The results showed that the number, position, and G-scores of PQSs were similar in Wuhan-hu-1 and Alpha, Beta, and Gamma variants. The main difference was observed in the Delta variant, in which a PQS was deleted at position 630 of the gene, which is the top-ranked highly conserved G-quadruplex. The Omicron variant had this PQS back at position 621 as it had acquired several mutations. In addition, there is also a unique R203M mutation, in the N protein of the Delta variant that leads to increased RNA packaging, replication, and severe COVID-19. We proposed that the R203M mutation has led to G>T substitution and loss of the top-ranked highly conserved PQS in the N gene of the Delta variant. Therefore, due to the loss of this important PQS or indeed an obstacle to viral replication, the Delta variant could exhibit higher reproduction and pathogenicity than other variants of concern.

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Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has spread across the world rapidly since the end of 2019 and has resulted in an epidemic of viral pneumonia, and a global health threat. Although the global scientific community has not found any effective antiviral treatment, researchers assist in creating novel COVID-19 diagnostic and treatment methods. SARS-CoV-2 as an enveloped RNA virus, along with other phylogenetically related species could infect huge numbers of vertebrates (Astuti, 2020; Díaz, 2020). Up to now, five SARS-CoV-2 variants of concern (VOCs) have been reported including B.1.1.7 (Alpha), B.1.351 (Beta), P.1 (Gamma), B.1.617.2 (Delta) and B.1.1.529 (Omicron). Emerging evidence revealed increased transmissibility and virulence as well as, reduced neutralization, which would be even more dangerous and troublesome. It should be noted that the SARS-CoV-2 genome undergoes frequent and quick mutation during its life which might serve as potential therapeutic targets against different SARS-CoV-2 variants. Each VOC is defined by a combination of point mutations across the SARS-CoV-2 genome. Although some mutations are common among different VOCs, there are some novel mutations observed in one individual variant (Telenti *et al*., 2022). Delta, as one of the most concerned

variants, is related to a greater risk of hospitalization, which shared some mutations with other variants, and owned its special mutations, which might explain the increase in transmission and virulence (Zhan *et al*., 2022). Under these circumstances, a better understanding of the VOC's genetic variations and their clinical implications is necessary to provide new aspects for antiviral treatments.

The SARS-CoV-2 genomic RNA is ~30 kb long encoding about 25 non-structural proteins (nsps) and accessory proteins and also, four structural proteins including spike (S), envelope (E), membrane (M), and nucleocapsid (N) (Chan *et al*., 2020; Huston *et al*., 2021). Exclusively, one of the most conserved and stable structural proteins is the N protein which is composed of 419 amino acids and is encoded by the ninth ORF of the virus (He *et al*., 2004). Nucleocapsid protein has multiple functions, participates in helical ribonucleoprotein formation in the packaging of the genomic RNA, regulation of RNA synthesis during viral replication and transcription, and control of metabolism in infected cells. Therefore, this protein is crucial for various steps of the viral life cycle (Chang *et al*., 2014; Narayanan *et al*., 2003).

Recent studies emphasized that secondary structural elements such as G-quadruplexes, as relatively highly conserved structures, could be used as potential antiviral targets (Zhai *et al*., 2022b). RNA G-quadruplexes (RG4) that can be formed in guanine (G) rich in RNAs, contain two or more layers of G-quartets constructed in a planar arrangement by Hoogsteen hydrogen bonding (Agarwala *et al*., 2015; Varshney *et al*., 2020). It can be suggested that RG4 might fold and stabilize the RNA motif appropriately to prevent viral replication, transcription, and translation (Abiri *et al*., 2021; Lyu *et al*., 2021; Metifiot *et al*., 2014; Ruggiero *et al*., 2021; Xu *et al*., 2021). There is increasing evidence that G-quadruplex structures play a regulatory role in gene transcription in humans and other organisms and are important potential therapeutic targets (Rhodes and Lipps, 2015; Zidanloo *et al*., 2016)

This study, for the first time, analyzed the putative G-quadruplexes-forming sequences (PQS) *in silico* in nucleocapsid gene of SARS-CoV-2 different VOCs and the PQSs possible impact on the packaging of the viral genome and viral life cycle. Therefore, it can be suggested that the G-quadruplex motifs presenting in the N gene, might play a fundamental role in regulating its function and serving as potential targets for SARS-CoV-2 treatment.

Methods

SARS-CoV-2 genomic sequences

The genomic sequence of the SARS-CoV-2 Wuhan Hu-1 RNA reference genome (MN908947) and other global variants of concern were downloaded from EpiCoVTM database of Global Initiative on Sharing All Influenza Data (GISAID) (https://www.gisaid.org/) using automatic search function feeding information for geographical location, SARS-CoV-2 lineage and sample collection and sequence reporting dates (up to 2023-19-07). Afterward, the N gene sequence of different strains was extracted for further investigation.

G-quadruplex mapping

G-quadruplex sequences of the N gene are mapped on the SARS-CoV2 genome via the website QGRS mapper software (https://bioinformatics.ramapo.edu/QGRS/analyze. php) (Kikin *et al*., 2006). PQS of a maximum length of 30 bases and loop consisting of any length from 0 to 30 base sequences are considered. As an output, this software gives the list of detected PQSs (unique and overlapping sequences) with the scoring data. The possibility of the G-quadruplex structure formation for each sequence was gained as G-scores. In addition, the position of each PQS sequence in the forward and reverse strands of the SARS-CoV2 genome was listed as software output.

Result

Identification of RNA G-quadruplexes in SARS-CoV-2 N gene.

To explore the potential involvement of RNA G-quadruplexes in SARS-CoV-2 VOCs, an *in silico* method with online software was used (Kikin *et al*., 2006) which offered a G-score for each PQS in the virus genome. The PQSs were predicted in the SARS-CoV-2 genome by the QGRS mapper algorithm. In the 29.9 kb genome

of SARS-CoV-2, the PQSs in the Nucleocapsid gene (nt. 28274-29533) of VOCs were analyzed. Consequently, any position with a higher probability of forming RNA G-quadruplex structures had a higher score so, the presence of potential sequence stretch that could form a G-quadruplex structure was listed in Table 1 (Supplement 1-6).

Table 1. Putative G-quadruplex sequences in Nucleocapsid gene of SARS-CoV-2 variants of concern

WHO nomenclature	Lineage	Date of designation	Emergence	QGRS/PQS position[a] (G-Score) [Overlaps not included]	QGRS/PQS position (G-Score) [Overlaps included]
Wuhan-hu-1	Reference	Dec, 219	China	508 (9), **630 (18)**, 850 (14), 961 (11)	508 (9), 508 (8), 608 (3)**, 630 (18)**, 850 (14), 859 (7), 961 (11), 961 (10)
Alpha (α)	B.1.1.7	Dec, 2020	UK	508 (9), **630 (18)**, 850 (14), 961 (11)	508 (9), 508 (8), **630 (18)**, 850 (14), 859 (7), 961 (11), 961 (10)
Beta (β)	B.1.351	Dec, 2020	South Africa	508 (9), **630 (18)**, 850 (14), 961 (11)	508 (9), 508 (8), 608 (3)**, 630 (18)**, 850 (14), 859 (7), 961 (11), 961 (10)
Gamma (P.1)	B.1.1.128	Jan, 2021	Brazil	508 (9), **630 (18)**, 850 (14), 961 (11)	508 (9), 508 (8), **630 (18)**, 850 (14), 859 (7), 961 (11), 961 (10)
Delta (δ)	B.1.617.2	May, 2021	India	178 (7), 508 (9), 850 (14), 961 (11)	178 (7), 183 (7), 508 (9), 508 (8), 850 (14), 859 (7), 961 (11), 961 (10)
Omicron (ο)	B.1.1.529	Nov, 2021	South Africa	73 (8), 499 (9), **621 (18)**, 841 (14), 952 (11)	73 (8), 88 (3), 499 (9), 499 (8), **621 (18)**, 841 (14), 850 (7), 952 (11), 952 (10)

a: nucleotide position

We found several PQSs in the N gene of the SARS-CoV2 and the VOCs with the acceptable potential to form a G-quadruplex structure. Even though PQSs were confirmed to be present near the transcription start site of many genes (Kikin *et al*., 2006; Perrone *et al*., 2017), the PQSs of the N gene were not located in these regions. Without considering overlapping sequences, there are four PQS in the N gene of VOCs except for the Omicron with five PQSs regions. If overlapping sequences are considered, up to nine PQS regions for SARS-CoV-2 VOCs are observed (Supplementary Material). These regions all fit the G2N1-7G2N1-7G2N1-7G2 formula, indicating the potential to adopt the non-canonical and metastable RG4 structures with 2-quartet (Ji *et al.*, 2021; Zhao *et al*., 2021). This analysis revealed that in all PQSs of the N gene, there are regions with four contiguous GG runs and lengths of 15-30 nucleotides (See Supplementary Supplement 1-6; also Table 1). The QGRS mapper results revealed that the number, position, and G-scores of PQSs were similar in Wuhan-hu-1 SARS-CoV-2 and the three VOCs Alpha, Beta, and Gamma. The differences were observed in the Delta variant in which a PQS at position 630 (RG-1) was deleted (Supplement 5). The Omicron variant exhibited this PQS again at position 621 due to the several mutations it acquired. This PQS showed the highest G-score (G-score =18) in both analyses considering overlap and not considering overlap (bold in Table 1, Supplement 6).

Discussion

SARS-CoV-2 has been circulating in the human population for three years and has infected hundreds of millions of people. Therefore, understanding the molecular mechanism that could affect its gene expression in host cells is a prerequisite for developing a successful and effective treatment for COVID-19.

This is the first study to analyze the presence of PQSs in different VOCs of SARS-CoV-2. Our result confirmed that the highest ranked PQS (RG-1, G-score = 18) was present at position 630 (621 for Omicron) in all VOCs except the Delta variant (Table 1). Recent studies identified G-quadruplex motifs in the SARS-CoV-2 genome and also found that some PQSs were well-conserved in a wide range of coronaviruses (Cui and Zhang, 2020; Panera *et al*., 2020; Ji *et al*., 2021). Also, Belmonte-Reche et al. identified PQSs with scores higher than 20, and conserved PQSs in SARS-CoV-2, SARS-CoV, and Bat-CoV (Belmonte-Reche *et al.*, 2021). In another study, Zhang et al. obtained 24 PQSs with three prediction tools nine of which were scattered on the negative strand (Zhang *et al*., 2020). According to the importance of G-quadruplex structures in promoter regions (Zidanloo *et al*., 2016), Maiti focused on those SARS-CoV-2 genes that have PQS sequences upstream at the putative promoter region and identified PQSs upstream of at least 16 genes of the virus (Maiti, 2022). It is noteworthy that the expression of

these genes might be controlled via genome rearrangements or stability and by preventing RNA splicing or translation (Machida *et al*., 2020). Although there are no such potential sequences upstream of the SARS-CoV-2 N gene, it is notable that the PQSs in any position of a gene can modify gene expression. Besides, the results of a recent study showed that the higher the number of PQS, the more the replication rate of the viruses is affected. Thus, the fact that SARS-CoV-2 contains fewer PQSs than SARS-CoV seems to be consistent with the fact that SARS-CoV-2 has a stronger replication capacity and faster spread (Zhai *et al*., 2022a).

Lately, seven conserved RG4 sequences have been reported in the genomes of SARS-CoV and SARS-CoV-2, three in the S coding region, two in the nsP1 coding region, and the other two in the nsP10 and N coding regions (Cui and Zhang, 2020). In addition, another study analyzed more than 200,000 SARS-CoV-2 genome sequences from five continents and found PQSs at six positions with a high degree of conservation of more than 90%. Interestingly one of them was a PQS at N protein-coding sequence (Carvalho *et al*., 2021) which is the top-ranked PQS of the N gene (RG-1) (Table 1). It has been confirmed that RG-1 can form a stable parallel G-quadruplex conformation with some ligands can interact and potentially over-stabilize it to significantly reduce protein levels of SARS-CoV-2 N protein by inhibiting viral genome expression both in vitro and in vivo and impairing the viral life cycle (Miclot *et al*., 2021; Mukherjee *et al*., 2022; Zhao *et al*., 2021).

Several mutations were observed in the N protein of SARS-CoV-2 VOCs, including D3L (aspartic acid to leucine substitution at position 3), R203K (arginine to lysine substitution at position 203), R203M (arginine to methionine substitution at position 203), G204R (glycine to arginine substitution at position 204), S194L (serine to leucine substitution at position 194) and S235F (serine to phenylalanine substitution at position 235), T205I, (threonine to isoleucine substitution at position 205). It is worth noting that R203M, G28881T substitution in the Delat genome, is a common mutation that occurred in the N protein and is significantly associated with the Delta variant (Ravi *et al*., 2022; Khetran and Mustafa, 2023). In another study, Li and his co-workers pointed out that the Delta variant may have a faster viral replication rate and is more infectious in the early stage of infection (viral RNA concentration is increased by a factor of 1000 in patients) (Li *et al*., 2022). In addition, several studies have found a link between the R203M mutation and severe COVID-19 (Alsuwairi *et al*., 2023; Mourier *et al*., 2022; Zhao *et al*., 2022). Interestingly, Syed et al., showed that the R203M mutation in the N gene of Delta variants leads to increased RNA packaging and replication (more than 50-fold) (Syed *et al*., 2021).

Conclusions

The RNA G-quadruplexes block viral replication in SARS-CoV-2 and could inhibit viral proliferation in SARS-CoV-2 VOCs (Fig. 1). We propose that the unique R203M mutation in the Delta variant could result in the loss of the highly conserved PQS (RG-1) of the N gene. The mutation R203M (G>T) is located at position 28881 of the Delta variant genome and led to the loss of one of the guanines in this region, which is involved in the formation of the G-quadruplex structure (Supplement 7). Therefore, the Delta variant may have effective and efficient replication and higher pathogenicity than other variants of concern due to the absence of this important PQS or an obstacle to viral replication (Fig. 2). Since the presence of this conserved PQS could play a regulatory role in the viral genome, the highest-ranking PQS in the N gene (RG-1) is an interesting topic for further research and could be the most attractive candidate for further investigation of SARS-CoV-2 gene expression. Exploring such roles by designing future experiments with PQS could be extremely useful in understanding whether these sequences modulate the expression of SARS-CoV2 genes and support viral amplification for their survival in human cells. Nevertheless, G-quadruplex nucleic acid structures are relatively difficult to study in vivo, and only a limited number of structures have been solved to date (Ji *et al*., 2021).

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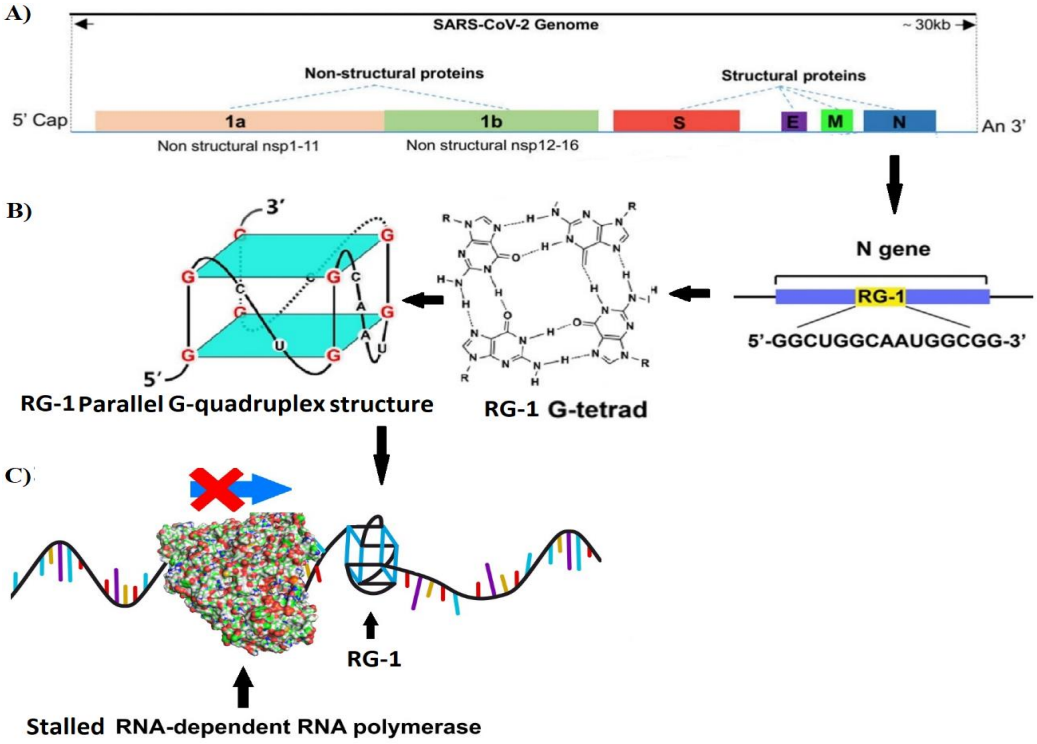

Fig. 1. RNA G-quadruplex could inhibit viral proliferation in SARS-CoV-2 VOCs: A) Schematic representation of the SARS-CoV-2 genome and the N gene. B) The RG-1 sequence in the N gene, the structure of its G-quartet, and its parallel G-quadruplex at position 630. C) Stalling the polymerase via the formation of a top-ranked G-quadruplex structure (RG-1) could reduce viral replication.

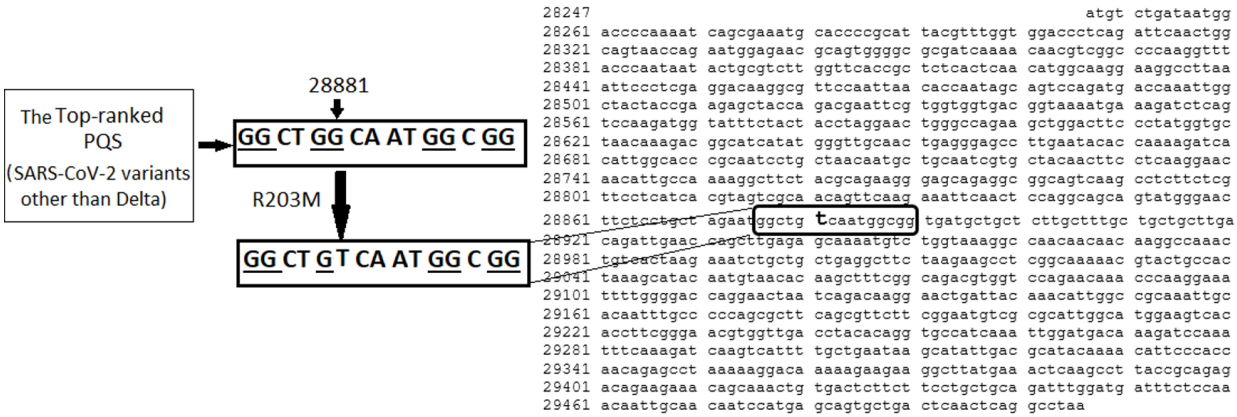

Fig. 2. R203M takes place in position 203 of the N protein in the Delta variant, which is nucleotide 288881 of the virus genome. It exactly overlaps with the formation of the top-ranked PQS. Guanine-rich sequences with four guanine repeats tend to fold intramolecularly into DNA quadruplexes. This guanine at 28881, plays an important role in the formation of the PQS, and the conversion of G to T reduces the possibility of the formation of a G-quadruplex structure.

Disclosure Statement

The authors declare that there is no conflict of interest. The author alone is responsible for the content of the paper.

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